Expression profiles of microRNAs encoded by the oncogenic Marek’s disease virus reveal two distinct expression patterns in vivo during different phases of disease

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Marek’s disease virus (MDV) is a long-recognized oncogenic herpesvirus, which induces lymphoma in its natural host that can be prevented by vaccination. MDV infection provides an excellent biological model for investigating the biology, genetics and immunology of viral oncogenesis. Recently discovered microRNAs (miRNAs) in the MDV genome have been suggested to have regulatory roles during MDV oncogenesis. We have examined the expression profiles of all 22 previously reported miRNAs encoded by MDV-1 in chickens artificially challenged with MDV-GX0101. We found that a subset of the miRNAs was differentially expressed during different phases of the developing disease. These miRNAs show early or late expression during disease progression, accompanied by obvious tissue-specific and differential expression patterns. This temporal and differential tissue distribution suggest that these miRNAs may perform different regulatory roles in switching from latency to lytic replication, immunosuppression, neoplastic transformation or other aspects of lymphoma formation. These reported in vivo expression profiles indicate the potentially functional MDV-1-encoded miRNAs that should be selected for further investigation of their functions in MDV oncogenesis.

INTRODUCTION

MicroRNAs (miRNAs) are recently discovered small non-coding RNAs with important post-transcriptional regulatory roles in various cellular processes, including development, differentiation, all aspects of cancer biology (Bartel, 2004; Filipowicz et al., 2008; Lee & Dutta, 2009) and also in the pathogenesis of a number of herpesviruses (Boss et al., 2009). Marek’s disease virus (MDV) is phylogenetically classified as a herpes virus of the subfamily Alphaherpesvirinae. The virulent strains of serotype 1 MDV (MDV-1) establish and maintain latent infections in their natural hosts and may finally cause a rapid-onset aggressive T-cell lymphoma, an important neoplastic immunosuppressive disease of poultry named Marek’s disease (MD) (Witter & Schat, 2003). The mechanism of MDV oncogenesis has historically been an important focus of research for virologists (Nair & Kung, 2004). Recently a large number of viral miRNAs have been reported to be encoded in the genomes of all three serotypes of MDV (Burnside et al., 2006; Yao et al., 2007, 2008, 2009; Waidner et al., 2009) and several recent studies suggest that some of the MDV-1-encoded miRNAs, such as miR-M4-5p and miR-M32-3p, have possible functions in MDV oncogenesis (Morgan et al., 2008; Lambeth et al., 2009; Zhao et al., 2009). Although there is no base sequence conservation, the genomic location of MDV-encoded miRNAs in the long or short repeat (R1, R3) regions of their genomes is conserved in all three serotypes of MDV. MDV genomes are primarily composed of a ‘unique long region’ (UL) and a ‘unique short region’ (US), and may finally cause a rapid-onset aggressive T-cell lymphoma, an important neoplastic immunosuppressive disease of poultry named Marek’s disease (MD) (Witter & Schat, 2003). The mechanism of MDV oncogenesis has historically been an important focus of research for virologists (Nair & Kung, 2004). Recently a large number of viral miRNAs have been reported to be encoded in the genomes of all three serotypes of MDV (Burnside et al., 2006; Yao et al., 2007, 2008, 2009; Waidner et al., 2009) and several recent studies suggest that some of the MDV-1-encoded miRNAs, such as miR-R4-5p and miR-M32-3p, have possible functions in MDV oncogenesis (Morgan et al., 2008; Lambeth et al., 2009; Zhao et al., 2009). Although there is no base sequence conservation, the genomic location of MDV-encoded miRNAs in the long or short repeat (R1, R3) regions of their genomes is conserved in all three serotypes of MDV. MDV genomes are primarily composed of a ‘unique long region’ (UL) and a ‘unique short region’ (US), and may finally cause a rapid-onset aggressive T-cell lymphoma, an important neoplastic immunosuppressive disease of poultry named Marek’s disease (MD) (Witter & Schat, 2003). The mechanism of MDV oncogenesis has historically been an important focus of research for virologists (Nair & Kung, 2004). Recently a large number of viral miRNAs have been reported to be encoded in the genomes of all three serotypes of MDV (Burnside et al., 2006; Yao et al., 2007, 2008, 2009; Waidner et al., 2009) and several recent studies suggest that some of the MDV-1-encoded miRNAs, such as miR-M4-5p and miR-M32-3p, have possible functions in MDV oncogenesis (Morgan et al., 2008; Lambeth et al., 2009; Zhao et al., 2009). Although there is no base sequence conservation, the genomic location of MDV-encoded miRNAs in the long or short repeat (R1, R3) regions of their genomes is conserved in all three serotypes of MDV. MDV genomes are primarily composed of a ‘unique long region’ (UL) and a ‘unique short region’ (US),

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The GenBank/EMBL/DDBJ accession numbers assigned to MDV-1-encoded miRNAs can be found in Table 1 and in the miRBase database (www.mirbase.org/cgi-bin/mirna_summary.pl?org=mdv1).
flanked by inverted repeats named ‘terminal/internal-repeat long regions’ (TRl/IRl) and ‘internal/terminal-repeat short regions’ (IRs/TRs) (Cebrià et al., 1982). The genes encoded by the UL and US regions are highly conserved among herpesviruses whereas virus-specific genes are mainly located in the inverted-repeat regions (Osterrieder et al., 2006). Thus the highly conserved viral miRNA genomic locations possibly imply their specific regulatory functions in MDV biology. All of the MDV-1-encoded miRNAs are focused in three gene clusters (Burnside et al., 2006; Yao et al., 2008; Luo et al., 2010), namely the Meq cluster, LAT cluster and one which is named the middle cluster (Mid cluster) for its location downstream of Meq cluster and upstream of LAT cluster (Fig. 1a). It has been suggested that the MDV-encoded miRNA clusters are processed from a single long primary miRNA and this has been partially verified by detecting a single amplicon containing the Meq cluster of miRNAs (Burnside et al., 2006).

Most MDV-1-encoded miRNAs are expressed at higher levels in splenic tumours and in a T-lymphoma cell line than in chicken embryo fibroblast cultures (CEFs) (Burnside et al., 2006; Yao et al., 2008). Although highly conserved in terms of sequence among oncogenic MDV-1 strains of different virulence, the Meq-clustered miRNAs are expressed at higher levels in lymphomas produced by very virulent plus (vv+) MDV than those produced by a less very virulent (vv) MDV strain, whereas the expression of LAT-cluster miRNAs is equal, implying that the Meq-clustered miRNAs may be more significant in MD pathogenesis (Morgan et al., 2008).

Recently, miRNA expression signatures in many cancers have been identified (Calin & Croce, 2006; Zhang et al., 2009), increasing our understanding of the connection between miRNA and cancer. The course of MD is well established as the ‘Cornell model’ (Calnek, 2001), which includes four phases: (i) the early cytolytic phase [2–7 days post-infection (p.i.)], (ii) the latent phase (7–10 days p.i. onwards), (iii) the late-cytolytic and immunosuppressive phase (18 days p.i. onwards) and (iv) the proliferative phase (28 days p.i. onwards) (Baigent & Davison, 2004). Previous studies on MDV-miRNA expression have focused on virus-infected CEFs, virus-transformed cell lines or virus-caused splenic tumours. Herein, we have investigated the in vivo expression profiles of all previously reported viral miRNAs encoded by MDV-1 in chickens challenged with the strain MDV-GX0101 during the late cytolytic and immunosuppressive phase, the proliferative phase and the late stages of gross tumour generation. We found that only a subset of MDV-1-encoded miRNAs were stably expressed in diseased birds and represented both temporal and tissue expression patterns that suggested differing regulatory roles in the pathogenesis of MD.

RESULTS

Establishment of MD using the virulent MDV strain GX0101

To investigate the in vivo expression profiles of MDV-1-encoded miRNAs, experiments were carried out using chickens infected with GX0101, a prevalent field strain of MDV-1 isolated from South China (Cui et al., 2010). The level of viraemia in peripheral blood lymphocytes of ten birds, randomly selected from the virus-infected or uninfected groups, was determined at 7, 14, 21, 28, 35, 60 and 90 days p.i. As shown in Fig. 2, GX0101 virus was detected in infected birds as early as 7 days p.i. The levels of viraemia increased during the following 3 weeks, peaking at 28 days p.i. at a mean value of 3950 p.f.u. ml\(^{-1}\) of peripheral blood, and then declining rapidly to remain at approximately 1000 p.f.u. ml\(^{-1}\) during the last 2 months of the experiment. No virus proliferation was observed in the negative controls. The growth rate of infected birds was strongly inhibited compared with the negative controls. In GX0101-challenged chickens the final mortality was 64.4% whereas no deaths were recorded in the negative control group. Additionally, 46.7% of GX0101-challenged chickens demonstrated gross tumours in diverse visceral organs. Thus the typical tumorigenic form of MD has been successfully established by GX0101 infection.

Clinical symptoms of diseased birds randomly selected for miRNA expression analysis

Two groups of five birds previously challenged with GX0101 virus were humanely euthanized at 18 and 36 days p.i., the late cytolytic and immunosuppressive phase and at the proliferative phase of MD, respectively, for examination by necropsy. Among all of the sacrificed birds, GX0101 infection resulted in severe atrophy of the central immune organs, including the thymus and bursa of Fabricius, but no obvious macroscopic pathological changes were observed in the other viscera. For subsequent analysis of miRNA expression, two birds were randomly selected from each of these two groups and designated chickens 1, 2 and 3, 4, respectively. At 90 days p.i. the experiment was terminated and a total of 32 surviving birds were sacrificed and examined. From these, six birds (chickens 5–10) that showed gross tumours were selected for analysis of viral miRNA expression at the late tumorigenic stages of infection. As well as severe atrophy of the central immune organs, these diseased birds showed clear macroscopic tumours. Tumours were seen only in the spleens of chickens 5 and 6, whereas chickens 7 and 8 had tumours in both spleen and liver. The remaining two birds, chickens 9 and 10, showed macroscopic tumours in multiple organs, including spleen, liver and proventriculus. Macroscopic tumours were seldom seen in the kidneys.

Characterization of the in vivo-expressed mature miRNAs encoded by MDV-1

Using the γ-\(^{32}\)P–ATP-labelled oligonucleotides listed in Table 1, the in vivo expression level of all of the previously reported MDV-1-encoded miRNAs were globally determined by Northern blot analysis. The diagram and details...
of the in vivo expression of the MDV-1-encoded miRNAs are shown in Fig. 1(b) and Table 2, respectively. The results showed that a total of 12 miRNAs, namely miRNA-M1-5p, M2-5p, M2-3p, M3-5p, M4-5p, M6-5p, M7-5p, M8-3p, M9-5p, M10-3p, M12-3p and M32-3p, were found to be transcribed, processed and ultimately matured into stable miRNAs for all or part of the whole course of the disease caused by MDV-GX0101 (Figs 3a and 4a). Although precursor miRNAs (pre-miRNAs) of miRNA-M1, miRNA-M4, miRNA-M5, miRNA-M6, miRNA-M8, miRNA-M9, miRNA-M11 and miRNA-M13 were transcribed in all the birds showing gross tumours, the previously reported miRNAs: miRNA-M1-3p, M4-3p, M5-5p, M5-3p, M6-3p, M8-5p, M9-3p, M11-5p, M11-3p and M13-3p were not detected at any time point post-challenge (Table 2). The lengths of all of the pre-miRNAs detected varied from 65–70 nt except for miRNA-M6-5p-3p, whereas all of the mat-miRNAs had lengths of 21–23 nt. These data indicate that only a fraction of the previously reported MDV-1-encoded miRNAs are stably expressed and occur as mat-miRNAs in vivo.

Temporal expression of MDV-1-encoded miRNAs derived from three gene clusters

For the further investigation of the dynamics of MDV-1-miRNA expression, tissue from eight organs (heart, liver, spleen, lung, kidney, proventriculus, thymus and bursa of Fabricius) were sampled from the pairs of diseased birds collected at the late cytolytic and immunosuppressive phase (18 days p.i.), the proliferative phase (36 days p.i.) and at the late stages of tumorigenesis (90 days p.i.). Northern blot analysis showed that all of the Meq-clustered mat-miRNAs, miRNA-M2-5p, M2-3p, M3-5p, M4-5p, M6-5p, M7-5p, M9-5p and M12-3p were expressed at some time during the disease and gave two distinct expression patterns (Fig. 3 and Table 2). As shown in Fig. 3(a), miRNA-M3-5p, M4-5p and M12-3p were detected in the
spleen as early as 18 days p.i. Then, slightly higher expression levels of these miRNAs were detected at 36 days p.i., and with progress to tumorigenesis, the expression levels of miR-M3-5p, M4-5p and M12-3p universally increased in all examined organs. The remaining three Meq-clustered mat-miRNAs, miR-M2-5p, M2-3p and M9-5p, were not detected in the diseased birds sampled at earlier infection stages (18 or 36 days p.i.) but were expressed in the diseased birds showing gross tumours (90 days p.i.). Compared with miR-M3-5p, M4-5p and M12-3p, the other three Meq-clustered mat-miRNAs, including miR-M2-5p, M2-3p and M9-5p, were expressed at obviously lower levels (Fig. 3b). For easily distinguishing these two groups of miRNAs showing distinct expression patterns in vivo, we named the former three Meq-clustered miRNAs as early expressed miRNAs while the latter three miRNAs were named late-expressed miRNAs.

Interestingly all the LAT-clustered mat-miRNAs, including miR-M6-5p, M7-5p, M8-3p and M10-3p, can also be classified into two groups (Fig. 4 and Table 2). Both miR-M7-5p and miR-M8-3p were detected in spleen as early as 18 days p.i., and then similarly to the early expressed Meq-clustered miRNAs, their expression levels similarly

### Table 1. MDV-1-encoded miRNAs and their corresponding antisense oligonucleotide probes used for Northern blot analysis

<table>
<thead>
<tr>
<th>No.</th>
<th>miRNA</th>
<th>Length (nt)</th>
<th>Accession no.</th>
<th>miRNA sequences (5’–3’)</th>
<th>Antisense oligonucleotide sequences (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>MDV1-miR-M1-5p</td>
<td>24</td>
<td>MI0005093</td>
<td>UGCUUGUUCACUGUCGCGCAUUAU</td>
<td>ATATGCGCGCAGTGAACAAGCA</td>
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<tr>
<td>02</td>
<td>MDV1-miR-M1-3p</td>
<td>22</td>
<td>MI0005093</td>
<td>AUGCUGCGCAUGAAAGCGCAA</td>
<td>TTCCGCTCCTTCATTGGGACACGT</td>
</tr>
<tr>
<td>03</td>
<td>MDV1-miR-M2-5p</td>
<td>26</td>
<td>MI0005094</td>
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<td>AAAACGACTACGGGCAAGATACAC</td>
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<td>MDV1-miR-M2-3p</td>
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<td>AAAGCATCTTCGCGGACCTGTC</td>
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<td>05</td>
<td>MDV1-miR-M3-5p</td>
<td>26</td>
<td>MI0005095</td>
<td>CAUCAAAUGUUAGAAACUCCUCGCGCU</td>
<td>AGGCGGAGGAGGTTTCACATTTCATG</td>
</tr>
<tr>
<td>06</td>
<td>MDV1-miR-M3-3p</td>
<td>27</td>
<td>MI0005096</td>
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<td>AAGAAGGGGTTCCGATAGACGATAATTAA</td>
</tr>
<tr>
<td>07</td>
<td>MDV1-miR-M4-3p</td>
<td>24</td>
<td>MI0005096</td>
<td>CGAUUGUUUUCAGAGCAUGACCU</td>
<td>AGGTCTATCTGTCAGAAACCTCG</td>
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<tr>
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<td>MDV1-miR-M5-5p</td>
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<td>AACAGTAGACGACAGATACAC</td>
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<tr>
<td>10</td>
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<td>11</td>
<td>MDV1-miR-M6-3p</td>
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<td>ACTGGTATCTTGGGAGGATCTC</td>
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<td>12</td>
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<tr>
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<td>TGCAAGCCACAAGCAGAACAA</td>
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<tr>
<td>14</td>
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<td>15</td>
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<td>MI0006987</td>
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</tr>
<tr>
<td>17</td>
<td>MDV1-miR-M10-3p</td>
<td>24</td>
<td>MI0006988</td>
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<td>18</td>
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<td>MI0006989</td>
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<tr>
<td>19</td>
<td>MDV1-miR-M11-3p</td>
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<td>MI0006991</td>
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<tr>
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<td>MDV1-miR-M32-3p</td>
<td>23</td>
<td>MI0008337</td>
<td>UGCUCGAGUCUGAGCAGUCAUA</td>
<td>TTGGATCTGCTACGACTGACAGA</td>
</tr>
</tbody>
</table>

http://vir.sgmjournals.org
Relative expression levels of miRNA are described as high (H), moderate (M) or low (L), respectively. miRNAs derived from the 5’ or 3’ arms in a single miRNA precursor are indicated by a ‘-5p’ or ‘-3p’ suffix as described by Yao et al. (2008) and ten of them, including MDV1-miR-M1-5p, M2-5p, M2-3p, M3-5p, M4-5p, M4-3p, M5-5p, M6-3p, M8-3p and M32-3p had been previously given different names by Burnside et al. (2006) or Morgan et al. (2008), respectively. UD, undetermined.

**Table 2.** Details of the *in vivo* expression level of MDV-1-encoded miRNAs in splenic tumours caused by virulent MDV-1 strains analysed by Northern blot and reported in the present or previous reports

Relative expression levels of MDV-1-encoded miRNAs in splenic tumours caused by virulent MDV-1 strains are shown as detectable (Y) or undetectable (N), respectively. Relative expressions of MDV-1-encoded miRNAs are compared to each other in the present or previous independent reports.

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Genomic location in miRNA clusters</th>
<th>In vivo expression details of MDV-1-encoded miRNAs in splenic tumours caused by GX0101*</th>
<th>Relative expression levels of MDV-1-encoded miRNAs in splenic tumours caused by virulent MDV-1 strains†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre-miRNAs Length (nt) Mat-miRNAs Length (nt)</td>
<td>GX0101*</td>
</tr>
<tr>
<td>(a) Early expressed miRNAs encoded by MDV-1</td>
<td>Mid</td>
<td>Y 65–70 Y 22–23</td>
<td>L L UD H H</td>
</tr>
<tr>
<td>MDV1-miR-M1-5p</td>
<td>Mid</td>
<td>Y 65–70 Y 22–23</td>
<td>M M M M M M M M</td>
</tr>
<tr>
<td>MDV1-miR-M1-3p</td>
<td>Meq</td>
<td>Y 65–70 Y 22–23</td>
<td>M M M M M M M M</td>
</tr>
<tr>
<td>MDV1-miR-M4-5p</td>
<td>Meq</td>
<td>Y 65–70 Y 22–23</td>
<td>M M M M M M M M</td>
</tr>
<tr>
<td>MDV1-miR-M7-5p</td>
<td>LAT</td>
<td>Y 68–70 Y 21–22</td>
<td>M M M M M M M M</td>
</tr>
<tr>
<td>MDV1-miR-M8-3p</td>
<td>LAT</td>
<td>Y 65–68 Y 21–22</td>
<td>M M M M M M M M</td>
</tr>
<tr>
<td>MDV1-miR-M12-3p</td>
<td>Meq</td>
<td>Y 60–65 Y 22–23</td>
<td>M M M M M M M M</td>
</tr>
<tr>
<td>(b) Late-expressed miRNAs encoded by MDV-1</td>
<td>Meq</td>
<td>Y 65–70 Y 21–22</td>
<td>M M M M M M M M</td>
</tr>
<tr>
<td>MDV1-miR-M2-5p</td>
<td>Meq</td>
<td>Y 65–70 Y 21–22</td>
<td>M M M M M M M M</td>
</tr>
<tr>
<td>MDV1-miR-M2-3p</td>
<td>Meq</td>
<td>Y 65–70 Y 21–22</td>
<td>M M M M M M M M</td>
</tr>
<tr>
<td>MDV1-miR-M6-5p</td>
<td>LAT</td>
<td>Y 110–120 Y 21–22</td>
<td>M M M M M M M M</td>
</tr>
<tr>
<td>MDV1-miR-M9-5p</td>
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<td>Y 65–68 Y 21–22</td>
<td>M M M M M M M M</td>
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<tr>
<td>MDV1-miR-M32-3p</td>
<td>Mid</td>
<td>Y 60–65 Y 21–22</td>
<td>M M M M M M M M</td>
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<tr>
<td>(c) Passenger miRNAs encoded by MDV-1</td>
<td>Mid</td>
<td>Y 65–70 N N</td>
<td>N UD L UD UD</td>
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<td>MDV1-miR-M1-3p</td>
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<td>MDV1-miR-M4-3p</td>
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<td>MDV1-miR-M6-3p</td>
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<td>Y 65–68 N N</td>
<td>N UD M M M M</td>
</tr>
<tr>
<td>MDV1-miR-M9-3p</td>
<td>Meq</td>
<td>Y 60–65 N N</td>
<td>N UD L UD UD</td>
</tr>
<tr>
<td>(d) Unstable miRNAs encoded by MDV-1</td>
<td>Mid</td>
<td>Y 65–70 N N</td>
<td>N UD L UD UD</td>
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<td>N UD L UD UD</td>
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<tr>
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<td>MDV1-miR-M11-5p</td>
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<tr>
<td>MDV1-miR-M11-3p</td>
<td>Mid</td>
<td>Y 65–68 N N</td>
<td>N UD L UD UD</td>
</tr>
<tr>
<td>MDV1-miR-M13-3p</td>
<td>LAT</td>
<td>Y 60–65 N N</td>
<td>N UD L UD UD</td>
</tr>
</tbody>
</table>

*Virulent MDV-1 strain GX0101 used in the present work. Precursor miRNAs (pre-miRNAs) and mature miRNA (mat-miRNAs) expressed or not in GX0101 caused splenic tumours and are shown as detectable (Y) or undetectable (N), respectively.*

†Relative expressions of MDV-1-encoded miRNAs are compared to each other in the present or previous independent reports.

‡Virulent MDV-1 strains used in the studies previously reported by Burnside *et al.* (2006), Yao *et al.* (2008), or Morgan *et al.* (2008), respectively.

increased in all tissues by 90 days, whereas miR-M6-5p and miR-M10-3p gave low expression only at the late stages of tumorigenesis (Fig. 4a, b). Compared to Meq-clustered and LAT-clustered miRNAs, both of the mat-miRNAs originating in the Mid cluster, MiR-M1-5p and miR-M32-3p, were found to be expressed at low levels throughout the course of the disease (Fig. 4a, b). Even so it is possible that miR-M1-5p can be classified as an early expressed miRNA since it does show slightly elevated expression in spleen at 36 days p.i., whereas miR-M32-3p should be classified as a late-expressed miRNA as it is only expressed at 90 days p.i.
Tissue-specific and differential expression of early or late-expressed miRNAs encoded by MDV-1

To check the reliability of the Northern blot analysis, detection of all of the MDV-1-encoded miRNAs was repeated in two further birds randomly selected at each time point post-challenge. Each of the investigated miRNAs showed similar expression levels in two repeat samples from the investigated chickens except for one diseased bird at 90 days p.i., chicken 8, in which all the miRNAs were universally expressed at very low or undetectable levels (data not shown). Globally, the expression levels of both early and late-expressed miRNAs showed an obvious relationship to the time-course of the disease (Figs 3 and 4) rather than to the \textit{in vivo} proliferation of MDV, as shown by the post-challenge viraemia levels in peripheral blood lymphocytes of GX0101-challenged chickens (Fig. 2).

Different levels of expression among the early expressed miRNAs were also found (Figs 3a and 4a). In relative terms, the \textit{in vivo} expression levels of miR-M3-5p and miR-M8-3p were higher than those of miR-M7-5p, M12-3p and M4-5p, whereas M1-5p showed the lowest level of expression (Figs 3b and 4b). The early expressed miRNAs showed a typical tissue-specific expression pattern in spleen and lung at the earlier stages of infection (18 and 36 days p.i.), were most abundantly expressed in spleen in chickens showing only
**Fig. 3.** In vivo expression profiles for Meq-clustered mature miRNAs (mat-miRNAs). (a) Northern blot analysis. Expression of each Meq-clustered mat-miRNA in two individual chickens randomly selected for each time point post-infection were detected separately. Only one representative chicken is shown here because of limited space. Tissue U6 snRNA served as the reference control. MK, DNA markers of 70, 24 and 22 nt were used to indicate the position of miRNA precursors or mat-miRNAs. (b) Relative tissue expression levels of Meq-clustered mat-miRNAs. Relative expression level, expressed as the ratio of Northern blot signal intensities of each mat-miRNA to U6 snRNA signal intensity, was quantified using the bio-software Quantity One. Means of the relative abundance of each mat-miRNA expressed in the same tissues of two chickens tested at each time point post-infection are shown. Ch #, chicken number; Ch #5/6-90 days p.i., diseased birds showing gross tumours only in spleen, Ch #7/8-90 days p.i., tumours in spleen and liver; Ch #9/10-90 days p.i., tumours in multiple visceral organs including spleen and liver together with proventriculus, respectively.
splenic tumours (chickens 5 and 6) or both splenic and liver tumours (chickens 7 and 8) at early stages of tumorigenesis, and then universally expressed in all the other organs, irrespective of the presence of gross tumours in multiple visceral organs (chickens 9 and 10). No tumour-specific miRNAs encoded by MDV-1 were observed at the late stages of tumorigenesis (90 days p.i.). In relative terms, the early expressed miRNAs showed generally higher expression levels in spleen, thymus and bursa of Fabricius than in heart, liver, lung, kidney and proventriculus at the late stages of tumorigenesis. The thymus gave the highest expression levels of early expressed miRNAs among the examined immune organs, as well as the lung, among the other tested visceral organs from chickens 9 and 10.

Compared with the early expressed miRNAs, the late-expressed miRNAs can only be detected at low levels at 90 days p.i., the late stages of MDV infection (Figs 3b and 4b). The late-expressed miRNAs showed a similar distribution to those of early expressed miRNAs, being most abundantly expressed in spleens of chickens showing only splenic tumours (chickens 5 and 6) or both splenic and liver tumours (chickens 7 and 8) at early stages of tumorigenesis, and then universally expressed in all the
Fig. 4. *In vivo* expression profiles of LAT- and mid-clustered mature miRNAs (mat-miRNAs). (a) Northern blot analysis. Expression of each LAT- or mid-clustered mat-miRNA in two individual chickens randomly selected for each time point post-infection were detected separately. Only one representative chicken is shown here because of space limitations. MK, DNA markers of 70, 24 and 22 nt were used to indicate the positions of miRNA precursors or mat-miRNAs. Tissue U6 snRNA served as the reference control. (b) Relative tissue expression levels of LAT- and mid-clustered mat-miRNAs. Relative expression levels of each mat-miRNA, expressed as the ratio of their Northern blot signal intensities to that of U6 snRNA, were quantified using the bio-software Quantity One. Means of relative abundance for each mat-miRNA expressed in the same tissues of two chickens tested at each time point post-infection are shown. Ch #, chicken number; Ch #5/#6-90 days p.i., diseased birds showing gross tumours only in spleen; Ch #7/#8-90 days p.i., tumours in spleen and liver; Ch #9/#10-90 days p.i., tumours in multiple visceral organs including spleen and liver together with proventriculus, respectively.
other organs at late stages of tumorigenesis (chickens 9 and 10). Among the visceral organs including heart, liver, lung, kidney and proventriculus and similarly to the early expressed miRNAs, the late-expressed miRNAs were most highly expressed in lung at late stages of tumorigenesis (chickens 9 and 10). However, in contrast to the early expressed miRNAs, the spleen, rather than the thymus, gave the highest expression levels for late-expressed miRNAs among the investigated immune organs. Whether the early expressed and late-expressed miRNAs encoded by MDV-1 are involved in different aspects of MDV pathogenesis and/or oncogenesis remains unknown.

DISCUSSION

Generally, biogenesis of miRNAs is initiated from primary transcripts which are processed into pre-miRNAs, then further processed to generate double-stranded miRNA duplexes before incorporation into the RNA-induced silencing complex to provide regulatory functions (Bartel, 2004). In most cases, only one strand of the miRNA duplexes serves as functional miRNA while the other strand is a ‘passenger’ miRNA. Occasionally, both strands may be functional (Bartel, 2004; Filipowicz et al., 2008). We found that both strands of miR-M2 were stably expressed at moderate levels during the late stages of disease, as previously described (Burnside et al., 2006; Yao et al., 2008). This is the only case among the MDV-1-encoded miRNAs of miRNA duplexes in which both strands were found to stably exist in vivo. However, in the present work, of the miRNAs (miR-M1-3p, M4-3p, M6-3p, M8-5p and M9-3p) previously reported to have low levels of expression (Burnside et al., 2006; Morgan et al., 2008; Yao et al., 2008), none were detected as mat-miRNAs at any stage of the disease caused by GX0101. This indicates that these five miRNAs are passenger miRNAs. Our results also showed that although pre-miRNAs of miR-M5-5p, M5-3p, M11-5p, M11-3p and M13-3p were effectively transcribed and processed, miRNAs derived from any strand of the hairpin structures have not been stably detected by Northern blot analysis.

According to the in vivo expression profiles, the total of 12 mat-miRNAs represented two groups, early or late-expressed miRNAs, with differing temporal and tissue expression patterns. The early expressed miRNAs were found to be expressed as early as 18 days p.i. in vivo, and then their expression globally and persistently increased during disease progression (from 36 days p.i. to the distinct stages of tumorigenesis observed at 90 days p.i.). However, the proliferation of MDV in GX0101-challenged chickens reached a peak at about 28 days p.i. and then declined and was maintained at a lower level for the remainder of the experimental period (from 36 to 90 days p.i.). This indicates that the increased expression of early expressed miRNAs has no obvious correlation to the proliferation and amount of MDV, but is possibly enhanced by their own gene expression regulatory elements. Contrary to the early expressed miRNAs showing increasing expression levels accompanying the time-course of disease, expression of the late-expressed miRNAs seems to correlate with macroscopic tumour appearance because all of them can only be detected at the late stages of tumour generation (90 days p.i.) rather than the late cytolytic and immunosuppressive phase (18 days p.i.) and the proliferative phase (36 days p.i.). Usually, at about 3 weeks post-infection, the latently infected lymphocytes are transformed into lymphoblastoid tumour cells. At 3–4 weeks post-infection, non-productive infected lymphocytes progressively migrate into the visceral organs and peripheral nerves, where they proliferate under the influence of unknown factors to form lymphomas (Baigent & Davison, 2004). Whether or not these two distinct groups of MDV-1-encoded miRNAs are involved in the regulation of this disease progression deserves further study.

MDV-1-encoded miRNAs are all located in the TR/IR regions, which are generally active in latently infected and tumour cells (Lee et al., 1975; Sugaya et al., 1990; Jones et al., 1992). The more abundant expression of MDV-1-encoded miRNAs in T-lymphoma cell lines and tumours compared with MDV-1-infected CEFs, suggested that some or all of them may play regulatory roles in MDV oncogenesis (Burnside et al., 2006; Yao et al., 2008). Some researchers have speculated that Meq-clustered miRNAs may play key roles in MDV pathogenesis because of their higher expression levels in vv+ MDV-induced tumours than those caused by vv MDV (Morgan et al., 2008). However, we found that both the late-expressed and early expressed miRNAs simultaneously exist in all three distinct gene clusters, suggesting that they have differing functional roles even though they are encoded in the same gene cluster. Interestingly, miR-M4-5p is one of the early expressed RNAs, has recently been characterized as a functional orthologue of miR-155, and possibly regulates the lymphoid malignancy and immune responses (Zhao et al., 2009). Another early expressed miRNA, miR-M3-5p, has recently been reported to significantly suppress cisplatin-induced apoptosis of DF-1 cells by directly downregulating the translation of Smad2, a critical component of the TGF-β signalling pathway (Xu et al., 2010). Considering that our present work also revealed that miR-M3-5p is early expressed and has the highest abundance during the whole course of disease in MDV-1-challenged hosts, it is possible that a portion of early expressed miRNAs may target cellular factors involved in antiviral responses, creating a cellular environment beneficial for viral latency, replication and/or oncogenesis. Whether all or some of these early expressed miRNAs have a similar function to miR-M4-5p or miR-M3-5p remains unknown.

Notably, the mid-clustered miRNAs showed the lowest expression levels in vivo compared with those located in the Meq- or LAT-clusters. Indeed, because of its low expression miR-M1-5p has previously been suggested to be non-essential in MDV pathogenesis (Burnside et al., 2006).
While the expression level of miR-M11 is low in MSB-1 cells (Yao et al., 2008), expression of miRNAs derived from any strand of its pre-miRNA was not detected in the present study. However, another of the mid-cluster miRNAs (miR-M32-3p) was expressed late in association with tumorigenesis at the lowest level of expression. This miRNA has recently been suggested to share a seed sequence with host miRNA miR-221 and possibly participates in the growth and proliferation of cancer cells (Morgan et al., 2010). Additionally, significant differential expression in vivo was also observed among LAT-clustered miRNAs, with miR-M7-3p and M8-3p being expressed early and in abundance, whereas miR-M6-5p and miR-M10-3p could only be detected at tumorigenic stages. The biological significance of these miRNAs needs further study.

MDV-1 is one of the oncogenic herpesviruses and provided the first demonstration of the efficacy of antiviral vaccination in the control of cancer in any species. As a consequence, experimental MDV infection provides an ideal model for investigating the possible regulatory roles of virus-encoded miRNAs in the biology, genetics and immunology of tumorigenesis. Presently, we have used MDV-GX0101 (Cui et al., 2010), a field-isolated strain of MDV-1 prevalent in South China that contains an LTR fragment of reticuloendotheliosis virus (REV) integrated into its genome, to establish a lymphoma model for investigating the in vivo expression profiles of MDV-1-encoded miRNAs. Although whether or not REV-LTR insertion affects the in vivo expression of MDV-1-encoded miRNAs is unknown as yet, our research may provide meaningful clues for further evaluating their functions, especially for the twelve miRNAs highlighted in this study. Based on the successful establishment of strategies for miRNA target prediction and experimental identification (Kuhn et al., 2008; Alexiou et al., 2009; Bartel, 2009), and the application of bacterial artificial chromosomes in research on MD pathogenesis (Schumacher et al., 2000; Petherbridge et al., 2003, 2004; Sun et al., 2009, 2010), focusing on those miRNAs shown to be expressed in vivo in this study should reveal more about the biological functions of MDV-1-encoded miRNAs.

**METHODS**

**Virus and cells.** This study used GX0101, a field strain of MDV-1 isolated from an egg production unit in Guangxi province of South China that contains an LTR fragment of reticuloendotheliosis virus (REV-LTR) in its genome (Cui et al., 2010). Virus was propagated in CEFs, harvested and stored in liquid nitrogen until used. Virus titre was measured using CEF monolayer cultures in 96-well plates. Briefly, a virus sample was serially diluted twofold in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% FBS (Gibco). The supernatant of CEF cells, maintained in 96-well plates, was removed, and then 100 μl of each dilution was transferred to eight wells. An equal volume of dilution medium was added to the last column to serve as a control. The plates were incubated at 37 °C in a 5% CO₂ incubator for 4 days prior to reading the end point by plaque formation. Virus titres (p.f.u. ml⁻¹) were conventionally calculated by multiplying the mean number of p.f.u. with the dilution factor of the highest dilution.

**Animal experiments.** One-day-old white leghorn specific-pathogen-free (SPF) chickens (Jinan SPF Egg & Poultry Co.) were used for the animal experiments. Birds were randomly divided into two groups and housed in isolators supplied with air under positive pressure filtration. For the experimental group, a total of 100 birds were each challenged with 1000 p.f.u. of GX0101 virus via abdominal cavity inoculation. Another group of 30 birds inoculated with an equal volume of normal CEFs served as negative controls. Post-infection, birds were inspected regularly for any clinical symptoms and mortality. At the end of 90 days, all surviving birds were humanely euthanized and examined for lesions at necropsy. The rates of cumulative mortality and tumour generation were used to evaluate the pathogenicity of the GX0101 virus.

**Determination of the viraemia.** To ensure that infection was successful, the level of viraemia in peripheral blood lymphocytes of GX0101-infected chickens was monitored for the duration of the study. Blood samples were collected in anticoagulant from ten randomly selected birds from the virus infected or uninfected groups at 7, 14, 21, 28, 35, 60 and 90 days p.i. One milliliter of blood in anticoagulant was mixed with 9 ml of DMEM medium and centrifuged at 500 x g for 5 min. White blood cells were collected and inoculated into two duplicate 35 mm plates containing confluent CEF monolayers. After 6 days, viral plaques were counted by indirect immunofluorescence assay (IFA) using mAb H19 as previously described with modifications (Lee et al., 1983). Briefly, infected cells were washed with PBS (pH 7.2) and fixed with ethanol/acetone (4:6, v/v) at room temperature for 10 min. After removal of the fixative, the cells were air-dried, rehydrated and then incubated with mAb H19 (1:1000) for 1 h at 37 °C. Following three washes, the cells were incubated with FITC-labelled goat anti-mouse secondary antibodies (Sigma) for 1 h. After washing, the viral plaques were counted under a fluorescence microscope.

**Extraction of total RNA from tissue samples.** For the detection of MDV-1-encoded miRNAs, tissue samples (heart, liver, spleen, lung, kidney, proventriculus, thymus, and bursa of Fabricius) were collected from randomly selected birds at 18, 36 and 90 days p.i. The tissues were ground in liquid nitrogen and total RNA extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Concentrations of the extracted total RNA were determined using a NanoDrop ND-1000 spectrometer (NanoDrop Technologies) and the integrity of the RNA was assessed using denaturing agarose-gel electrophoresis.

**Small RNA enrichment.** To improve the sensitivity of detection for miRNAs, low-molecular-mass RNAs <200 nt in the extracts were enriched by polyethylene glycol (PEG) precipitation. Briefly, approximately 1 mg of tissue total RNA was diluted in 400 μl diethyl pyrocarbonate (DEPC)-treated water, and mixed with 50 μl of 50% (v/v) PEG-8000 (Sigma) and 50 μl of 5 M NaCl solution, kept on ice for 2 h, and then centrifuged at 8000 x g for 10 min at 4 °C. The supernatants were transferred to new tubes for phenol extraction and ethanol precipitation. Finally, the small RNAs were redissolved in DEPC-treated water. The concentration of the enriched small RNAs was measured as described above and they were stored at −70 °C until used.

**Oligonucleotides.** According to the sequences of previously reported MDV-1-encoded miRNAs, a total of 22 antisense oligonucleotide probes listed in Table 1 were designed for Northern blot analysis using the bio-software Primer Premier 5.5 (Premier). Oligonucleotides were synthesized and purified by Sangon Co. (Shanghai, China). The probe 5'-GCTAATCTTCGTGTATCGTTC-3', for detecting the chicken U6 snRNA, was used as a reference control.
Northern blot analysis. Northern blot analysis was performed as previously described (Wang et al., 2004), with some modifications. Briefly, a 10 μg aliquot of low-molecular-mass RNAs from each tissue was size-fractionated by using denaturing 12% polyacrylamide/8 M urea gel electrophoresis (PAGE) and then electrophotoretherally transferred on to Hybond-N+ membranes (Amersham Biosciences) using a semi-dry blotting apparatus (Bio-Rad), followed by UV cross-linking for 3 min (1200 mJ, Stratalinker; Stratagene). DNA oligonucleotides complementary to the MDV-miRNAs were 5’-end labelled with γ-32P-ATP (Yahui Co. Beijing) using T4 Polynucleotide Kinase (TaKaRa). The membranes were prehybridized for at least 1 h in hybridization buffer (750 mM NaCl, 750 mM Na2HPO4•2H2O (trisodium citrate dihydrate), 20 mM Na2HPO4•12H2O (pH 7.2), 7% SDS, 0.2% Ficoll 400, 0.04% polyvinylpyrrolidone and 0.04% BSA, pH 7.0) and then hybridized with γ-32P-ATP-labelled oligonucleotides overnight at 42 °C. After being washed three times with 1× SSPE (10 mM phosphate buffer, pH 7.4, 149 mM NaCl, 1 mM EDTA) containing 0.1% SDS at room temperature, the membranes were exposed to a phosphor screen for 24 h. Finally, the hybridization results were visualized by using a Storm 820 phosphorimager (GE Healthcare). Based on the Northern blot signals, the relative expression of each miRNA was obtained from the ratio of intensities of MDV-1-miRNAs to that of chicken U6 snRNA using the bio-software Quantity One (Bio-Rad).

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