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MicroRNAs as biomarkers in the host response to influenza A
virus infection in humans and animals

THESIS

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To my parents, sisters and wife
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Summary

“MicroRNAs as biomarkers in the host response to influenza A virus infection in humans and animals”

Mohamed Samir Ahmed Mohamed

Influenza A virus (IAV) is a negative sense, single-stranded RNA virus that belongs to the family of Orthomyxoviridae. In addition to the seasonal epidemics, it caused three human pandemics in the last century (1918 A/H1N1, 1957 A/H2N2, and 1968 A/H3N2). In 2009, the world was confronted with the fourth pandemic caused by a triple reassortant H1N1 virus. The highly pathogenic avian influenza (HPAI) viruses of the subtype H5N1 are unique in that they are highly lethal to birds and are able to cross the species barrier and infect humans. MicroRNAs (miRNAs) are small non-coding RNAs (sncRNAs) that are able to fine-tune gene expression. The modulation of miRNAs under pathological conditions raises the possibilities of utilizing them as host biomarkers to pinpoint the disease in question and track its progress. This applies similarly to IAV at the farm level, where these small molecules might aid sensing the very early cases before the outbreaks spread widely.

The objectives of my thesis were to investigate the importance of miRNAs as components of the host response to IAVs in humans and animals and to identify candidate miRNAs that might be used as future biomarkers.

Before starting the experiments, I performed a literature search with the aim to review the up-to-date known roles of host-encoded miRNAs in infectious viral diseases of veterinary importance and the potential of using small non-coding RNAs to limit and/or prevent infectious viral diseases in livestock of veterinary importance. This resulted in two review articles (Manuscript I and II).

In the first part of the experimental work (section 4.1 in Unpublished Data), I used reverse transcriptase real-time PCR (RT-qPCR) to validate the results of next generation sequencing (NGS; specifically, small RNA sequencing, small RNA-seq) with regard to the relative expression levels of a set of miRNAs. Those miRNAs have been previously identified by Dr. Matthias Preuße (Helmholtz Center for Infection
Research) to be associated with susceptibility to PR8 H1N1 in murine system. As a result, both approaches agreed to a high degree, indicating that RT-qPCR is a valid method to confirm the results of RNA-seq.

In the second part (Manuscript III), I established in vitro assays comprising human cell lines to study the cell type-specific expression of miRNAs following stimulation with toll-like receptor (TLR) agonists and after infection with 2009 swine-origin influenza virus wild type (S-OIV wt) and S-OIV NS PR8, a reassortant variant of it that carries the NS segment of the PR8 H1N1 virus. The findings indicated that S-OIV NS PR8 triggered a cell-dependent alteration of certain miRNA (e.g. miR-223 and miR-155) as compared to the S-OIV wt. The data also show that while the infection with both viruses caused higher expression of miRNAs in human lung epithelial (A549) than in macrophage-like (dTHP-1) cell lines, the TLR agonists (LPS and R848) displayed the reverse pattern.

In the third part of my thesis (Manuscripts IV and V), I aimed to analyze the miRNA responses to the HPAI virus H5N1 in a natural host. Therefore, a native duck breed (derived from Anas platyrhynchos) was experimentally infected with A/chicken/Faqous/amn12/2011(H5N1) virus, an Egyptian isolate that, according to my phylogenetic analyses, belongs to the recently emerging clade 2.2.1.2. The initial viral characterization revealed that brain, compared to other organs notably lung, exhibited much lower viral transcription, growth rate and associated tissue-pathology. As these results suggested an organ-specific host response to HPAI H5N1 virus infection, small RNA-seq. was employed to test the hypothesis if there is a unique miRNA response in these organs. As a result, the overall degree of miRNA reprogramming was higher and manifested earlier in lung than in brain. In both organs, the magnitude of the miRNA response reached the peak during the late infection stage (72-120 hpi), following the peak of viral transcription, arguing that the miRNA reprogramming is part of the host tissue response to viral transcription and, consequently, replication. I also identified common and organ-specific miRNAs that have a characteristic clustering pattern, including miR-183, miR-194, miR-205 and miR-215. Generally, gene ontologies such as T-helper cell differentiation and Positive regulation of JUN kinase activity were commonly enriched in both organs. Although certain pathways were enriched in both organs such as MAPK and Jak-
STAT signaling pathways, their enrichment degree was higher in lung than in brain. Considered together, these results propose the involvement of miRNAs in the organ-specific response to HPAI H5N1 and that their regulatory roles might shape the replication of the virus in different duck tissues. Furthermore, this study suggests possibilities for the future use of miRNAs as candidate biomarkers in IAV infection. In the last part of my thesis (section 4.2 in Unpublished Data), I established a human lung tissue explant (HLTE) model to study the impact of single reassortment of IAV NS segment on viral replication and differential host miRNA expression in a human tissue ex vivo. This approach might narrow the gap between current infection models and the actual human infection. For this purpose, I used the two viruses that I mentioned in Manuscript III (S-OIV wt and the S-OIV NS PR8). The initial establishment indicated that the HLTE model could support the replication of both viruses. However, the NS reassortment augmented viral transcription, especially at 8 and 48 hpi. This was associated with increased IFN-α mRNA and protein levels. It was also found that S-OIV NS PR8 caused a consistent higher degree of endothelial damage and epithelial delamination when compared to the S-OIV wt.

In summary, my results suggest that miRNAs are key players in the host response to various IAV strains. They exhibit cell type- and organ-specific expression and possibly function. Studying the miRNA response to HPAI H5N1 in ducks, a natural host, will help to identify key processes in disease susceptibility and, possibly, transmission to humans and further underscoring the potential value of miRNAs as host biomarkers in IAV infection.
Deutsche Zusammenfassung

“MicroRNAs als Biomarker der Wirtszellantwort auf Influenza A Infektion in Menschen und Tieren”

Mohamed Samir Ahmed Mohamed


Meine Doktorarbeit zielte darauf ab, die Bedeutung der miRNAs in der Wirtszellantwort auf IAV in Menschen und Tieren zu untersuchen und bestimmte miRNAs zu identifizieren, die als potentielle Biomarker nützen könnten. Vor den ersten Experimenten habe ich eine ausgiebige Literatursuche gemacht, um einen Überblick über die aktuell bekannte Rolle der Wirts-miRNAs in viralen Infektionskrankheiten, die für die Veterinärmedizin bedeutend sind, zu bekommen. Ein weiteres Ziel der Literaturrecherche war, Perspektiven und Hindernisse in dem Gebrauch von sncRNAs, insbesondere miRNAs, zur Kontrolle und Prävention von viralen Krankheitsausbrüchen mit Auswirkungen auf die Nutztierhaltung zu
identifizieren. Diese Recherchen ergaben zwei Übersichtsarikel (Manuscript I und Manuscript II).

In dem ersten Teil der experimentellen Arbeit (Abschnitt 4.1 in Unpublished data) habe ich mittels Reverse Transkriptase Echtzeit-PCR (RT-qPCR) die durch Sequenzierungstechnologie der Nächsten Generation (next generation sequencing, NGS) gewonnenen Daten überprüft, und zwar in Bezug auf die relativen Expressionsspiegel eines ausgesuchten Sets von miRNAs. Diese ausgewählten miRNAs wurden zuvor von Dr. Matthias Preuß (Helmholtz Zentrum für Infektionskrankheiten) im Zusammenhang mit einer Krankheitsempfänglichkeit gegenüber PR8 H1N1 in Mäusen identifiziert. Das Ergebnis war eine hohe Übereinstimmung der beiden Herangehensweisen, was dafür spricht, dass RT-qPCR eine geeignete Methode ist, um NGS-Ergebnisse zu bestätigen.

organspezifische Wirtsantwort auf HPAI H5N1 Infektion gibt, wurde nun NGS (small RNA sequencing, small RNA-seq) eingesetzt, um die Hypothese zu testen, dass in diesen Organen spezifische miRNA-Antworten nachzuweisen sind. Als Ergebnis dieser Untersuchung war zu sehen, dass in den Lungen die miRNA Umprogrammierung insgesamt stärker ausfiel und früher stattfand, als im Gehirn. Der Maximalwert der miRNA-Antwort wurde in beiden Organen im späten Infektionsstadium (72-120 hpi) erreicht, nach dem Höchstwert der viralen Transkription. Daraus lässt sich schließen, dass die miRNA-Umprogrammierung einen Teil der Wirtsantwort im Gewebe auf die virale Transkription, die Replikation, darstellt.


weiteres Ergebnis waren verstärkte Endothelzellschäden und Epithelzellablösungen durch S-OIV NS PR8, verglichen mit dem S-OIV wt.

Zusammengefasst lassen meine Ergebnisse darauf schließen, dass miRNAs eine Schlüsselrolle in der Wirtsantwort auf unterschiedliche IAV Typen spielen. Sie weisen zellart- und organspezifische Expression und Funktionen auf. Die Untersuchung der miRNA-Antwort auf HPAI H5N1 in einem natürlichen Wirt, d.h. der Ente, ist hilfreich, um Schlüsselprozesse von Krankheitsempfänglichkeit und Übertragbarkeit auf Menschen zu identifizieren. Des Weiteren weisen die Ergebnisse auf die vielversprechende Rolle der miRNAs als wirtsbezogene Biomarker bei IAV Infektionen hin.
1. Introduction

1.1. Influenza A virus (IAV)

1.1.1. Epidemiology and significance of IAVs

Infection with seasonal influenza viruses is estimated to cause 25000-50000 annual deaths per year worldwide. In addition to several outbreaks, 5 human IAV pandemics have been reported (Fig. 1). The pandemic potential of IAV has been mainly linked to its efficient inter-human transmission [5]. After 1-2 days of infection, the symptoms of IAV present as chills, fever, loss of appetite and aches. Diarrhea is not a common symptom in IAV cases. However, it has been reported in few H5N1 cases [6]. Seasonal and low pathogenic IAVs often cause upper respiratory tract symptoms with low fatality rates, whereas the highly pathogenic strains (e.g. H5N1) can cause severe disease, possibly due to the preference to infect the lower respiratory tract [5].

Figure 1. Influenza A virus pandemics throughout the history. So far, five IAV pandemics have been documented. Pandemics, as depicted here, are the ones that occurred over a large geographical area and involved multiple countries. N/A: non available. Adapted from [2]. Copyright, Cell Press.
1.1.2. Morphology of IAVs

Influenza A viruses belong to the family *Orthomyxoviridae*, which is classified into 3 different genera: A, B and C. Influenza A viruses (IAVs) are capable of infecting a wide variety of mammals and birds. Influenza B and C viruses preferentially infect humans with very low infection rate in animals [7]. The morphology of IAV is shown in fig. 2. Under the electron microscope, IAV appears pleomorphic (spherical; 120 nm diameter and filamentous; 300 nm in length). The virus has a negative sense genome (≈13,600 nucleotide) that is divided into 8 segments [8]. These 8 segments encode 11 proteins (Table 1). The virus lipid envelope has two exposed viral proteins, hemagglutinin (HA) and neuraminidase (NA). HA represents ≈ 80% of the envelope proteins, whereas NA accounts for 17%. The matrix protein M2, an ion channel transmembrane protein (16-20 particle per virion) [9], is located underneath the envelope. The M1 protein represents the internal lining of the whole virion sphere and constitutes the bridge between the viral envelope and the viral ribonucleoproteins (vRNPs). The vRNP complex consists of the 8 viral RNA segments coated with the nucleoprotein (NP) together with the polymerase complex (PB1, PB2 and PA). A small amount of NS2 protein is also present in the vRNP complex [10]. All viral segments have 5′ and 3′ untranslated regions (UTRs) of variable length. The last 13 (AGUAGAAAACAAGG) and 12 (UCG(U/C)UUUCGUCC) nucleotides (nt) of the 5′ and 3′ ends, respectively, are highly conserved among IAV strains [11].

*Figure 2. Schematic representation showing the morphology of IAV.* Description of the figure can be found in the text above. Adapted from [3]. Copyright, Nature Publishing Group.
<table>
<thead>
<tr>
<th>Viral segment</th>
<th>Length (nts)</th>
<th>Length (a.a.)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymerase basic protein 2 (PB2)</td>
<td>2341</td>
<td>759</td>
<td>mRNA cap recognition</td>
</tr>
<tr>
<td>Polymerase basic protein 1 (PB1)</td>
<td>2341</td>
<td>PB1 → 757</td>
<td>PB1: RNA elongation, endonuclease activity</td>
</tr>
<tr>
<td>PB1-F2</td>
<td></td>
<td>PB1-F2 → 87</td>
<td>PB1-F2: Pro apoptotic activity</td>
</tr>
<tr>
<td>Polymerase acidic (PA)</td>
<td>2233</td>
<td>716</td>
<td>Protease activity</td>
</tr>
<tr>
<td>Hemagglutinin (HA)</td>
<td>1778</td>
<td>550</td>
<td>Receptor binding</td>
</tr>
<tr>
<td>Nucleoprotein (NP)</td>
<td>1565</td>
<td>454</td>
<td>Nuclear import regulation</td>
</tr>
<tr>
<td>Neuraminidase (NA)</td>
<td>1431</td>
<td>454</td>
<td>Virus release from the cells</td>
</tr>
<tr>
<td>Matrix protein 1 (M1)</td>
<td>1027</td>
<td>M1 → 252</td>
<td>M1: Virus budding, nuclear export</td>
</tr>
<tr>
<td>Matrix protein 2 (M2)</td>
<td></td>
<td>M2 → 97</td>
<td>M2: Uncoating</td>
</tr>
<tr>
<td>Non-structural protein (NS1)</td>
<td>890</td>
<td>NS1→230</td>
<td>NS1: Interferon antagonist</td>
</tr>
<tr>
<td>Non-structural protein or nuclear export protein (NS2/NEP)</td>
<td></td>
<td>NS2→121</td>
<td>NS: Nuclear export of RNA</td>
</tr>
</tbody>
</table>

1 The lengths of the nucleotide and protein sequences are approximate, as they differ among IAV strains. Abbreviations: nts, nucleotides; a.a., amino acids.

1.1.3. IAV replication cycle

According to the HA and NA proteins, IAV strains are serologically classified into many subtypes. The HA has 16 subtypes (H1–H16) and NA has 9 subtypes (N1–N9). Additionally, avian influenza viruses can be classified according to the induced pathology in the natural host into low pathogenic avian influenza (LPAV) and high pathogenic avian influenza (HPAI) viruses.

The life cycle of IAV is summarized in Fig. 3. The infection starts with inhalation of viral particles, which then land on the mucus lining of the upper respiratory tract. Those virions which escape the clearance mechanisms bind to a sialic acid receptor (α-2,3-linkage in animals or α-2,6-linkage in humans) through the HA protein [12]. In ≈ 20 min after infection, the viral particles are endocytosed and enclosed within the endosome. The internal acidification of the endosome promotes conformational changes in the viral HA protein that allow its adhesion to the inner endosome.
membrane. In parallel, the interior of the virus becomes acidified via the activity of the M2 protein. The low pH inside the virus assists in the dissociation of the M1 protein from the vRNPs, which are then released into the cytoplasm. The nuclear localization signals (NLSs) that are present within the NP, PB1, PB2 and PA segments mediate their binding to nucleus importin-α and -β proteins, which facilitate their trafficking to the nucleus, where IAV transcription and replication take place. Transcription starts with the activity of RNA-dependent RNA polymerases (PB1 and PB2). The virus does not provide the primer needed for this process. Instead, a process called “cap snatching” initiates in which PB2 recognizes the cellular 5’ mRNA cap (1–13 nts) and then PA, by its endonuclease activity, “steals” this cap. The PB1 mediates the transcription, which produces a population of capped and polyadenylated + sense viral mRNA (vmRNA). HA, NA, NP, PB1, PB2 and PA are transcribed in a monocistronic manner. By contrast, the M and NS segments are transcribed by splicing in a polycistronic manner. In the cytoplasm, the 6 monocistronic vmRNA are translated to HA, NA, NP, PB1, PB2 and PA proteins, whereas the other 2 vmRNA segments give rise to M1, M2, NS1 and NS2 proteins. The first synthesized viral proteins are HA, NA and M2, which, by the help of apical signals, are directed to the cell surface waiting for the virus assembly. The other proteins (M1, PA, PB1, PB2, NP and NS2) are redirected to the nucleus where they are required for either replication or nuclear export of newly synthesized vmRNA. Replication of the virus is mediated by the polymerases, which act on the viral RNA (vRNA) to produce a + sense RNA strand (cRNA) as a template for producing more – sense virus progeny. The switch of polymerases from vmRNA synthesis (transcription) to cRNA and vRNA synthesis (replication) is believed to be mediated through virally encoded small RNAs [13]. Later in infection, M1 promotes the recruitment of vRNPs to the apical part of the cells where packaging of the viral particles occurs, followed by their release from the cells via the activity of NA [8, 9, 14]. The whole IAV cycle takes place within 5–8 hours (h). With successive replication cycles, the virus titer peaks around 48–72 hours post infection (hpi). Usually, the infection resolves in 5–7 days post infection (dpi) with complete epithelial regeneration taking place within 1 month. Generally, the virus can be isolated between 1–7 dpi [15].
1.1.4. Host factors involved in IAV infection

The infected cells can sense the viral RNA via multiple pattern recognition receptors (PRRs) such as toll-like receptor (TLR) 7 and retinoic acid-inducible gene-1 (RIG-1) [16, 17]. Binding of viral genome to these receptors leads to production of interferon and activation of nuclear factor κ-B (NF-κB). This, in turn, promotes the production of inflammatory chemokine and cytokine.

1.1.4.1. Chemokines

Upon IAV infection, chemokines are elicited from virus-infected cells and bind their corresponding receptors on leukocytes to help their recruitment to the site of inflammation [18]. IAV-infected epithelial cells produce IL-8, regulated on activation normal T cells expressed and secreted (RANTES or CCL5), and monocyte chemotactic protein-1 (MCP-1 or CCL2). IAV-infected monocytes/macrophages
produce macrophage inflammatory protein-1α (MIP-1α), MIP-1β, MIP-3α, monocyte chemotactic protein-1 (MCP-1), RANTES, MCP-3 and CXCL10) [19, 20].

### 1.1.4.2. Cytokines

Type I Interferon (IFN-α/β) represents the most commonly induced cytokine upon IAV infection. Compared to macrophages and dendritic cells, the lung epithelium is a poor producer of IFN-α/β. Upon binding to their cognate receptor (IFNAR) on the cell surface, the downstream factors Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) are activated and phosphorylate the IFNAR, leading to the recruitment of signal transducer and activator of transcription (STAT) proteins followed by their phosphorylation, dimerization and translocation to the nucleus. Different STAT proteins define the resultant state. Dimerization of STAT1, STAT2 and the interferon regulatory factor 9 (IRF9) results in the formation of interferon-stimulated gene factor 3 (ISGF3), which produces an anti-viral response, most commonly by the production of interferon stimulated genes (ISGs). On the other hand, the STAT1 homodimer binds to the gamma-activated sequences (GASs), leading to a proinflammatory response. The STAT3 homodimer can lead to repression of inflammation [21].

Infection with IAV is characterized by multi-cellular interactions. The proinflammatory cytokines IL-6, IL-1β and TNF-α are mostly produced by infected macrophages [22, 23], whereas the anti-inflammatory homeostatic counter-regulatory mechanisms of the lung are mediated through a variety of mechanisms. Among them is the interaction of CD200, a surface glycoprotein on epithelial cells, with CD200 R, a receptor on macrophages and dendritic cells [24]. Their interaction is known to inhibit the recruitment of immune cells, TNF-α and IL-6 production, and thus confers an anti-inflammatory state [25]. IL-10, which is produced from CD8+ T cells under the effect of IL-2 (produced from CD4+ T cells) and IL-27 (produced from macrophages), is considered to be another anti-inflammatory factor [26, 27]. IL-18 together with IFNα/β produced from macrophages triggers the production of IFN-γ from natural killer cells (NK) and the development of a Th1-type immune response [28].
1.1.5. Host range of IAVs

IAVs have been circulating in a wide variety of mammalian species including humans, and in birds. The mammalian-adapted viruses or some of their genes have their origin in viruses that were maintained in aquatic birds [29]. For IAVs to be endemic, they need time to adapt and colonize a new host. Any combination of the 16 HA (H1–H16) and 9 NA (N1–N9) subtypes can be found and co-circulated among aquatic and wild migratory birds. Equines have been reported to be infected with H3 and H7 subtypes [30, 31]. H3N8 and H3N2 have been identified in dogs [32, 33]. H1 and H3 subtypes have been transmitted among pigs, which constitute mixing vessels for IAV strains [34, 35]. While the human population has experienced several infection events with H1, H2 and H3 subtypes, H5N1 can sporadically infect humans, but its capacity to sustain inter-human transmission is limited [36]. Fortunately, this host range barrier remains largely intact, thus protecting the human population from widespread infection with H5N1 viruses. However, if this barrier ever fails, a pandemic with potentially high mortality could be the result [37]. Hosts and laboratory models of IAVs are shown in Table 2.

In contrast to chicken and turkey, duck has traditionally been considered resistant to H5N1 infection. This implies that they exhibit mild diseases. However, in late 2002, a shift in the ecology of H5N1 virus in ducks has been reported in that certain H5N1 isolates became lethal in this species [38, 39]. Outbreaks of HPAI H5N1 have been reported in wild birds in western China [40, 41] and Korea [42, 43]. In parallel, in vivo experiments reflected the same pattern for post-2002 H5N1 isolates [44-46]. The virulence of H5N1 HPAI viruses is a multifactorial feature. Understanding of the host response in the natural hosts is important for more than one reason. First, it would allow unraveling the complex disease mechanism and elements of the immune system that restrict zoonotic transmission of HPAI viruses. Second, it would assist in developing novel diagnostics and devise more efficient control measures in the natural hosts. Third, it would enable further comparative analyses of the host response between the natural reservoir and the spillover hosts. In this regard, researchers have paid great attention to study the host gene response and viral virulence factors. Of note, in recent years increasing attention has been paid to the
contributions of microRNAs (miRNAs) in the host response in various aspects of IAV infection.

### Table 2. Host and laboratory models of IAVs.

<table>
<thead>
<tr>
<th>Host Categories</th>
<th>Type of host</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Natural</td>
<td>Spillover</td>
</tr>
<tr>
<td>Wild birds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aquatic (gull, gadwall, common</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>teal, etc.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbits (eagles, falcon, great</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>horned owl, etc.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Domestic animals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avian (chicken, domestic duck,</td>
<td></td>
<td>√</td>
</tr>
<tr>
<td>turkey, domestic geese, etc.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mammals (pig, horse, dog, camel,</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>etc.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undomesticated animals (wild</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>pig, marine seal, mink, whale</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Humans</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>Non-human primates (cynomologus,</td>
<td></td>
<td>√</td>
</tr>
<tr>
<td>rhesus and pig-tailed macaques)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laboratory animals (mice¹, ferrets,</td>
<td></td>
<td>√</td>
</tr>
<tr>
<td>cotton rat, guinea pig, etc.)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ Require prior adaptation (adaptive host).

### 1.2. MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are a class of non-coding RNAs ≈ 22 nts in length that are able to regulate gene expression in plants and animals post-transcriptionally [63, 64]. The regulatory function of miRNAs spans a wide spectrum of biological processes from development to oncogenesis [65, 66]. They constitute key players in different aspects of host-virus cross talk [67]. While miRNAs can be encoded from genomes of some viruses (in particular DNA viruses such as herpes viruses), there is consensus that influenza viruses do not encode miRNAs. In this thesis, I therefore mainly focus on host-encoded miRNAs.
1.2.1. Nomenclature of MiRNA

The names of miRNAs as depicted in miRBase, the most common database of annotated miRNA sequences [68], start with 3 letters that signify the species (e.g. hsa for humans, homo sapiens). In the old nomenclature (miRBase1-17), the degraded miRNA strand had a star (*) above the name while the mature strand did not. From miRBase18 onward, most of the mature miRNAs have been designated 5p whereas the degraded strand has been designated 3p. As an exception, the guide strand of miR-223 is the one with 3p at the end. The nomenclature of 5p and 3p is assigned arbitrarily in some cases when the data are not sufficient to determine which sequence is more abundant. Another rule is that MiR-223, with the capital “R”, refers to the mature form, whereas mir-223, with lowercase “r”, indicates either the miRNA gene or the stem-loop sequence of its pri-miRNA. MiRNAs that have identical mature sequences, but are produced from distinct pre-miRNA and/or from different genomic loci (chromosomes) are numbered sequentially (e.g. 1, 2, 3, etc.) after the name of the same miRNA. On the other hand, adding letters (e.g. a, b, c, etc.) after the name of the same miRNA indicates that they are produced from the same chromosome, but processed from two different precursor and nonetheless differ in one or two nucleotides only [68].

1.2.2. MiRNA biogenesis and mechanism of action

In order to create the mature miRNAs, several steps occur in the nucleus and cytoplasm (Fig. 4). No matter whether they are present in single form or as clusters on the genome, miRNAs are transcribed by RNA polymerase II together with its associated transcription factors [69]. This results in primary miRNA (pri-miRNA), which forms long (over 1 kb) stem-loop structures. The pri-miRNA is processed in the nucleus by the Drosha enzyme and its co-factor DGCR8, to release hairpin-like RNA of ≈ 65 nts in length called precursor miRNA (pre-miRNA) [70]. After that, the pre-miRNA is exported to the cytoplasm by exportin 5 [71]. There, Dicer cleaves the pre-miRNA near the terminal loop, liberating a duplex of miRNA (guide strand) and miRNA* (passenger or degraded strand) [72]. The duplex is then unwound by a helicase enzyme, thus producing two strands. The strand that has an unstable
terminus at the 5’end and that carries a U as a first nucleotide mostly acts as a guide strand [73, 74]. The process of selection occurs during uploading of the mature strand and the associated Argonaut (AGO) proteins onto the RNA induced silencing complex (RISC). The non-selected passenger strand is quickly degraded, resulting in an accumulation of the guide strand in the total miRNA pool. In terms of function, miRNA binds the 3’ UTR of their target mRNA through imperfect complementarity, causing decreased translation of the mRNA into the encoded protein. [64]. In a few cases, such as miR-196 in targeting Hoxb8 mRNA, perfect miRNA-mRNA pairing causes cleavage of the mRNA, thus affecting the gene at the mRNA level [75].

1.2.3. Potential roles of miRNA as diagnostic and prognostic biomarkers

The changes in the pattern of miRNA expression during and after infection suggest that miRNAs can be used to diagnose a disease, to assess its severity, and to predict the subsequent response of the host to the disease, i.e. the clinical course [76]. Cancer was found to be associated with changes in miRNA expression [77, 78]. Additionally, miRNAs demonstrated a great importance to diagnose inflammatory rheumatic diseases [79], sepsis [80, 81], and heart failure [82]. With regard to IAV infection, patients infected with pandemic (H1N1) 2009 demonstrated a specific
miRNA response, in which 41 miRNAs were differentially expressed miRNAs in their peripheral blood mononuclear cells (PBMCs) [83]. Tambyah et al identified 14 blood-deregulated miRNAs that can discriminate between healthy and H1N1-infected patients [84]. More recently, a comparative study suggested that a higher serum level of miR-150 is an indicator of poor outcomes in patients infected with severe H1N1 infection if compared to its counterpart with milder disease [85]. As prerequisites of using miRNAs as biomarkers, a comparison between miRNAs levels in healthy tissue versus that in the diseases should be conducted. Furthermore, fast, reliable and reproducible methods need to be implemented. A high sensitivity of the methodologies is critical for identifying miRNAs with low abundance. NGS is a major approach for miRNA profiling studies [86, 87]. Among several things that NGS can do that other approaches cannot, is the direct access to the sequences without prior knowledge of the gene structure. Therefore, it can be applied to species for which full genome sequences are not available [88]. Nevertheless, several issues might influence the results of miRNA profiling, such as the type of biosample and quality of preanalytical sample preparation [89], and hence should be taken into consideration. A detailed description of different aspects of using miRNAs as biomarkers is provided in “Manuscript II”.

1.2.4. MiRNAs in IAV infection

miRNAs are known to be involved in multiple physiological and pathological processes in human lung [90]. Moreover, several miRNA members are known to regulate cytokine genes [91], anti-viral immune-related molecules [92, 93], and virus recognition receptors [94]. Therefore, it is reasonable to assume that host-encoded miRNAs can affect the pathogenesis and outcomes of IAV infection. Indeed, in vivo approaches have been used to address the roles of miRNAs in different aspects of IAV infection. It has been claimed that tissue-specific miRNAs might influence the lung versus tracheal tropism of H5N3 and H3N2 in chicken and dog [95, 96]. A series of experiments in which mice and macaque models were used put forward the assumption that differential miRNA expression might be associated with increased
IAV virulence [97-100]. In the experiment done by Choi et al, it was found that miR-147-3p, miR-151-5p, miR-155-3p and miR-223-3p were higher in lung of mice infected with mouse adapted H5N2 virus than those infected with low pathogenic H5N2 virus. Mechanistically, they observed that miR-223-3p and miR-151-5p could promote viral replication evidenced by low viral titers, high survival rates and weight gain in the mice inoculated with anti-miR-223-3p and anti-miR-151-5p. In contrast, the mice treated with anti-miR-147-3p and anti-miR-155-3p showed a reduction in body weight with a tendency of increased viral titers, suggesting the anti-viral activities of these miRNAs. Li et al. suggested that miRNAs might be involved in the differential susceptibility of chicken and duck to HPAI H5N1 infection [101]. In this experiment, the authors showed that, in contrast to duck, the miRNA repertoire of chicken spleen, thymus and bursa changed their dynamics upon infection with more miRNA showing up-regulation than down-regulation. They also identified that in the spleen of infected chicken, miR-2188-5p, miR-34c-5p, miR-200b-5p, miR-122-5p and miR-146b-5p were predicted to target genes in the B-cell receptor (BCR) pathway. However, in the spleen of infected ducks, only miR-122-5p, which was down regulated, was predicted to target RASGRP3 gene, an effector molecule in the BCR pathway. Using a murine model, host-encoded miRNAs have been proposed as biomarker for the susceptibility to H1N1 virus [102]. An association between miRNAs and IAV pathogenesis at acute and recovery phases has also been proposed in experiments involving mice and pigs [103-106]. In parallel, several studies analyzed the potential of miRNAs as biomarkers for IAV in humans [83-85, 107]. Taken together, these studies suggest the importance of miRNAs as regulators of gene expression in host-IAV interaction.

A update on the current state of research addressing various roles of host-encoded miRNAs in infectious viral diseases in their natural animal host as well as in relevant in vivo laboratory models is reviewed in Manuscript I.
2. Objectives

In Objective 1, I reviewed the literature to determine the current known roles of host-encoded miRNAs in viral infectious diseases in animals of veterinary importance (Manuscript I, P. 28). Additionally, I performed a literature search to summarize the up-to-date laboratory trials geared towards clinical applications of sncRNAs (miRNA, small interfering RNA (siRNAs) and short hairpin RNA (shRNA)) to diagnose and combat viral infectious diseases that affect animals of veterinary importance and may thus impact animal and human health (Manuscript II, p. 83).

In Objective 2, I validated the results of RNA-seq with regard to the differential expression level of selected miRNAs and measured the expression level of certain mRNA genes using the reverse transcriptase quantitative real time PCR (RT-qPCR). These miRNAs were previously identified in H1N1 PR8-infected lungs of DBA/2J (susceptible) and C57BL/6J (resistant) mice. The results are shown in section 4.1 of Unpublished Data (p. 247).

In Objective 3, I analyzed the cell-type and viral-strain-specific expression of selected miRNAs using different human cell lines. TLR ligands (LPS and R848) were used as stimulants. Furthermore, wild-type swine origin IAV H1N1 2009 pandemic (H1N1 pdm) (S-OIV wt) and a reassortant variant of it (S-OIV NS PR8), which was created by inserting the NS segment of the PR8 strain, were used as infection model viruses. The results are shown in Manuscript III (p. 125).

Infection with H5N1 virus still a major problem in the poultry population worldwide and ducks have been known to be the main reservoir of the virus. Given the involvement of miRNAs in IAV-host cross talk, Objective 4 of this thesis was to characterize miRNA populations ("miRNomes") in lung and brain of H5N1-infected and control ducks (Anas platyrhynchos) using small RNA-seq approach. Firstly, the replication dynamics of the HPAI H5N1 isolate used and the associated histopathological lesions were studied in a time course experiment (Manuscript IV, p.158). Total RNA was then extracted from lung and brain and subjected to small
RNA-seq for profiling of small non-coding RNAs. The resulting data was analyzed with OASIS modules and other bioinformatics tools in order to identify small RNA biomarkers for pulmonary vs. CNS involvement in HPAI infection in ducks (Manuscript V, p. 190).

Humans are known to be susceptible for IAV infection. In humans, the highly pathogenic strains cause serious complications and high fatalities. **Objective 5** of this thesis was dedicated to study the differences in miRNA expression upon infecting explanted human lung tissue with S-OIV wt or the reassortant S-OIV NS PR8, with the goal to investigate the potential role of cellular miRNAs as biomarkers for IAV infection in humans. Therefore, firstly, I adapted an *ex vivo* system based on healthy human lung tissue explants (HLTEs), and then pieces of this tissue were infected with the two viral strains for comparison. Viral transcription and expression of host inflammatory cytokines and chemokines were measured; in addition, a semi-quantitative histological score was applied to compare the degrees of tissue lesions caused by the infection with both viruses (*section 4.2* in *Unpublished Data*, p. 259).
3. Results

3.1. Manuscript I
(in preparation)

Title
Host-encoded microRNA associated with viral infectious diseases of veterinary importance: general aspects and role in pathobiology

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Abstract

The discovery of small regulatory non-coding RNAs is a major advance in the field of genomics. MicroRNAs (miRNAs) are endogenous RNA molecules, approximately 21-25 nucleotides in length that regulate gene expression, mostly at the post-transcriptional level. MiRNA profiling technologies have made it possible to identify and quantify novel miRNAs and to uncover their potential roles in disease pathogenesis. Although miRNAs have been extensively investigated in human viral infections, their implications in viral diseases affecting animals are much less understood. The number of annotated miRNAs in different animal species is growing continuously, suggesting even more important roles in regulating the host-pathogen relationship. In this review, we present an overview of synthesis and function of miRNAs and an update on the current state of research on host-encoded miRNAs in the genesis of viral infectious diseases in their natural animal host as well as in relevant in vivo laboratory models and in vitro systems, where models for natural infections do not exist or are cumbersome to perform.

Key words
Animals; infectious diseases; influenza A virus; miRNAs; viruses; veterinary science
1. Introduction and brief history

The discovery of non-coding RNAs such as long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) has begun to direct more and more attention to these potentially very powerful regulatory molecules. The first miRNA was discovered in 1993 in an experiment on the timing of embryonic development of different larval stages of the worm *Caenorhabditis elegans* (*C. elegans*). In this experiment, Lee and colleagues observed that the RNA transcribed from the *lin-4* locus did not encode a protein, but instead silenced the gene encoding Lin-14, an important protein in the larval embryonic development [1]. Since then, the number of studies on miRNAs has been rising at an amazing rate. Indeed, the number of publications that describe roles of miRNAs in animal diseases has been growing exponentially (PubMed search, data not shown). MiRNA-encoding genes comprise 1-5% of the animal genome but regulate up to 30% of all protein-coding genes [2, 3]. It is being recognized that their regulatory roles are much more sophisticated than initially thought, owing to the cooperativity (i.e. more than one miRNA species can target the same mRNA) and the multiplicity of their targets (i.e. one miRNA can target hundreds of mRNA species) [4]. MiRNAs have been shown to play important roles in essentially all biological processes, and the differential expression of host miRNAs during infection [1, 5] supports the idea that they may constitute key players in the host response to invading pathogens. This review presents an update on miRNA biogenesis and profiling, discusses some of the challenges encountered when studying them in animals, and summarizes current knowledge of the roles of miRNAs in viral infectious diseases in their respective natural animal hosts. In addition to this, information obtained from *in vivo* laboratory models, including mice and non-human
2. MicroRNA biogenesis and mechanisms of action

MiRNAs are non-coding single-stranded oligoribonucleotides roughly 22 nucleotides (nts) in length. Their biogenesis and mode of action is illustrated in Fig 1, but the reader is also referred to excellent recent reviews of miRNA biogenesis, e.g., ref. [6]. MiRNAs can be transcribed from within protein-coding genes (intragenic miRNAs), from dedicated miRNA coding genes (intergenic miRNAs), or from genes encoding other ncRNA classes such as small nucleolar RNA (snoRNA) and IncRNAs. More than half of vertebrate miRNA genes lie in introns, implying that most miRNA are co-expressed with specific host mRNAs [7], although specific transcription start sites for miRNA coding sequences also exist [8]. While the majority of miRNAs genes are, physically, separated on the genome [9, 10], many functionally-related miRNA genes often, but not always, reside in clusters within the genome [11], probably because they are processed from the same polycistronic transcript [5]. When they have their own promoter, miRNA genes are transcribed mainly by RNA polymerase II and, rarely, by RNA polymerase III [12] to form primary miRNAs (pri-miRNAs) [13], which are then folded to produce hairpin structures. Each hairpin structure consists of a 32 nt-long imperfect stem and a large terminal loop. The enzyme Drosha and its co-
factor DiGeorge Syndrome Critical Region 8 (DGCR8) cleave the 22 nts downstream of
the stem to yield 60 nt-long precursor miRNAs (pre-miRNA) [13]. The pre-miRNAs
are then exported to the cytoplasm via exportin 5 where the terminal loops are
excised by Dicer and Tar RNA Binding Protein (TRBP) to produce short imperfect
miRNA duplex intermediates [14]. This duplex is then unwound by a helicase into
two miRNA strands. One strand (guide strand) is incorporated into the RNA-induced
silencing complex (RISC) to target mRNAs, and the other strand (miRNA* or
passenger strand) is degraded. In addition to their cytoplasmic location and function,
mature miRNAs can also be found in the nucleus [15-17]. Profiling studies of
fractionated cells revealed the enrichment of miRNAs in the nucleus of multiple cell
lines derived from various origins [15, 18, 19]. Examples of those miRNAs are miR-
320, miR-373 and miR-29b [16, 20, 21]. The driving force of this cytoplasmic-nuclear
shuttle, at least for miR-29b, is thought to be a miRNA-associated hexanucleotide
terminal motif (AGUGUU), which increases the stability of the miRNA [16]. The
presence of miRNAs in the nucleus suggests that they may participate in regulating
transcription or splicing of target transcript rather than their classic regulatory roles
as a translation repressors or mRNA degradation factors. Accumulating evidence
indicates that nuclear miRNAs might both repress [20, 22, 23] and activate
transcription [21, 24]. Taken together, these studies suggest that the scope of
miRNA functions has become broader than previously thought, including additional
epigenetic modifications such as histone modification and DNA methylation [25].
Apart from the classic biogenesis pathway, Dicer or Argo-2-independent (non-
canonical) pathways have also been described [26]. For instance, mirtrons are
miRNAs that are produced from introns by the splicing machinery instead of the
Drosha processing [27]. The maturation of some miRNAs (for example miR-451) is Dicer independent, but Argo-dependent. This is possibly because the stem part of its pre-miRNA is too short (17 base pair) to be processed by Dicer [28]. MiRNAs mainly act by repressing mRNA translation and, less commonly, by inducing mRNA degradation. Usually, the seed region (7 or 8 consecutive nts at the miRNA 5' end) binds to complementary sequences in the 3' untranslated region (UTR) of the target mRNA [29], but miRNAs can also target other sequences such as the 5'-UTR, the promoter, or coding sequences. Remarkably, some miRNAs have been implicated in actually stimulating translation [30, 31]. The half-life of miRNA ranges from hours to days [32] and is regulated by several mechanisms, including degradation by small RNA degrading nucleases (SDNs), extrusion from the cell in exosomes or microvesicles, and sequestration by circular RNAs (“miRNA sponges”) [33].

3. Update on microRNA identification and computational prediction of their targets in animals

In vertebrates, miRNAs have been studied most extensively in humans and mice, in part because much fewer miRNAs have been annotated or made publicly available in other organisms, including animals of veterinary importance [34]. Fig. 2 shows the number of currently annotated mature and immature miRNAs in different animal species of veterinary importance, as well as in humans and mice according to miRBase version 21 [34]. Humans and mice had the highest number of annotated miRNAs, followed by chicken. In chicken, in-situ hybridization, cloning and deep sequencing approaches have all been used to define the miRNA population [35, 36].
MiRNA expression profiling has assisted in identifying miRNAs that regulate a range of biological processes. There are several established and emerging methods for measuring miRNA expression profiles in biological samples. The commonly used approaches include reverse transcription quantitative real-time PCR (RT-qPCR), microarrays, and RNA sequencing (RNA seq) [37]. In the process of discovering novel miRNAs, the analysis of profiling data follows the same principle in that the generated reads have to be mapped to the reference genome of the concerned species. In this context, the lack of a published reference genome always represents a limitation. The miRNAs from such species have been identified by homology searches where the deep sequencing reads are aligned against the genome of the most closely related species [38]. Indeed, most bovine miRNAs have been identified in this way, and a similar method was used to identify goat and sheep miRNAs [39]. Researchers from China recently characterized miRNA species in skin and ovarian tissue of ducks [40, 41]. An alternative strategy was used in these studies. First, the reads were filtered and then mapped by blast alignment to all known mature chicken miRNA sequences present in miRBase. The sequences that were found to correspond to chicken miRNAs were removed, and the remaining ones were mapped to miRNAs in other species. This strategy has also been applied to the Chinese hamster [36]. There are several computational resources for the identification of miRNAs and their targets [42]. Along these, miReader is a newly launched bioinformatics tool for the discovery of novel miRNAs that can be used to identify miRNAs that are not annotated in miRBase, yet without the need for reference genomic sequences or homologous genomes. It shows a high degree of accuracy in a wide range of animal species [38]. The prediction and validation of
miRNA targets are essential steps in their regulatory function. In this regard, the imperfect complementarity between the miRNAs and their mRNA targets represents a major challenge because of potentially false positive predictions. Nevertheless, various online target-prediction tools have been developed. Prominent tools include target scan [43], miRanda [44], PITA [45], and RNA hybrid [46]. Most of these tools rely on basic principles such as miRNA/mRNA pairing, cross-species conservation of the mRNA 3’ UTR, and the free energy required to form the duplex [47, 48]. Kiriakido indicated that the agreement of mRNA targets predicted for a set of 79 miRNAs by several target prediction tools was only in the range of 10-50% [49]. One reason for this may be the divergent use of the degree of conservation, which, e.g., may be used but not directly incorporated into the score (as in PITA), or not used at all (as in RNA22) [50]. However, these tools differ by several key features. Some variations are independent of the algorithms such as using different UTR databases for prediction, cross-species comparison, or alignment artifacts. Variations that are related to the prediction algorithms themselves include the number of nucleotides involved in pairing (canonical, marginal and atypical seed-matched sites) [51], the method used to measure 3’ UTR conservation, the accessibility to the UTR and the statistical approach used [52]. Some attempts have been successful to use the mRNA targets to predict biological pathways that are regulated by a set of miRNAs. For instance, the DNA intelligent analysis (DIANA) tool [53] offers the miRPath server, which can create hierarchical clusters of miRNAs and pathways based on the levels of the predicted miRNA/mRNA interactions. Generally, a major limitation of the available web tools is that they include a limited number of species (e.g. human, mouse, *C. elegans*), leaving many animal species underrepresented, in
particular those of veterinary importance. However, some tools (e.g. Target scan, miRDP and microcosm) include some species of veterinary importance such as chicken, dog and cow [43, 54, 55]. The growing list of annotated miRNAs in species of veterinary importance undoubtedly warrants better inclusion of such species in the on-line prediction algorithms. Considering the probability of obtaining false positive and negative results using the on-line prediction algorithms, it seems critical to confirm miRNA function using experimental work. The function of miRNA can be validated by several experimental approaches. As one example of many, the luciferase reporter assay can detect miRNA effects on a target at the mRNA level [56]. In this approach, the wild-type or mutated 3’UTR of a predicted mRNA target are cloned up or downstream of the luciferase gene promoter. Reduced luciferase activity derived by the binding of a miRNA to the respective 3’UTR is considered a positive result. On the other hand, immunoblotting can be used to detect the effect of a miRNA on its targets at the protein level. For a detailed analysis of procedures used for the experimental validation of miRNA targets, we recommend references [57-59].

4. Role of microRNAs in viral infectious diseases of animals

Considering the implication of miRNAs in nearly all biological processes, links between miRNAs and disease status are expected. Earlier reports suggested that miRNAs are involved in the regulation of inflammatory pathways as well as adaptive and innate immunity, stress factors and cytokine signaling [60]. Diseased tissues may show unique miRNA expression patterns, which subsequently might affect virus replication and/or survival. For instance, miRNAs might promote virus replication by
direct pairing with virus-derived transcripts, as has been shown in the case of miR-122 and hepatitis C virus [61] and miR-939 and influenza A virus (IAV) [62]. In contrast, miRNAs may restrict the replication of the virus, as exemplified by miR-32 in the context of primate foamy virus type 1 infection [63]. Indeed, our understanding of the mechanistic associations and implications of miRNAs in animal viral infectious diseases is still far from complete. Here, we review the literature that describes the expression of miRNAs in the context of viral infectious diseases that affect farm and pet animals, with an emphasis on infections in the natural host. Additionally, we discuss miRNA expression in infectious viral diseases in laboratory models and in vitro where there are no sufficient data involving the natural host.

4.1. Role of miRNAs in infections of the natural host

4.1.1. Influenza A virus (IAV)

Infection with IAV has a negative impact on the poultry and swine industries, and on human public health in that, it is able to cross species barriers and adapt to the human host. The differences in pathogenesis of various IAV strains are attributed to both viral and host factors. Yet, there remains an urgent need for diagnostic markers to sense the very early phases of IAV outbreaks at the farm level. The first trial to emphasize the effect of miRNAs on IAV pathogenesis in a species of veterinary importance attempted to define miRNA populations in lung and trachea of commercial Leghorn chickens experimentally infected with H5N3 virus [64] (Table 1). Some miRNAs were up-regulated in both lung and trachea and others showed a tissue-specific pattern. For instance, miRNA-206 was more highly expressed in infected than in non-infected lungs, while the reverse was reported for
trachea. These findings suggest that specific host miRNA regulatory mechanisms might exist in response to IAV infection and substantiate the fact that these miRNAs affect the in situ replication of the virus [64]. The same research group subsequently confirmed that host cellular miRNAs following H5N3 virus challenge could lead to different results depending on the host genetic background [65]. They proved that host-encoded miRNAs were modulated differentially in the lungs of broiler and layer chickens (Table S1). In broilers, more miRNAs were up- than down-regulated, whereas this was reversed in layers. Only two miRNAs, miR-1599 and miR-1416, were consistently regulated independent of chicken breed. Since the study showed a breed-dependent effect on miRNA expression, the authors proposed that miRNA expression is linked to immunity. Some of the identified miRNAs have predicted target sites in immune-related genes such as IL17RD, ARL11, CHMP2B, POU1F1, PDHB and HIF1AN. Indeed, broiler chickens have weak short-term humoral immunity, whereas layers possess a long-term humoral immune response and strong cellular immunity, which goes in line with the fact that layers have a longer life expectancy [66]. Along with the immunity-related miRNAs, Li and his colleagues claimed that miRNAs account for part of the immune-related differences between chicken and ducks upon H5N1 infection. They showed that, in contrast to duck, the miRNA repertoire of chicken spleen, thymus and bursa changed their dynamics upon infection with more miRNAs showing up-regulation than down-regulation (Table S1). A set of spleen-specific miRNAs were found to target genes in the B-cell receptor pathway [67]. This study highlighted the value of miRNAs in the differential susceptibility to IAV infection between chicken and ducks. In pig, miRNAs were found to be dysregulated in the lungs after aerosol challenge with reassortant IAV
(H1N2). Some miRNAs were up-regulated 1, 3, and 14 days after infection (miR-15a); others were expressed late (miR-21, miR-206 and miR-451) or transiently up-regulated (miR-223), whereas miR-146 was transiently down-regulated (Table 1). These miRNAs target several inflammation-related molecules [68]. In a recent experiment that also involves pig, Jiang et al. showed that miRNAs of piglet pulmonary alveolar macrophages differed in expression during acute (4 days post challenge) and recovery (7 days post challenge) phases of IAV (H1N1) infection. Most of the miRNAs at 4 days (70 miRNAs) were down-regulated, presumably allowing an increase in their target genes that participate in the host defense against viruses. Then, 3 days later, the expression levels of most miRNAs returned to normal with subsequent normal expression of immune genes during the recovery phase (Table S1) [69]. In another study, Tan et al., showed that most miRNAs are down-regulated at 7 and 15 days after PR8 strain (H1N1) infection in mice [70]. The isolation of H3N8 in 2005 from infected dogs in the United States, and the identification of H3N2 in 2007 from dogs in Korea and China marked the emergence of canine influenza virus [71, 72]. In 2014, Rong Zaho et al. conducted an experiment in which they profiled the miRNA expression patterns in lung and trachea of beagles experimentally infected with H3N2 virus [73]. In this study, 34 and 45 miRNAs were differentially expressed between infected and non-infected groups in lung and trachea, respectively (Additional table 1). In addition, 99 miRNAs were differentially expressed between infected lung and trachea. Interestingly, while infected lungs showed higher expression levels of miRNAs than the non-infected ones, the reverse was reported in trachea, indicating a tissue-specific signature of miRNA and suggesting that these miRNAs may play different roles in different organs. The
 divergence in the results obtained from various studies might be due to different strains or models used. Other publications support the notion of miRNA involvement in various diseases, using *in vitro* models [74-77]. Considered together, these observations suggest that a specific host miRNA response is associated with IAV infection and could contribute to the pathogenesis of IAV including its tissue/cell tropism and host preference.

4.1.1.1. Influenza A virus infection as an example of cross-species conservation of host-encoded miRNAs

All IAV subtypes primarily originated in wild birds that are classified under orders Anseriformes and Charadriiformes [78]. Their migration and aquatic nature enable both the maintenance of IAV strains as well as the emergence of novel strains in spillover hosts. While mice can be infected with IAV only after serial passages [79], domestic chicken, swine, and humans are among the main transmission reservoirs [80]. Species-dependent variation in the host response to IAV has been reported, including in chicken and ducks [81]. Despite their potential roles in inter-species differences in host responses to infections, a global view of cross-species expression, conservation and functionality of miRNAs is incomplete and spread across several studies. In order to obtain an overview of shared and distinct miRNAs in an infectious disease that affects both animals of veterinary importance and humans, we reviewed the literature on miRNAs regulated upon IAV infection in humans [77, 82, 83], mice [84-89], chicken [64, 65, 67] and pig [68, 69], extracted all miRNAs (separately for each species) that have been shown to be differentially regulated upon IAV infection (Table S2), and selected those miRNAs that are
regulated in all four species. Naturally, this analysis needs to be interpreted knowing that its negative predictive value may be high due to publication bias and differences in the numbers of studies done in the various hosts; on the other hand the positive predictive value likely is high. As shown in Fig. 3A, three miRNAs (miR-18a-5p, miR-223-3p, and miR-451-3p) were differentially regulated in all four species, suggesting a common IAV infection-related signature. Limiting the analysis to the three natural hosts (humans, chicken, and pig) revealed an additional four consistently regulated miRNAs (miR-18b-3p, miR-22-5p, miR-30a-3p, and miR-155-3p). The expression pattern and tissue specificities for these miRNAs are listed in Table 1 and Table S1. These seven miRNAs are related to diverse biological processes. Among them, miR-223 and miR-155 have been reported to regulate various aspects of immune responses, notably during IAV infection. An association between miR-223 and IAV virulence has been proposed in several studies. Among these, Li et al observed an up-regulation of miR-223 in mice infected with the lethal r1918 pandemic H1N1, but not the less virulent Tx/91 strain. The authors also reported that miR-223 can indirectly repress the transcription factor CREB, which is responsible for maintaining cell survival and growth [90], indicating that the lethal IAVs can induce cellular apoptosis via increasing the level of miR-223 [84]. Luciferase-based reporter assay was utilized by Haneklaus et al. to show that overexpression of miR-223 tends to reduced luciferase expression in a vector containing the wild type 3’ UTR of NOD-like receptor P3 (NLRP3), but not the one, that contains a mutated version of the 3’ UTR of NLRP3, implying that miR-223 can target NLRP3 with subsequent reduction in the inflammasome [91]. In another study, miR-223 has been shown to be up-regulated in lung of mice experimentally infected with H5N2 virus with more induction
in the more virulent strain compared to the less virulent one. Subsequent administration of anti-miR-223 to these mice reduced IAV titer and increased both animal survival rate and weight gain at 1, 3 and 5 days post inoculation. [92]. The role of miR-223 in inflammation, immunity and cancer has been reviewed extensively [93-95]. MiR-155 has been found to regulate both innate and adaptive arms of the immune system. On the one hand, CD8+ T cells transduced with the MigR1 vector overexpressing miR-155 showed a higher expansion and proliferation rate compared to the negative controls, suggesting a role for this miRNA in the T cell response [96]. On the other hand, overexpressing and blocking miR-155 in a murine macrophage cell line indicated that miR-155 can target the suppressor of cytokine signaling (SOCS), a negative regulator of IFN-α, thereby trigger type 1 IFN signaling [97] and fight against invading pathogens. In support of this, Choi et al found that mice injected with anti-miR-155-3p displayed a dramatic loss in body weight and succumbed to IAV infection within 8 dpi with high viral titers in their lungs [92]. These studies suggest that IAV may exploit host miRNAs for its own benefits. The pathogenesis of IAV involves effector molecules that converge to form interacting signaling pathways [98]. To test the hypothesis that there are common miRNA-regulated functional pathways that are induced upon IAV infection, we utilized the bioinformatics tool DIANA miRPath v.2.0 [53] to predict pathways that might be regulated by these miRNAs. For this purpose, we chose the smaller set of miRNAs regulated also in the mouse (an adaptive, well studied host for IAV) and the murine functions of the DIANA tool, as it does not support analyses of chicken and pig miRNAs, and also because the mouse is the one of the four species with the best experimental evidence for miRNA function at the organismal level. Fig. 3B shows
the pathways regulated by two of these three miRNAs, i.e. miR-18a-5p and miR-223-3p (miR-451-3p is not included in the DIANA tool). Among the identified pathways, endocytosis and the PI3K-Akt, MAP kinase, mTOR, and TGF-β signaling pathways are known to be associated with IAV-host interaction. The PI3K pathway is mainly involved in apoptosis. Binding of the NS1 gene of IAV to the P85β regulatory subunit activates the PI3K leading to the phosphorylation of the downstream effector molecule Akt, which further phosphorylates both caspase 9 and GSK-3β, thereby suppress the apoptosis and prolong virus infection [99]. Hirata et al suggested that inhibition of Akt kinase activity may have therapeutic advantages for IAV infection by mediating inhibition of viral entry and replication [100]. Besides the PI3K pathway, MAP kinases have been reported to promote IAV ribonucleoprotein capsid trafficking and virus production [101] and to regulate the production of RANTES, a chemokine that is released by lung epithelial cells and alveolar macrophages [102]. Among the identified pathways, the TGF-β signaling pathway is known to be activated by the IAV neuraminidase. The authors claimed that the modulation of TGF-β activity during IAV infection influences viral titers and disease outcome in experimentally infected mice, suggesting a significant role for TGF-β signaling in IAV pathogenesis. Interestingly, this study postulated that the inability of H5N1 virus to trigger the TGF-β cascade might explain the improper host immune response and the exaggerated immune pathology seen in many H5N1 cases [103]. Clathrin-mediated endocytosis is a perquisite for IAV entrance into the target cells [104]. For further description of the implication of IAV-associated pathways, we recommend references [98, 105]. The remaining 12 pathways are mainly related to cancer, but we cannot rule out any possible roles for these
pathways in IAV infection, as cancer-related pathways also tend to be involved in the host response to infection. To gain further insight into the cross-species conservation of functionally related miRNAs, we then used the ClustalX version 2.0 software [106] to align the sequences of the stem-loop (premature) forms of miR-223 and miR-155 in humans, mice, chicken and pig (Fig. 3C). The degree of conservation was higher in the mature miRNA than in the rest of the stem-loop sequence. In terms of sequence identity, the mature forms of human miR-223 and miR-155 were more closely related to the corresponding sequences in mice than in chicken and pig. The seed region was 100% identical among the four species studied. Shared seed sequences might indicate shared miRNA targets, lending further support to the notion that these miRNAs play similar roles in these species in the host response to IAV infection. Additional functional implications of these shared miRNAs in the course of IAV infection are discussed in a separate manuscript (Samir & Pessler, in press by *Frontiers in Veterinary Science*).

### 4.1.2. Infectious bursal disease virus (IBDV)

The infectious bursal disease virus (IBDV) is the etiologic agent of infectious bursal disease, which is a highly contagious disease that predominantly affects the bursa of Fabricius in birds [107]. Although vaccination efforts have contributed to the reduction of the overall impact of this disease in poultry [108], several challenges remain to be overcome [109]. Multiple cell line-based studies demonstrated inhibitory roles for miRNAs against a variety of IBDV proteins [110-114]. However, the first study that involved a natural host for IBDV (leghorn chicken), was done only recently [115]. In this study, the authors identified a pool of significant
differentially expressed miRNAs that are induced upon infection. Among these miRNAs, gga-miR-9* showed a strong pattern of differential expression, being regulated at 2, 4, 12, 24 hpi. These authors provided evidence miR-9* can promote IBDV replication by repressing the production of IFN.

4.1.3. Marek's Disease virus (MDV)

Marek's disease, or Gallid herpesvirus 2 (GaHV-2) infection, is a highly contagious viral neoplastic disease in chicken [116]. The disease has been remained a major concern in poultry owing to the continual emergence of new virulent strains [117]. The virus causes T cell lymphosarcoma in chicken and turkey [118]. There has been significant progress in our understanding or the roles played by MDV-encoded miRNA in the pathogenesis of the virus [119-121]. On the other hand, in vitro approaches have also implicated the involvement of host-encoded miRNAs in this context [122-127]. However, few studies have addressed this role in vivo. In a study conducted by Stik et al [128], white leghorn chicken experimentally inoculated with RB-1B strain were used to study the relation between cellular miRNAs induction and the oncogenic nature of MDV. The authors reported that gga-miR-21 was up-regulated in the infected chicken compared with the uninfected ones from 7 dpi onward. Chicken inoculated with the oncogenic strain of MDV had a higher induction rate of gga-miR-21 than those injected with CVI988, a non-oncogenic vaccine strain. These data highlight the role of gga-miR-21 in the host response to MDV and suggest a potential role for this miRNA in the development of MDV-associated tumor. Using microarray, Tian et al analyzed the association between miRNAs and MDV. The authors identified 58 down-regulated and 6 up-regulated miRNAs between
infected and non-infected MDV susceptible chickens line (line 7). Whereas none of the miRNAs were differentially expressed significantly between the infected and non-infected MDV resistant chicken line (line 6). The candidate target genes regulated by the differentially expressed miRNAs were identified in several immune related pathways, such as NF-κB signaling, T cell and B cell receptor signaling providing a hint that miRNAs might influence the genetic susceptibility of chicken to MDV infection through controlling the immune responses [129].

4.1.4. Avian leukosis virus (ALV)

Avian leukosis virus (ALV) belongs to the genus Alpharetrovirus of the Retroviridae family. This virus is capable of inducing the formation of various types of tumors, including hemangioma and myelocytoma [130]. MiRNAs have been shown to be associated with tumors through regulating cell proliferation and apoptotic processes [131]. Among the studies that highlight miRNA-ALV relationships, one that was conducted by Li et al., who identified a down regulation of gga-miR-375 in the liver of ALV-J-infected chicken compared to the control animals and that its overexpression led to a significant inhibition in the proliferative capacity of DF-1 cells (chicken fibroblast) [132]. These findings suggest that gga-miR-375 may play a role as a tumor suppressor during ALV infection. In another study, microarray analysis of liver tumors harvested from ALV-J-infected chicken indicated that four miRNAs were found to be differentially expressed. These include gga-miR-221 (up-regulated) and gga-miR-193a, gga-miR-193b and gga-miR-125b (down-regulated). Gene ontology and pathway analyses of these miRNAs showed that the MAPK signaling pathway and Wnt signaling pathway may be linked to ALV-
J-triggered tumorigenesis [133]. More recently, studying the spleen miRNome in ALV-J- infected chickens revealed that 167 miRNAs were differentially expressed between the infected and control groups. Target prediction analysis indicated that miR-18a, miR-19a can engage \textit{THBS1}. They also identified a relation between miR-200b and its target gene \textit{FN1}, which has been known to be involved in ovarian cancer [134]. Along the same line, overexpression of miR-23b in chicken embryo fibroblast caused a significant reduction in the expression of \textit{IRF1}, which further down-regulated the expression of \textit{IFNβ}, suggesting a significant role for miR-23b in ALV pathogenesis [135]. Collectively, these studies underscore the importance of miRNAs in the pathogenesis of ALV.

4.2. Role of miRNAs in laboratory models (\textit{in vivo})

4.2.1. Venezuelan equine encephalitis virus (VEEV)

VEEV is an equine disease that can be transmitted to humans via a mosquito vector and cause lethal inflammation of brain tissue. Mortality rates in equines have been estimated at 19–83%, but are below 1% in humans [136]. Reports suggest that miRNA expression is highly regulated upon VEEV infection of neurons and glial cells. Bhomia et al. [137] were the first to describe miRNA expression patterns in a mouse model of VEEV infection. Twenty-five miRNAs were up- and 4 were down-regulated in brain tissue collected after 48 and 72 h (Table S1). Mmu-miR-155 showed >5-fold higher expression at both time points, suggesting that it might serve as an indicator of VEEV infection. These miRNAs may play important roles in modulating gene expression and neuronal degeneration in the brain following VEEV infection.
4.2.2. Rabies virus (RV)

Rabies virus (RV) is a neurotropic virus that can infect the central nervous system (CNS) and lead to death in many cases of human infection. Canines constitute the main reservoir, but nearly all warm-blooded animals can contract the infection. The disease is transmissible from dogs to humans. Every year, it causes 55000 human deaths worldwide [138]. Thus, controlling the disease in domestic dogs has important implications for public health. In mice challenged with RV, a strong modulation in the expression of miRNA molecules was observed (Table 1), and the differentially expressed miRNAs are thought to be involved in several immune-associated signaling pathways [139]. In another study conducted in 2008, miR-133, which is specifically expressed in skeletal muscle, was predicted to bind to both N and G transcripts of rabies virus [140]. This might be a plausible explanation for the lengthy dormant state of this virus in skeletal muscle during the early phase of infection and before the continuation of its journey through the nervous system.

4.2.3. Prion protein-related diseases

The prion protein is the causative agent of bovine spongiform encephalopathy (BSE; “mad cow disease”) in cattle, feline spongiform encephalopathy in cats, scrapie in sheep and Creutzfeldt-Jakob disease (CJD) in humans. It is a group of fatal neurodegenerative disorders caused by an abnormally folded form of the naturally occurring cellular prion protein, PrPS\(^c\). Over the last 15 years, intense efforts have been undertaken following the appearance of a new prion disease that is transmissible to humans, variant Creutzfeldt–Jakob disease (VKJD). Ingestion of meat from bovine spongiform encephalopathy (BSE)-infected cattle or scrapie
infected sheep is the source of infection [141]. There is compelling evidence of a role played by miRNAs in the pathogenesis of the prion protein. Brain of mice infected with mouse-adapted scrapie showed a unique expression pattern of 15 miRNAs that might act during prion-induced neuro-degeneration. Most of them were up-regulated more than 2.5 fold (Table 1). Among these, only miR-128 had previously been shown to be dysregulated in other neurodegenerative diseases, suggesting a pattern specific for a family of closely related diseases [142, 143]. To determine whether miRNA dysfunction is involved in prion disease pathogenesis, the authors used a BSE-infected cynomolgus macaque model to confirm that miR-342-3p was up-regulated in the brain [144]. Moreover, the authors confirmed that hsa-miR-342-3p was up-regulated in brain samples of human type 1 and type 2 sporadic CJD, suggesting that miR-342-3p may be a biomarker of prionopathies in animals and humans, but this needs further exploration. This also indicates that this miRNA might affect pathogenesis in different species. In another study, Taganov et al proposed a role for miR-146a as a potent modulator of microglial function by controlling the activation state during prion induced neurodegeneration [145]. In general, miR-146a can directly down-regulate the production of pro-inflammatory cytokines by acting as a negative-feedback effector of the inflammatory signaling pathway initiated by NF-κB [146]. A coordinated deregulation of miRNAs seen in prion diseases may be a response to the abnormal accumulation of PrPSc, leading to signaling pathways that include alterations in neurotransmitter receptors and protein degradation, resulting in the ultimate failure of neuronal function.
4.3. Role of miRNAs in animal viruses (selected *in vitro* studies)

In addition to the above-mentioned *in vivo* models, there are several examples of *in vitro* trials that have unraveled multiple roles of cellular miRNAs in different scenarios of animal viral diseases of veterinary importance. One prominent example can be seen in the oncogenic miR-155 [147]. MiR-155 was initially identified as a gene that was activated by promoter insertion at a common retroviral integration locus in B-cell lymphomas called B-cell Integration Cluster (*BIC*) [148]. B cell lymphoma express greater levels of miR-155 together with *BIC* RNA than the control cells. This is because miR-155 and *BIC* RNA are processed from the same primary transcript [149]. By generating *bic/miR*-155 deficient mice, Rodriguez et al provided evidences that absence of *BIC/miR*-155 resulted in lung fibrosis that mimics the picture of complicated autoimmune diseases, reduced amount of immunoglobulin M and low levels of *IL-2* and *IFN-γ*, suggesting a key role for miR-155 in regulating the immune response [150]. Extensive studies have been performed to clarify the role of miR-155 in animal tumor viruses. One example is the reticuloendotheliosis virus strain T (REV-T). Infection of chicken embryo fibroblasts and REV-T-induced B-cell lymphomas demonstrated elevations in miR-155 levels. The authors implemented functional studies to reveal that miR-155 can target JARID2 mRNA (a cell cycle regulator), reflecting the role of miR-155 in promoting cell survival [151]. In a comparable *in vitro* approach, previous studies highlighted the role of miR-181a in MDV-induced lymphoma. In this regard, transfecting miR-181a mimic into MYBL1 cells, a Marek's disease lymphoma cell line, resulted in the reduction of MYBL1 (*v-myb myeloblastosis viral oncogene homolog-like 1*) protein. The study also showed an inhibitory effect of gga-miR-181a on cell proliferation [152]. A similar role was
reported for miR-26a in MDV infection, where it inhibited lymphoma cell proliferation by targeting NEK6 [124]. Another instance is gga-miR-1650, which interacts with the 5' UTR of ALV-J, as evidenced by the suppression of luciferase-reporter vectors carrying the 5' and 3' UTRs of the virus [153]. In bovine medicine, bovine viral diarrhea is a significant economic disease of cattle. The disease is endemic in many parts of the world [154]. The role of miRNA in bovine viral diarrhea infection has been illuminated in two recent studies. In a first one, the authors reported that miR-29b can bind to the 3' UTR of 2 key apoptosis-related genes, caspase-7 and nuclear apoptosis-inducing factor-1, causing their down regulation in Madin-Darby bovine kidney cells. They also showed that miR-29b could target the viral envelope glycoprotein with subsequent suppression of viral replication [155]. In the second study, the same researchers proved that lentivirus-mediated overexpression of the miR-29b precursor reduced replication of bovine viral diarrhea virus in Madin-Darby bovine kidney cells by down-regulating the expression of ATG14 and ATG9A, two important autophagy-associated proteins [156]. These studies emphasize that miR-29b can modulate the pathogenesis of bovine viral diarrhea virus using different mechanisms. In the same direction, PK-15 cells were used to screen for differentially expressed miRNAs during foot and mouth disease virus (FMDV) infection [157]. The analysis revealed that 172 known miRNAs and 72 novel potential miRNAs were differentially expressed, the majority of which were down-regulated. Bioinformatic analyses predicted that the target mRNA genes of these differentially expressed miRNAs were involved in pathways such as cytokine receptor signaling, NOD-like receptor signaling and Toll-like receptor signaling. Another example from the porcine field can be seen in the infection with porcine
reproductive and respiratory syndrome virus (PRRSV). Recently, it was found that PRRSV infection triggered miR-24-3p expression, which down-regulates heme oxygenase-1 mRNA with an overall increase in PRRSV replication in the MARC-145 cell line [158]. On the contrary, miR-26a can reduce the replication of PRRSV virus by activating type I interferon-signaling pathways and augmenting the production of IFN-stimulated genes [159]. A virus inhibitory effect can be also seen for miR-506, via mediating suppression of CD151 mRNA, which is the receptor of PRRSV [160]. Among the in vitro approaches, swine testis cells infected with transmissible gastroenteritis virus-infected uniquely expressed 59 miRNAs. These studies clarified that the differentially expressed miRNAs are involved in multiple cellular and metabolic processes as well as in immune-related functions [161].

5. Perspectives

Several miRNAs have been identified that may affect pathogenesis and outcome of viral infectious diseases of veterinary importance. Nevertheless, many open questions remain. Inter-host differences in susceptibility to a given viral infection are often due to differences in mRNA and, subsequently, protein expression [162], and the role of miRNAs in this regard has received much less attention. Regarding roles of miRNAs in animal susceptibility to viral diseases, highly pathogenic avian influenza (HPAI) is a well-studied example. While infection is asymptomatic in waterfowl, humans and chicken are more susceptible with a concurrent strong inflammatory response and high tissue cytokine levels [163]. In parallel, pathogenicity of HPAI H5N1 viruses varies among various breeds of ducks [164]. In
the same context and as a deviation from the normal ecology of the virus, some recent isolates of H5N1 proved to be lethal for waterfowl species including ducks [165]. Given these facts, comparing the expression patterns of host-encoded miRNAs in response to HPAI in different hosts might explain their difference in susceptibility to the viral infection and help to identify additional elements of the host response that affect disease severity. One research area of great scientific interest and of clinical importance as well is to investigate the role of miRNAs in the host response to viral and bacterial co-infections, such as IAV / *S. pneumoniae* in humans and IAV / *Escherichia coli* in chicken and ducks [166], as the role of host miRNAs in modulating potential synergies between the two pathogens should be investigated, as it may be substantial. Human populations witness the emergence of zoonotic diseases, in particular those caused by viruses that cause varying numbers of human fatalities. It is important to invest more efforts to delineate the role of miRNAs that are associated with these zoonotic viral diseases, especially when considering their cross-species conservation. This will improve our understanding of the complex nature of zoonotic pathogens as well as their potential to further establish inter-human transmission.

6. Conclusions

The discovery of small non-coding RNAs was a turning point in biology. The role of miRNAs has grown with an unprecedented speed from research on worms to a wide variety of physiological and pathological processes in humans and animals. With genome-wide profiling techniques and the tools of bioinformatics, considerable information about miRNAs and their role in animal viral diseases is now available.
The current experimental data on the role of miRNAs in host-virus interaction upon infection of a natural host, in laboratory models and in cell-based systems, indicate that miRNAs can contribute to both pathogenesis and clinical outcome of many diseases affecting animal populations on the individual and farm level (Fig 4). However, a general conclusion on the role of miRNAs cannot be drawn yet. One possibility is that they favor the host as part of the anti-viral response. This could occur in two ways. First, miRNAs could silence viral transcripts through sequence-specific binding, and thus protein expression. Second, they could indirectly modulate host transcripts in a way that creates a less favorable condition for virus propagation and survival. Vice versa, host miRNAs may be beneficial for the viral pathogens if they tend to increase their replication or survival. Finally, they can be beneficial for both sides as in the case of latent viruses. The role of miRNAs in veterinary medicine is receiving more and more attention. Given the global efforts to annotate novel animal-specific miRNAs in online databases (for instance miRBase) together with high-throughput profiling approaches and functional studies, researchers in the field of animal viral infectious diseases will continue to improve their understanding of the role played by miRNAs in infections that threaten animal health and cause economic losses.

List of abbreviations

ALV, avian leucosis virus; BIC, B-cell Integration Cluster; BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; CNS, central nervous system; DGCR8, DiGeorge Syndrome Critical Region 8; DIANA, DNA intelligent analysis; GaHV-2, Gallid herpesvirus 2; HPAI, highly pathogenic avian influenza; IBDV,
infectious bursal disease virus; IAV, influenza A virus; IncRNAs, long-noncoding RNAs; MDV, marek’s disease virus; miRNAs, microRNAs; NLRP3, NOD-like receptor P3; nts, nucleotides; PRRSV, Porcine reproductive and respiratory syndrome virus; pre-miRNA, precursor miRNA; pri-miRNA, primary miRNA; RISC, RNA-induced silencing complex; RNA seq, RNA sequencing; RT-qPCR, reverse transcription quantitative real time PCR; RV, rabies virus; REV-T, reticuloendotheliosis virus strain T; SDN, small RNA degrading nucleases; snoRNA, small nucleolar RNA; SOCS, suppressor of cytokine signaling; TRBP, Tar RNA Binding protein; VEEV, Venezuelan equine encephalitis virus; VKJD, variant Creutzfeldt–Jakob disease; 3'-UTR, 3’ untranslated region.

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Authors’ contributions

M. Samir conceived the project, did the literature search, wrote the initial draft of the manuscript and prepared the figures and tables. L. Vaas participated in the bioinformatics analyses and edited the manuscript. F. Pessler oversaw the project, edited the manuscript, and takes responsibility for the integrity of the data.
Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Differentially expressed host-encoded miRNAs in animal viral infectious diseases of veterinary interest in natural infection and laboratory models. The column “Tissue” refers to the tissue in which the respective profiling study was conducted. Most of the listed miRNAs are non-tissue specific. A (+) sign next to a miRNA indicates that this miRNA have been reported previously to be specific in the corresponding tissue in the column “Tissue” (human data [167]). The column “Function” refers to the function of all miRNAs contained in the corresponding miRNAs column.

<table>
<thead>
<tr>
<th>Disease reservoir</th>
<th>Disease</th>
<th>miRNAs</th>
<th>Function</th>
<th>Tissue</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle, sheep, goat, cat</td>
<td>Prion protein related diseases</td>
<td>MiR-342-3p, MiR-320, let-7b, MiR-328, MiR-128, MiR-139-5p, MiR-146a.</td>
<td>MiR-338-3p and MiR-337-3p</td>
<td>Regulation of pro-inflammatory cytokine production</td>
<td>Brain</td>
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<tr>
<td>Horse</td>
<td>Venezuelan equine encephalitis virus (VEEV)</td>
<td>Various (n=24)</td>
<td>Various (n=43)</td>
<td>Cell death, apoptosis and inflammation</td>
<td>Brain</td>
</tr>
<tr>
<td>Layer chickens</td>
<td>Influenza A virus (H5N3)</td>
<td>MiR-1a, MiR-140, MiR-449</td>
<td>MiR-181a</td>
<td>Regulation of host and viral</td>
<td>Lung nd trachea</td>
</tr>
<tr>
<td>Layer</td>
<td>Virus</td>
<td>MiRNAs</td>
<td>Effect</td>
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<tr>
<td>Layer chickens</td>
<td>Influenza A virus (H5N3)</td>
<td>MiR-445, miR-34b, miR-34c, miR-1a-1, miR-1a-2, miR-1b, miR-449, miR-140</td>
<td>Regulation of host and viral gene expression</td>
<td>Trachea</td>
<td>[64]</td>
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<td>Broiler chickens</td>
<td>Influenza A virus (H5N3)</td>
<td>MiR-449b, miR-460a, miR-206, miR-301 and miR-</td>
<td>Regulation of host and viral gene expression</td>
<td>Lung</td>
<td>[65]</td>
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<tr>
<td>Dog and carnivore</td>
<td>Rabies</td>
<td>MiR-1894-5p, miR-290-3p, miR-1901, miR-207, miR-1896, miR-715, miR-3470b, miR-146b*, miR-203, miR-770-5p</td>
<td>Targeting RIG-1 like receptor, specifically RIF3 a target for miR-203 and TRIM25, a target for miR-207.</td>
<td>Brain</td>
<td>[139]</td>
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<td>Pig</td>
<td>Influenza A virus (H1N2)</td>
<td>MiR-21, miR-15a, miR-206, miR-451, miR-</td>
<td>Inflammation</td>
<td>Lung</td>
<td>[68]</td>
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</table>

1 A complete list of miRNAs is provided in Table S1.
Table S1. Differentially expressed host-encoded miRNAs in animal viral infectious diseases of veterinary interest in natural infection and laboratory models. Column tissue refers to the tissue where the respective profiling study has been conducted. Most of the miRNAs are non-tissue specific. A (+) sign besides a miRNA indicates that this miRNA have been reported previously to be specific in the corresponding tissue in column tissue tissue (human data [65]). The column “function” refers to the function of all miRNAs contained in the corresponding miRNA column.

<table>
<thead>
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<th>Disease reservoir</th>
<th>Disease / pathogen</th>
<th>MiRNAs</th>
<th>Tissue</th>
<th>Function</th>
<th>Ref.</th>
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<tbody>
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<td>miR-155, miR-542–5p, miR-300, miR-203, miR-879, miR-320, miR-27a, miR-136, miR-135a, miR-449a, miR-672, miR-297b-5p, miR-409–3p, miR-126–3p, miR-27b, miR-574–3p, miR-101a, miR-135b, miR-331–3p, miR-339–5p, miR-704, miR-743a, miR-409–5p, miR-501–3p.</td>
<td>miR-455, miR-376b, miR-193, miR-690, miR-129–5p, let-7, miR-181a-1, miR-154, miR-381, miR-15a, miR-27a, miR-801, let-7e, miR-668, miR-106b, miR-206, miR-330, miR-381, miR-30e, miR-30a, miR-322, miR-93, miR-720, miR-598, miR-154, miR-382, miR-10a, miR-33+, miR-125b+, miR-26b, miR-27a, miR-30b, miR-26b, miR-488, miR-30e, miR-126–5p, miR-381, miR-370, miR-217, miR-194, miR-195, miR-24 and miR-801.</td>
<td>Brain</td>
<td>Cell death, apoptosis and inflammation</td>
</tr>
<tr>
<td>Broiler chickens</td>
<td>H5N3</td>
<td>gga-miR-153, gga-miR-34a, gga-miR-202, gga-miR-32, gga-miR-211, gga-miR-19b, gga-miR-18a, gga-miR-18b, gga-miR-155, gga-miR-15a, gga-miR-223, gga-miR-30b, gga-miR-142–3p, gga-miR-106, gga-miR-20a, gga-miR-146a, gga-miR-20b, gga-miR-29a, gga-miR-29c, gga-miR-24, gga-miR-7b, gga-miR-17-5p, gga-miR-23b, gga-miR-17-3p, gga-miR-92.</td>
<td>gga-miR-449b, gga-miR-460a, gga-miR-206, gga-miR-301 and gga-miR-187.</td>
<td>Lung</td>
<td>Impact host and viral gene expression</td>
</tr>
<tr>
<td>Layer chickens</td>
<td>Chickens</td>
<td>Impact immune-related genes</td>
<td></td>
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<tr>
<td>H5N1</td>
<td>gga-miR-429-3p, gga-miR-23b-3p, gga-miR-24-3p, gga-miR-223+, gga-miR-34c-5p, gga-miR-9-5p, gga-miR-499-5p, gga-miR-29b-3p, gga-miR-199-5p, gga-miR-184-3p, gga-miR-22-3p, gga-miR-146b-5p, gga-miR-365-3p, gga-miR-205a, gga-miR-1b-3p, gga-miR-144-3p, gga-miR-204, gga-miR-144-5p, gga-miR-29a-3p, gga-miR-193b-3p, gga-miR-451, gga-miR-2188-5p, gga-miR-7b, gga-miR-133a-3p, gga-miR-122-5p, gga-let-7i-5p, gga-miR-499-5p, gga-miR-23b-3p, gga-miR-30b-5p, gga-miR-200b-3p, gga-miR-100-5p, gga-miR-455-5p, gga-miR-223-3p, gga-miR-203, gga-miR-1329-5p, gga-miR-429-3p, gga-miR-126-5p, gga-miR-30a-3p, gga-miR-31-5p, gga-miR-21-5p, gga-miR-142-3p, gga-miR-214, gga-miR-455-3p, gga-miR-10b-5p, gga-miR-193b-3p, gga-miR-22-5p, gga-miR-146a-5p, gga-miR-146b-5p, gga-miR-1b-3p, gga-miR-144-5p, gga-miR-1329-3p, gga-miR-153-3p, gga-miR-205a+, gga-miR-199-3p, gga-miR-1306-3p, gga-miR-2188-5p, gga-miR-126-3p, gga-miR-21-3p, gga-miR-451, gga-miR-221-5p, gga-miR-204, gga-miR-29a-3p, gga-miR-7b, gga-miR-144-3p, gga-let-7a-5p, gga-miR-144-3p, gga-miR-2188-5p, gga-miR-365-3p, gga-miR-204, gga-miR-222b-3p, gga-miR-451, gga-miR-22-3p, gga-miR-144-5p, gga-miR-22-5p, gga-miR-21-5p, gga-miR-147, gga-miR-1329-5p, gga-miR-203, gga-miR-146b-5p, gga-miR-21-3p, gga-miR-10b-5p, gga-miR-206, gga-miR-1329-3p, gga-miR-122-5p, gga-miR-429-3p, gga-miR-34c-5p, gga-miR-200a-3p, gga-miR-200b-3p, gga-miR-7b, gga-miR-375</td>
<td>H1N1</td>
<td>Spleen, thymus and bursa</td>
<td>Impact immune-related genes</td>
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<tr>
<td>Pig</td>
<td>ssc-miR-155-3p, ssc-miR-95, ssc-miR-221-3p, ssc-miR-9-2, ssc-miR-9-1, ssc-miR-196b-5p, ssc-miR-7, ssc-miR-9-2, ssc-miR-1, ssc-miR-424-3p, ssc-miR-365-5p, ssc-miR-542-5p, ssc-miR-450b-5p, pulmonary alveolar macrophage</td>
<td>[69]</td>
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</tr>
<tr>
<td>9-1, ssc-miR-221, ssc-miR-4331, ssc-miR-196b-5p, ssc-miR-4334-3p, ssc-miR-486</td>
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| ssc-miR-146b, ssc-miR-193a-5p, ssc-miR-542-3p, ssc-miR-30c, ssc-miR-505, ssc-miR-1306-5p, ssc-miR-374b-3p, ssc-miR-18a, ssc-miR-425-3p, ssc-miR-1306-3p, ssc-miR-425-5p, ssc-miR-92a, ssc-miR-1839-5p, ssc-miR-1307, ssc-miR-22-5p, ssc-miR-193a-3p, ssc-miR-128, ssc-miR-219, ssc-miR-191, ssc-miR-27b-5p, ssc-miR-30b-5p, ssc-miR-24-1-5p, ssc-miR-30a-3p, ssc-miR-27a, ssc-miR-2320-5p, ssc-miR-99a, ssc-miR-500, ssc-miR-935, ssc-miR-30b-3p, ssc-miR-664-5p, ssc-miR-30a-5p, ssc-miR-450b-3p, ssc-miR-339-5p, ssc-miR-125b, ssc-let-7c, ssc-miR-362, ssc-miR-423-5p, ssc-miR-205, ssc-miR-320, ssc-miR-149, ssc-miR-27b-3p, ssc-miR-146b, ssc-miR-424-5p, ssc-miR-328, ssc-miR-1, ssc-miR-423-3p, ssc-miR-326, ssc-miR-23a, ssc-miR-532-3p, ssc-miR-30c, ssc-miR-664-3p, ssc-miR-503, ssc-miR-30d, ssc-miR-505, ssc-miR-744, ssc-miR-574, ssc-miR-542-3p, ssc-miR-1343, ssc-miR-365-3p, ssc-miR-193a-5p, ssc-miR-450c-5p, ssc-miR-450a, ssc-miR-450b-5p, ssc-miR-365-
<table>
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<th>Dog (beagles)</th>
<th>H3N2</th>
<th>Lung and trachea</th>
</tr>
</thead>
<tbody>
<tr>
<td>cfa-miR-122, cfa-miR-127, d’a-miR-133a, cfa-miR-133c, d’a-miR-134, cfa-miR-135a-5p, cfa-miR-145, cfa-miR-182, cfa-miR-183, cfa-miR-1838, cfa-miR-190b, cfa-miR-206, cfa-miR-34b, cfa-miR-34c, cfa-miR-365, cfa-miR-378, cfa-miR-381, cfa-miR-96, cfa-miR-127, cfa-miR-134, cfa-miR-135a-5p, cfa-miR-136 cfa-miR-184, cfa-miR-190b, cfa-miR-206, cfa-miR-22 cfa-miR-30a, d’a-miR-323, cfa-miR-34b, cfa-miR-34c cfa-miR-376a, cfa-miR-380, cfa-miR-381, cfa-miR-410 cfa-miR-494, cfa-miR-497, d’a-miR-551a, cfa-miR-708, cfa-miR-758, cfa-miR-9, cfa-miR-96</td>
<td>cfa-miR-107, cfa-miR-1, cfa-miR-139, cfa-miR-142, cfa-miR-144, cfa-miR-146b, cfa-miR-190a, cfa-miR-221, cfa-miR-222, cfa-miR-23a, cfa-miR-27a, cfa-miR-29a, cfa-miR-345, cfa-miR-451, cfa-miR-7, cfa-miR-1307, cfa-miR-138a, cfa-miR-139, fa-miR-142 cfa-miR-146b, cfa-miR-147, cfa-miR-150, cfa-miR-19b cfa-miR-20b, cfa-miR-223, cfa-miR-23b cfa-miR-371, cfa-miR-451, cfa-miR-660, cfa-miR-7</td>
<td>[168]</td>
</tr>
</tbody>
</table>
Table S2. Host-encoded miRNAs that are commonly expressed upon IAV infection in humans, mice, chicken and pig. Individual miRNAs were retrieved from the literature. The literature search was conducted with PubMed, using “influenza” AND “miRNA” OR “microRNA” as key words.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Tissue/organ</th>
<th>MicroRNAs</th>
<th>IAV strain</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken</td>
<td>Lung and trachea</td>
<td>gga-miR-1a, gga-miR-140, gga-miR-449, gga-miR-181a.</td>
<td>H5N3</td>
<td>[64]</td>
</tr>
<tr>
<td></td>
<td>Trachea</td>
<td>gga-miR-445, gga-miR-34b, gga-miR-34c, gga-miR-1a-1, gga-miR-1a-2, gga-miR-1b, gga-miR-449, gga-miR-140, gga-miR-181a.</td>
<td>H5N3</td>
<td>[64]</td>
</tr>
<tr>
<td></td>
<td>Immune-organs (Spleen, thymus and bursa of fabrius)</td>
<td>gga-miR375, gga-miR7b, gga-miR200b-3p, gga-miR200a-3p, gga-miR34c-5p, gga-miR429-3p, gga-miR122-5p, gga-miR1329-3p, gga-miR206, gga-miR10b-5p, gga-miR21-3p, gga-miR146b-5p, gga-miR203, gga-miR1329-5p, gga-miR147, gga-miR21-5p, gga-miR22-5p, gga-miR144-5p, gga-miR22-3p, gga-miR451, gga-miR222b-3p, gga-miR204, gga-miR2188-5p, gga-miR144-3p, gga-let-7a-5p, gga-miR223, gga-miR184-3p, gga-miR383-5p, gga-miR2954, gga-let-7j-5p, gga-miR144-3p, gga-miR29a-3p, gga-miR221-5p, gga-miR126-3p, gga-miR1308-3p, gga-miR199-3p, gga-miR205a, gga-miR153-3p, gga-miR1b-3p, gga-miR146a-5p, gga-miR193b-3p, gga-miR455-3p, gga-miR214, gga-miR142-3p, gga-miR31-5p, gga-miR30a-3p, gga-miR126-5p, gga-miR455-5p, gga-miR100-5p, gga-miR301b-3p, gga-miR30b-5p, gga-miR218b-3p, gga-miR23b-5p, gga-miR499-5p, gga-miR128-3p, gga-miR29c-3p, gga-miR20b-3p, gga-miR133a-3p, gga-miR199-5p, gga-miR29b-3p, gga-miR9-5p, gga-miR24-3p, gga-miR16c-5p, gga-miR106-5p, gga-miR301b-5p, gga-miR92-5p, gga-miR18b-5p, gga-miR363-3p, gga-miR20b-5p.</td>
<td>H5N1</td>
<td>[67]</td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>gga-miR153, gga-miR34a, gga-miR202, gga-miR32, gga-miR211, gga-miR19b, gga-miR18a, gga-miR18b, gga-miR155, gga-miR15a, gga-miR223, gga-miR30b, gga-miR142-3p, gga-miR106, gga-miR20a, gga-miR146a, gga-miR20b, gga-miR29a, gga-miR29c, gga-miR24, gga-miR7b, gga-miR17-5p, gga-miR23b, gga-miR17-3p, gga-miR16, gga-miR144-5p, gga-miR22-3p, gga-miR146a, gga-miR20b, gga-miR155, gga-miR1b, gga-miR142-3p.</td>
<td>H5N3</td>
<td>[65]</td>
</tr>
<tr>
<td>Mice</td>
<td>Lung</td>
<td>mmu-miR5099, mmu-miR21a-3p, mmu-miR6931-5p, mmu-miR6968-5p, mmu-miR7002-5p, mmu-miR5128, mmu-miR711, mmu-miR7671-3p, mmu-miR7003-5p, mmu-miR6908-5p, mmu-miR3093-3p, mmu-miR6988-5p, mmu-miR6910-5p, mmu-miR6909-5p, mmu-miR3087-5p, mmu-miR1946a, mmu-miR1946b, mmu-miR2137, mmu-miR21a-5p, mmu-miR3473a, mmu-miR5622-3p, mmu-miR3473e, mu-mmu-miR3473b, mmu-miR7666-3p, mmu-miR147-3p, mmu-miR92a-1-5p, mmu-miR3473f, mmu-miR6980-5p, mmu-miR7667-3p, mmu-miR7a-5p, mmu-miR6955-3p, mmu-miR7052-5p, mmu-miR30c-1-3p, mmu-miR34b-3p, mmu-miR92b-3p, mmu-miR149-5p, mmu-miR375-3p, mmu-miR34c-3p, mmu-miR449a-5p, mmu-miR449c-5p, mmu-miR411-3p, mmu-miR431-5p, mmu-miR744-3p, mmu-miR205-5p, mmu-miR208a-5p, mmu-miR299a-3p</td>
<td>H1N1, H1N1(Tx/91), Pan H1N1, H5N2</td>
<td>[84, 85, 169, 170]</td>
</tr>
<tr>
<td>Spleen</td>
<td>mmu-miR15b-3p, mmu-miR24-2-5p, mmu-miR331-3p, mmu-miR124-3p, and mmu-miR337-5p, mmu-miR333-3p, mmu-miR124-3p, mmu-miR337-5p, mmu-miR375-3pand, mmu-miR1298-5p</td>
<td>H1N1(A/Swine/GD/2/12)</td>
<td>[87]</td>
<td></td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>mmu-miR331-3p, mmu-miR124-3p, and mmu-miR337-5p</td>
<td>H1N1 (PR8) and H3N2</td>
<td>[89]</td>
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<tr>
<td>Peripheral blood mononuclear cells (PBMCs)</td>
<td>hsa-miR-146b-5p, has-miR-148a, hsa-miR-150, hsa-miR-31, hsa-miR-155, has-miR-29a, has-miR-29b, hsa-miR-342-5p and hsa-miR-886-3p</td>
<td>H1N1 (2009)</td>
<td>[171]</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>Whole blood</td>
<td>hsa-miR122*, hsa-miR1224-3p, hsa-miR1248, hsa-miR1249, hsa-miR1258, hsa-miR125a-3p, hsa-miR1260, hsa-miR1270, hsa-miR1297, hsa-miR1304, hsa-miR130b*, hsa-miR145*, hsa-miR146b-3p, hsa-miR150, hsa-miR152, hsa-miR181a-2*, hsa-miR200b, hsa-miR204, hsa-miR208b, hsa-miR210, hsa-miR221*, hsa-miR222*, hsa-miR223*, hsa-miR224*, hsa-miR298, hsa-miR299-5p, hsa-miR29b-1*, hsa-miR301b, hsa-miR324-3p, hsa-miR335*, hsa-miR363*, hsa-miR374b, hsa-miR377, hsa-miR377*, hsa-miR454, hsa-miR483-5p, hsa-miR490-3p, hsa-miR491-3p, hsa-miR492, hsa-miR498, hsa-miR513a-3p, hsa-miR513b, hsa-miR516b, hsa-miR525-5p, hsa-miR542-3p, hsa-miR548d-5p, hsa-miR548e, hsa-miR553, hsa-miR556-3p, hsa-miR585, hsa-miR589</td>
<td>H1N1</td>
<td>[82]</td>
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miR590-5p, hsa-miR597, hsa-miR600, hsa-miR601, hsa-miR618, hsa-miR625*, hsa-miR627, hsa-miR630, hsa-miR635, hsa-miR659, hsa-miR664, hsa-miR675, hsa-miR767-5p, hsa-miR874, hsa-miR890, hsa-miR920, hsa-miR921, hsa-miR92a, hsa-miR92a-2*, hsa-miR934, hsa-miR941, hsa-miR944, hsa-miR98, hsa-miR99a, let-7a, let-7a*, let-7b, let-7b*, let-7c, let-7d*, let-7e, let-7f, hsa-miR100, hsa-miR106a, hsa-miR106b, hsa-miR107, hsa-miR1246, hsa-miR125a-5p, hsa-miR125b, hsa-miR125b-1*, hsa-miR125b-2*, hsa-miR1261, hsa-miR1265, hsa-miR1284, hsa-miR1285, hsa-miR1287, hsa-miR1290, hsa-miR1299, hsa-miR130b, hsa-miR138-2*, hsa-miR140-3p, hsa-miR146a, hsa-miR148b, hsa-miR149*, hsa-miR151-3p, hsa-miR151-5p, hsa-miR15b, hsa-miR16-2*, hsa-miR17, hsa-miR17*, hsa-miR181a, hsa-miR183, hsa-miR183*, hsa-miR185, hsa-miR185*, hsa-miR186, hsa-miR18a, hsa-miR18a*, hsa-miR18b, hsa-miR1908, hsa-miR191, hsa-miR192, hsa-miR200c, hsa-miR208a, hsa-miR21, hsa-miR22, hsa-miR222, hsa-miR223, hsa-miR23a, hsa-miR25, hsa-miR26a, hsa-miR26b, hsa-miR28-5p, hsa-miR29a, hsa-miR300, hsa-miR302e, hsa-miR30a, hsa-miR30b, hsa-miR30c, hsa-miR30d, hsa-miR30e*, hsa-miR32*, hsa-miR320a, hsa-miR320b, hsa-miR320c, hsa-miR320d, hsa-miR324-5p, hsa-miR331-3p, hsa-miR339-3p, hsa-miR339-5p, hsa-miR340, hsa-miR342-3p, hsa-miR342-5p, hsa-miR345, hsa-miR34b, hsa-miR361-5p, hsa-miR362-5p, hsa-miR363, hsa-miR375, hsa-miR378, hsa-miR421, hsa-miR422a, hsa-miR423-3p, hsa-miR423-5p, hsa-miR425, hsa-miR425*, hsa-miR444, hsa-miR448, hsa-miR486-5p, hsa-miR487b, hsa-miR501-5p, hsa-miR519d, hsa-miR519e, hsa-miR519e*, hsa-miR520d-5p, hsa-miR532-5p, hsa-miR574-3p, hsa-miR574-5p, hsa-miR576-3p, hsa-miR625, hsa-miR628-3p, hsa-miR629, hsa-miR629*, hsa-miR656, hsa-miR665, hsa-miR668, hsa-miR720, hsa-miR744, hsa-miR765, hsa-miR766, hsa-miR92b, hsa-miR93, hsa-miR93*.
| Serum | hsa-miR1290, hsa-miR1275, hsa-miR1260, hsa-miR574-3p, hsa-miR454, hsa-miR148a, hsa-miR539, hsa-miR223, hsa-miR142-5p, hsa-miR485-3p, hsa-miR548c-5p, hsa-miR17, hsa-miR484, hsa-miR652, hsa-miR660, hsa-miR20b, hsa-miR511, hsa-miR26b, hsa-miR210, hsa-miR489, hsa-miR22*, hsa-miR15a*, hsa-miR106a, hsa-miR31-5p, hsa-miR194, hsa-miR139-5p, hsa-miR193a-5p, hsa-miR29a, hsa-miR24, hsa-miR140-5p, hsa-miR28-3p, hsa-miR151-3p, hsa-miR192, hsa-miR190b, hsa-miR143, hsa-miR425, hsa-miR101, hsa-miR146b-5p, hsa-miR26a, hsa-miR425*, hsa-miR25, hsa-miR186, hsa-miR191, hsa-miR9*, hsa-miR494, hsa-miR323-3p, hsa-miR34a, hsa-miR125a-5p, hsa-miR152, hsa-miR19b, hsa-miR532-5p, hsa-miR32-4p, hsa-miR106b, hsa-miR886-5p, hsa-miR744, hsa-miR451, hsa-miR130a, hsa-miR200c, hsa-miR625*, hsa-miR532-3p, hsa-miR720, hsa-miR338-5p, hsa-miR324-3p, hsa-miR203, hsa-miR30a-3p, hsa-miR345, hsa-miR27a, hsa-miR30e-3p, hsa-miR664, hsa-miR340, hsa-miR495, hsa-miR21, hsa-miR335, hsa-miR181a, hsa-miR218, hsa-miR223*, hsa-miR93*, hsa-miR151-5p, hsa-miR27b, hsa-miR199a-5p, hsa-miR505, hsa-miR93, hsa-miR144*, hsa-let-7g, hsa-miR130b, hsa-miR128, hsa-miR132, hsa-miR331-3p, hsa-miR375, hsa-miR125b, hsa-miR148b, hsa-miR19b-1*, hsa-miR122, hsa-miR486-5p, hsa-miR409-3p, hsa-miR142-3p, hsa-miR150, hsa-let-7b, hsa-miR541*, hsa-miR221, hsa-miR649, hsa-miR92a, hsa-miR518f, has-hsa-miR155, hsa-miR301a, hsa-miR486-3p, hsa-miR10a, hsa-miR642, hsa-miR654-3p, hsa-let-7e, hsa-miR548c-3p, hsa-miR29b, hsa-miR548p, hsa-miR1243. | H7N9 |
|---|---|
| Lung | ssc-miR21, ssc-miR15a, ssc-miR206, ssc-miR451, ssc-miR223, ssc-miR146. | H2N1 |
| Pig | ssc-miR424-3p, ssc-miR542-5p, ssc-miR365-5p, ssc-miR450b-5p, ssc-miR450a, ssc-miR450c-5p, ssc-miR193a-5p, ssc-miR365-3p, ssc-miR1343, ssc-miR542-3p, ssc-miR574, ssc-miR74, ssc-miR505, ssc-miR30d, ssc-miR503, ssc-miR664-3p, ssc-miR30c, ssc-miR532-3p, ssc-miR23a, ssc-miR326, ssc-miR423-3p, ssc-miR1, ssc-miR32, ssc-miR424-5p, ssc-miR146b, ssc-miR27b-3p, ssc-miR149, ssc-miR320 | H1N1 swine influenza virus | [83] [172] [69]
miR205, ssc-miR423-5p, ssc-miR362, ssc-miR125b, ssc-miR339-5p, ssc-miR450b-3p, ssc-miR30a-5p, ssc-miR664-5p, ssc-miR30b-3p, ssc-miR935, ssc-miR500, ssc-miR99a, ssc-miR230-5, ssc-miR27a, ssc-miR30a-3p, ssc-miR24-1-5p, ssc-miR30b-5p, ssc-miR27b-5p, ssc-miR191, ssc-miR219, ssc-miR128, ssc-miR193a-3p, ssc-miR22-5p, ssc-miR1307, ssc-miR1839-5p, ssc-miR92a, ssc-miR425-5p, ssc-miR1306-3p, ssc-miR425-3p, ssc-miR18a, ssc-miR374b-3p, ssc-miR1306-5p, ssc-miR486, ssc-miR4334-3p, ssc-miR196b-5p, ssc-miR4331, ssc-miR221, ssc-miR9-1, ssc-miR9-2, ssc-miR7, ssc-miR196b-5p, ssc-miR9-1, ssc-miR9-2, ssc-miR221-3p, ssc-miR95, ssc-miR155-3p, ssc-miR505, ssc-miR30c, ssc-miR542-3p, ssc-miR193a-5p, ssc-miR146b, ssc-miR450b-5p, ssc-miR542-5p, ssc-miR365-5p, ssc-miR424-3p, ssc-miR1, ssc-miR365-3p, ssc-miR424-3p, ssc-miR450a, ssc-miR574, ssc-miR450c-5p, ssc-miR744, ssc-miR542-5p, ssc-miR532-3p, ssc-miR365-5p, ssc-miR193a-5p, ssc-miR1343, ssc-miR450b-5p, ssc-miR139-5p, ssc-miR149, ssc-miR542-3p, ssc-miR935, ssc-miR503, ssc-miR2320-5p, ssc-miR30b-3p, ssc-miR1306-5p, ssc-miR374b-3p, ssc-miR99a, ssc-miR505, ssc-miR219, ssc-miR30d, ssc-miR664-3p, ssc-miR92a, ssc-miR664-5p, ssc-miR23a, ssc-miR128, ssc-miR181b, ssc-miR125b, ssc-miR425-3p, ssc-let-7c, ssc-miR423-5p, ssc-miR24-1-5p, ssc-miR320, ssc-miR148a-3p, ssc-miR27b-3p, ssc-miR15b, ssc-miR423-3p, ssc-miR500, ssc-miR1277, ssc-miR185, ssc-miR362, ssc-miR18b, ssc-miR326, ssc-miR22-5p, ssc-miR30c, ssc-miR345-5p, ssc-miR143-3p, ssc-miR4331, ssc-miR4332, ssc-miR4334-3p, ssc-miR708-5p, ssc-miR7
Figures

Figure 1. The classic endogenous miRNA pathway and mechanisms of action. In the nucleus, pri-miRNA is transcribed by RNA polymerase II and further processed by the Drosha enzyme to produce pre-miRNA, which is then transported into the cytoplasm. There it is cleaved by the Dicer enzyme into the miRNA duplex. The guide strand is uploaded onto the RNA-induced silencing complex (RISC) to regulate gene expression by causing either target mRNA degradation or translation repression. Cytoplasmic-nuclear shuttle is possible for some miRNAs (e.g. miR-29b, miR-370 and miR-320). Adapted from Wikimedia commons (“Difference DNA RNA-EN.SVG”).
Figure 2. Numbers of currently annotated mature and immature miRNAs in selected animal species. The most recent numbers of immature (black bars) and mature (grey bars) miRNAs in animals of veterinary importance are plotted on the y-axis. Values for humans and mice are shown for comparison. Data were obtained from miRBase version 21.
Figure 3. miRNAs that are regulated in IAV infection in humans, chicken, pig, and mice. (A) Venn diagram depicting miRNAs that are differentially and commonly expressed in the four species. (B) Heat map showing the pathways regulated by two of the three miRNAs regulated in all four species. (C) Sequence alignment of the premature forms of two well characterized miRNA that are regulated in all four species (pre-mir-223) or only in the natural hosts only (pre-mir-155) (5' to 3' orientation). While the mature form of these miRNAs is highly conserved across the 4 species (represented by stars above the sequences), the premature form shows a lesser degree of conservation. The seed region of each miRNA is highlighted in grey.
Figure 4. Schematic representation of infectious viral diseases of farm and pet animals (in natural hosts and in laboratory models) where miRNAs have been reported to play a role. Examples of these diseases are bovine spongiform encephalopathy (BSE); feline spongiform encephalopathy (FSE); rabies; influenza A virus (IAV); infectious bursal disease virus (IBDV); Marek’s disease virus (MDV); Avian leukosis virus (ALV); scrapie and Venezuelan equine encephalitis (VEE). Adapted from Manz et al. 2013, J Virol, (13): 7200-9.
3.2. Manuscript II
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Title
Small non-coding RNAs associated with viral infectious diseases of veterinary importance: potential clinical applications

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Abstract

MicroRNAs (miRNAs) represent a class of small non-coding RNA molecules that can regulate mRNAs by inducing their degradation or by blocking translation. Considering that miRNAs are ubiquitous, stable, and conserved across animal species, it seems feasible to exploit them for clinical applications. Unlike in human viral diseases, where some miRNA-based molecules have progressed to clinical application, in veterinary medicine this concept is just starting to come into view. Clinically, miRNAs could represent powerful diagnostic tools to pinpoint animal viral diseases and/or prognostic tools to follow up disease progression or remission. Additionally, the possible consequences of miRNA dysregulation make them potential therapeutic targets and open the possibilities to use them as tools to generate viral disease-resistant livestock. This review presents an update of pre-clinical studies on using small non-coding RNAs to combat viral diseases that affect pet and farm animals. Moreover, we discuss the possibilities and challenges of bringing these bench-based discoveries to the veterinary clinic.

Keywords
Animals, animal viruses, biomarkers, clinical application, infectious diseases, miRNA, small non-coding RNAs, veterinary science
1. Introduction

Small non-coding RNAs (sncRNAs) are classes of short RNAs, which do not encode proteins, but rather perform regulatory functions by engaging target transcripts through sequence-specific interactions. Among these, miRNAs are single-stranded molecules roughly 22 nucleotides (nts) in length (2). The regulatory network and function of miRNAs are based on the fact that more than one miRNA species can target the same mRNA (cooperativity) and that one miRNA can target hundreds of mRNA species (multiplicity) (3). The binding of miRNAs to the 3’ untranslated region of particular mRNA leads to either mRNA degradation or protein translation repression (4). MiRNAs can be highly regulated both in pattern and degree of expression across multiple animal diseases. Targeting hundreds of host and pathogen encoded genes, a single miRNA can influence the gene networks essential for development and progression of a disease condition (5). This, coupled with their high degree of conservation, has made miRNAs attractive candidates for clinical application to combat pathogenic animal viruses. Being highly stable, they can be used as disease biomarkers (6). The availability of chemically synthesized miRNA mimics and agonists and vector-based RNA interference (RNAi) technology raised the idea of therapies based on non-coding RNA and made it feasible to utilize these approaches to create genetically modified animal breeds that are resistant to certain viral pathogens. In this review, we summarize the current state of laboratory studies geared towards clinical applications of small non-coding RNAs (miRNA, small interfering RNAs [siRNAs] and short hairpin RNAs [shRNAs]) to diagnose and combat viral diseases that affect animals of veterinary importance and may thus impact animal and human health.
2. MiRNAs as candidates for clinical application to combat animal viral diseases

2.1. Potential biomarkers

The emerging correlation between miRNA expression and disease pathogenesis and outcomes suggests the potential use of miRNAs as biomarkers. In the first report that described the role of a miRNA as a diagnostic and prognostic marker in humans, Takamizawa et al. demonstrated that in patients with lung cancer, lower let-7 levels predicted a significantly worse prognosis after potentially curative resection (7). The intense use of advanced genomic technologies has resulted in rapid progress in human personalized medicine, where biomarker studies play a central role. Similar research interest has been emerging in veterinary medicine, albeit with some delay. Indeed, Henry et al. reported in 2010 that biomarker studies in veterinary medicine were still lagging behind those in humans (8). Biomarker research in the field of veterinary medicine focuses on the health and welfare of farm and companion animals as well as broader aspects such as the biosafety of animal-derived food and milk production. Generally, the potential applications for biomarkers in veterinary clinics include diagnosis, staging, prognosis and monitoring responses to therapy. Although several well-established biomarkers have been recognized for a number of veterinary viral diseases, there are still many barriers. As one example of many, lack of specificity has been recorded when using acute phase proteins (APPs) as biomarkers in pig, horse and cattle suffering from inflammatory conditions that may have infectious etiologies, such as foot- and -mouth disease virus (FMDV) infection, porcine reproductive and respiratory syndrome virus infection, pneumonia, arthritis, enteritis, and post-castration inflammation (9-11). The concentration of some
available biomarker molecules is affected by animal age (12). Thus, there is a need for additional, improved biomarkers for animal diseases. There is growing recognition that miRNAs may provide a specific signature that reflects the existence of a given clinical state. In this regard, the results of profiling the fluctuation of miRNA expression levels in infected organs, tissues or single cells compared to uninfected ones throughout the course of a disease might reflect severity and outcome of the disease, including the likelihood of response to a given therapy. As biomarkers, miRNAs represent ideal candidates owing to their biological and clinical relevance, practicality, and consistent correlation with disease activity. The biological rational behind using miRNAs as biomarkers arises from their involvement in diverse physiological and pathological processes. MiRNAs are extremely practical, with many advantages over other currently used biomarkers. For instance, there are efforts to develop them into diagnostics for the differentiation between viral and bacterial infections, each of which typically requires different interventions such as quarantining versus feeding of antibiotics. The smaller number of identified miRNAs (1), compared to the approximately 30 000 protein-encoding genes currently known, implies that computational approaches dealing with miRNAs would be simpler and would require fewer resources than proteomics- or mRNA-based approaches. Another merit of miRNAs is their resistance to degradation by ribonucleases. For instance, they are stable in formalin-fixed, paraffin-embedded tissue (FFPE) independent of formalin fixation time and duration of tissue block storage (13). In contrast, mRNAs are highly fragmented and unstable in FFPE, which is problematic when FFPE is the only available sample type or when long storage of FFPE blocks has led to mRNA degradation (13). MiRNAs can be detected in a large number of
easily accessible samples such as tissue biopsies, whole blood, blood cells, cerebrospinal fluid, saliva, urine, and other body fluids. Circulating miRNAs have proven to be highly resistant against RNAse activity, extreme pH and temperature, and certainly more so than mRNAs. This is, at least in part, because they are often contained in lipid vesicles (microvesicles and exosomes) or bound by RNA-binding proteins (6). Additionally, miRNAs resist prolonged exposure to room temperature and repeated freezing/thawing cycles. Some miRNAs may be uniquely expressed only in specific body fluids, as exemplified by miR-224 (plasma/serum), miR-637 (tears), miR-193b (breast milk), and miR-508-5p (seminal fluid) (14). As opposed to miRNAs, proteins are a much more complex family of molecules due to use of alternate reading frames, splice variants and various post-translational modifications, and many proteins of interest are of low abundance and/or may display major sequence variations among clinically relevant species (6).

2.1.1. MicroRNAs as shared biomarkers in human and animal disease

In order to assess the role of miRNAs as a class of shared biomarkers, it is important to investigate the cross-species conservation and regulation of the same miRNA species or miRNA family. Most of the annotated miRNAs are evolutionarily conserved among a variety of organisms, particularly in their mature form, suggesting that the majority of miRNAs constitute a large class of predominantly orthologous or homologous molecules. As exemplified by miR-146b-5p, cross-species variation in miRNA sequence is typically observed in one or two nucleotides in the periphery of the mature form and in its 3´ untranslated region (UTR), i.e. away from the highly conserved seed region (Fig 1). This unique conservation pattern
might be attributed to the conservation of their genomic origin. It has been reported
that a consensus motif of 7-8 nucleotides upstream and downstream of the pre-
mRNA hairpin was found to be conserved among nematodes (15). Researchers
from Slovenia and the USA have put together a catalogue to describe the integrated
assembly of intragenic miRNAs and their host genes in humans, mouse, and chicken
(16). They showed that several miRNA genes were located within homologous
areas, which implies that miRNA co-localization, co-expression and potential co-
regulation may be conserved broadly across evolution and thus be applicable to both
animal and human diseases. In the same context, previous studies reported that 300
canine miRNAs are homologs of annotated human miRNAs, and that miRNA
clusters are usually conserved between humans and dogs (17). Using next
generation sequencing, Li et al. indicated that miRNAs in immune organs of chicken
and duck were about 99% conserved (18). To gain further insights into miRNAs that
are shared between species and might be used as common biomarkers, we selected
a group of miRNAs that are commonly expressed upon Influenza A virus (IAV)
infection in humans and chicken. These miRNAs were further analyzed with
miRviewer (19), a database that includes all known miRNAs of currently annotated
animal genomes. William Pearson's aligning program was used to assess the
degree of conservation of mature miRNAs between the two species (Table 1). The
percentage of sequence identity was further confirmed by the Bioedit sequence
alignment editor (20). Indeed, there is a high degree of conservation of most miRNAs
between the two species (Table 1). For some miRNAs, there are sequence
differences between humans and chicken in the form of deletion or addition of extra
nucleotides, but these are mostly located outside the seed region. We speculate that
conserved miRNAs might be the most promising candidates for universal biomarkers that may help in simultaneously pinpointing a given disease state in both species. In contrast, the non-conserved miRNAs might have the least contributory role as universal biomarkers, but may play roles in more species-specific aspects of disease pathogenesis and outcomes. Apart from the sequence conservation of miRNAs, the presence of the same miRNA signatures in both humans and animals upon contracting the same infectious disease supports the concept of common biomarkers. Indeed, our previous analysis showed that three miRNAs (miR-18a-5p, miR-223-3p, and miR-451-3p) were commonly expressed upon IAV infection in humans, pig, chicken, and mice. An additional four miRNAs (miR-18b-3p, miR-22-5p, miR-30a-3p and miR-155-3p) were commonly expressed in IAV infection when only the natural hosts (humans, chicken and pig) were considered (Samir et al., manuscript submitted). Taken together, these data indicate that cross-species comparisons of human and animal miRNA expression profiles as well as their conservation could provide unique opportunities to exploit miRNAs as universal biomarkers and also underline both commonalities and differences in pathology of the same disease in different species.

2.1.2. Limitations in using miRNAs as biomarkers to combat viral diseases

While using miRNAs as novel biomarkers in the veterinary field represents a promising concept, it comes with unique challenges. One challenge is the presence of miRNA isomers (isomiRs), i.e. forms of miRNA that differ slightly from the annotated mature sequence. They are likely created by the enzymatic addition of
adenine, cytidine or uridine and/or imprecise cleavage by the enzymes Dicer or Drosha (21). Observational studies have shown that isomiRs can be regulated upon infection and hence are biologically and functionally meaningful (22). There is some functional overlap between miRNAs and their isomers (23). However, most miRNA annotation tools ignore these isomers by considering them as either noise or sequencing artifacts. The presence of isomiRs might affect miRNA stability and repression capability (24) and therefore reduce their value as biomarkers. When looking for miRNAs as circulating biomarkers, it is important to consider the low miRNA yield (1-10 ng/µl) in body fluids such as plasma, serum and urine (25). While some studies suggested that plasma contains a higher miRNA concentration than serum (26), a growing body of evidence has indicated that using serum as a biological sample for miRNA biomarker studies might be biased (27). This is because the stress that blood cells are exposed to during coagulation results in the release of nucleic acids, including miRNAs into the serum, which may change the true repertoire of circulating serum miRNA giving rise to biased values. With this in mind, the lack of correlation in detection of some miRNAs in plasma and serum is not unexpected. Prior centrifugation of the blood and hemolysis might affect the amount and stability of the target miRNA and require some modifications in the isolation protocols (26). Moreover, difficulties in miRNA extraction can compromise yield and quality (28). Considering that miRNAs are differentially expressed among different animal breeds (29, 30), it is plausible that miRNA levels may differ among different animal breeds if they contract the same disease. This is also apparent among humans where the expression of some miRNAs was found to be related to ethnicity. In this regard, receiver operating characteristic (ROC) curve analysis
indicated that let-7c predicted the onset of breast cancer with an area under the curve (AUC) of 0.99 in African Americans while having an AUC of only 0.78 in Caucasians. On the other hand, the best predictor in Caucasians was miR-589 with an AUC of 0.85 (31). This holds true for other biomarkers as well. Two reports documented a significant breed effect on the level of plasma NT-proBNP, a diagnostic marker in dogs with degenerative mitral valve disease (32, 33).

2.2. RNA interference (RNAi) as a promising tool for therapeutic intervention

RNAi is a form of post-transcriptional gene silencing that can function in a broad range of eukaryotic species. Fighting animal viruses with RNAi can be mediated by using siRNA or miRNAs, although the origin of both molecules is different. While miRNAs are endogenously produced throughout two processing steps in nucleus and cytoplasm, siRNA can be exogenously introduced directly into the cytoplasm as a double strand (34). Once in the cytoplasm, both miRNA and siRNA pass through the same processing steps where they are digested by the Dicer enzyme to form a duplex. Only one strand of this duplex is translocated into the RNA induced silencing complex (RISC) to mediate its function (35). While siRNA forms a perfect complementarity with its target mRNA, causing its cleavage, miRNAs tend to bind to their mRNA targets less perfectly leading to repression in translation. Historically, laboratory-based experiments of using RNAi to block the replication of animal viruses started early on, namely in 2003 against IAV (36). Harnessing miRNAs for therapeutic use will rely on using gain and loss of function and is linked to the expression level of the miRNA (35). MiRNAs that are beneficial for the virus and are up-regulated upon infection might be blocked using classic or modified anti-miRNAs.
(37). In this regard, antagomiRs (cholesterol conjugated anti-miRNAs) have been used *in vitro* and *in vivo* (38). Chemically modified nucleotides such as locked nucleic acid (LNA) and other modifications have made it conceivable to design more stable and specific oligonucleotides. In an *in vivo* system, reports stated that the effect of using LNA proved to be long-lasting and safe, as neither toxicity associated with LNA nor histopathological changes were detected (39). Although there are attempts to down-regulate the Dicer or Drosha enzymes as indirect ways to block miRNAs, this mechanism should be strictly controlled since blocking these enzymes will affect the entire miRNA population (40). In cases where miRNAs tend to inhibit virus replication, a therapeutic approach could be to over-express these miRNAs or to restore their levels. In this context, synthetic miRNA mimics resembling mature miRNAs that could be recognized by RISC would be a suitable tool (40). The *in vivo* delivery of miRNA modalities to specific cells has remained a substantial barrier. Using viruses or virus-like vectors might be innovative approaches since viruses have evolved over many generations to infect certain cells and to deliver foreign RNA, including miRNA, in a tissue- and cell-specific manner (41). Viral vectors can express pri-miRNA or pre-miRNA-like structures or even mature miRNA. Here, RNA viruses of both nuclear and cytoplasmic origin have been utilized (42). miRNAs may have advantages over siRNAs as therapeutic candidates. In spite of having off-target effects, miRNAs bind to their targets with partial complementarity (43) and, thus, likely tackle the high rate of mutation seen in many viruses better than siRNAs. Also, siRNAs can trigger interferon production as part of a cellular stress response pathway that can cause translation arrest, growth inhibition and cytotoxicity (44). In contrast to the shRNA approach, the use of miRNAs enables the expression of
multiple miRNAs from a single transcript as compared to only one in regular shRNA vectors. Indeed, transfection of cells with two different shRNAs may lead to competition of the two for transport and incorporation into the RISC, resulting in a reduction in shRNA processing and activity (45). Despite reports on efficient silencing of genes using RNAi, differences in the efficacy of a given vector between experiments have been reported. This might be due to inefficient cellular uptake of the RNAi and may also depend on the cell type. What follows is an overview and update of the \textit{in vitro} and \textit{in vivo} experiments aiming at evaluating the potential use of small RNAs, including miRNAs, as a treatment option against viral diseases that affect animals of agricultural and/or economic importance.

\subsection{2.2.1. Influenza A virus (IAV)}

Infection with IAV is a worldwide problem that affects both human and animal health (46, 47). The presence of multiple viral genotypes and the possibilities of antigenic shift and drift continue to raise concerns about the pandemic potential (48, 49). Current influenza vaccines and therapies have proved to be inefficient to combat the continuously evolved IAV strains due to the occurrence of antigenic variation within influenza virus genomes due to point mutations (drift) or re-assortment (shift) (50, 51). The emergence of resistant virus strains added another limitation to anti-IAV therapies (52). RNAi formulated in an appropriate agent would offer the potential for a new therapy by targeting viral transcripts. Furthermore, inserting a let-7b response element within the H1N1 genome created an attenuated strain that conferred protection in mice against challenge with a lethal strain, suggesting that the attenuated strain might serve as a live-attenuated vaccine (53). Around 13 500
possible siRNA target sites are present in the IAV genome. Recent reports described the usefulness of methods and procedures to select highly effective influenza-specific siRNAs in cell culture, mice, and ferrets (54). Using in silico approaches, Raza and colleagues identified five conserved amino acid sequences, three in the hemagglutinin (HA) gene (RGLFGAIAGFIE, YNAELLV and AIAGFIE) and two in the neuraminidase (N) (RTQSEC and EECSYP) gene, which might provide potential RNAi-based therapeutic targets in various IAV strains (55). RNA interference has been shown to be effective in suppressing IAV replication both in vitro and in vivo.

For instance, transfecting MDCK cells with siRNA specific for nucleoprotein (NP, nucleotide positions 1496–1514) or polymerase acidic (PA, nucleotide positions 2087–2106) mRNA sequences inhibited IAV replication (36). Moreover, a mixture of siRNAs specific for highly conserved regions of NP and PA can protect mice from lethal challenge with IAV of the H5 and H7 subtypes (e.g., (56)). SiRNA against the matrix 2 (M2) gene exhibited similar or slightly higher reduction in virus replication in MDCK cells and in human HEK293 cells (57). Likewise, IAV titers in MDCK cells and in embryonated eggs were reduced more than 50-fold and 100-fold, respectively, when shRNA targeting the polymerase basic 1 (PB1) gene was transfected in vitro and in vivo using a liposome-encapsulated pSIREN/PB1 vector. In mice, the survival rate ranged between 50% and 100% (58). In another experiment, siRNA targeting a region of the M1 gene between nt 331 and 351 was found to be the most effective in inhibiting M1 protein translation in cell lines. Inhibiting the viral M1 protein using this siRNA caused an 80% reduction in viral titers in supernatants of siRNA-transduced MDCK cells at 6, 8 and 10 hpi. Furthermore, virus budding ability was reduced by 40%, suggesting the ability of siRNA targeting the M1 protein to suppress
IAV replication (59). Another report demonstrated the efficacy of anti-NP and anti-PA shRNAs in reducing IAV titers in MDCK cells and in avian CH-SAH cells. Significant decreases of up to 80% in the levels of IAV NP mRNA and up to 370-fold in viral titer were observed in the CH-SAH cells. The approach also worked well in MDCK cells, as demonstrated by significant decreases up to 90% in the level of viral mRNA, and up to 106-fold in IAV infective titer. Furthermore, the authors identified a novel, highly efficient and conserved RNAi target site in the viral NP gene, which can be used in antiviral cocktails of shRNAs to prevent IAV escape from RNAi silencing (60). Zhou and colleagues investigated the silencing effect of M2 and NP-specific siRNAs on IAV (H5N1, H1N1 and H9N2) replication in cell lines and mice (61). In the cell lines, a 0.51-1.63 TCID$_{50}$ reduction in virus titers was observed, and delivery of pS-M48 and pS-NP1383 significantly reduced lung virus titers in the infected mice (16 to 50-fold reduction in titer) and partially protected them from lethal IAV challenge. As an alternative approach, targeting host cell genes that are crucial for IAV replication can be conducted to control the virus. Expression of $\alpha_2, 3$-linked (avian-type) and $\alpha_2, 6$-linked (human-type) sialic acid (SA) receptors on host tissues is considered one of the host range and tissue tropism determinants of influenza viruses. An siRNA duplex was used to inhibit IAV binding and internalization via silencing $ST6GAL1$ gene that encodes the $\beta$-galactoside $\alpha$-2,6-sialyltransferase I ($ST6Gal I$), a protein important in SA receptor formation (62). In addition, targeting cellular proteases has been discussed as a method to suppress IAV replication. Rogers and colleagues studied pulmonary miRNA expression in mice infected with the IAV H5N1 strain and verified that furin, a member of the convertase family that mediates cleavage of hemagglutinin, is a target gene for miRNAs upon H5N1
infection (63). This highlights the importance of using miRNAs as potential therapeutic agents against IAV.

### 2.2.2. Venezuelan equine encephalitis virus (VEEV)

Venezuelan equine encephalitis virus (VEEV) belongs to the genus alphavirus in the family Togaviridae. This virus is still endemic in many parts of the world and is considered an emerging disease threat in other parts as well as a potential biological weapon (64). So far, there are no US Food and Drug Administration (FDA) approved drugs or vaccines against VEEV. Thus, developing artificial miRNAs that can be used to control VEEV infection is a step in the right direction. Indeed, VEEV has been targeted efficiently by siRNA (65). Most recently, it was shown that targeting the viral non-structural protein-4 (nsp-4) region with miRNAs in BHK-21 cells efficiently inhibited viral replication, with artificial miR-3 having the greatest effect (66). This study indicated that these artificial miRNAs merit further testing in animal models for antiviral therapies against VEEV infection.

### 2.2.3. Foot-and-mouth disease virus (FMDV)

Foot-and-mouth disease (FMD) is a highly infectious viral disease that usually affects cloven-hoofed animals. The direct impact of an FMD outbreak includes great losses to agricultural production and disruption of local economies, while the indirect effects lie in the disease control measures at both local and global levels and the high cost of disease control and prevention programs. FMDV has an RNA genome and many serotypes, and targeting conserved viral genes such as 3D, VP4 and 2B is a major aim in order to control FMD (67). The use of peptide-conjugated morpholino oligomers (PPMOs) and miRNAs with sequences complementary to various
segments of the FMDV genome effectively blocked viral replication in cell culture models (68). Likewise, DNA vector-based RNAi technology can specifically suppress the expression of the VP1, 3D, VP4 and 2B genes and thus inhibit viral replication in vivo and in vitro (67, 69). Using adenovirus-based vectors to express siRNA molecules in cell lines and mice, Kim et al. suggested to apply RNAi treatments before and after infection with FMDV (70). Treatment after FMDV infection inhibited viral replication effectively, but a combination of treatment before and after infection gave the best results in pig kidney cells, IBRS-2 cells and in suckling mice, as evidenced by lower viral titers in cell lines and higher survival rates of the treated mice. These experiments did reveal that the RNAi method took considerable time to induce a silencing effect, which ranged from 24 to 48 h (71, 72). This is considered a limitation when attempting to control certain rapidly spreading contagious diseases, including FMD, as viral spread will be faster than the inhibitory action of the RNAi. Finally, the use of artificial miRNAs (amiRs) resulted in specific silencing of reporter genes fused to FMDV target sequences (73).

2.2.4. Classical swine fever virus (CSFV)

Classical swine fever virus (CSFV) can cause a hemorrhagic disease in pigs characterized by disseminated intravascular coagulation, thrombocytopenia and immunosuppression (74, 75). CSFV has been recognized for nearly 200 years and now appears to have been eradicated in Europe and North America due to vaccinations and other control measures. The first study of using siRNA in blocking CSFV replication was conducted in 2008 (76). Three siRNA molecules targeting different regions of the CSFV Npro and NS5B genes were prepared and transfected
into PK-15 cells. They caused a 4-12 fold reduction in viral genome copy number. In another study, synthetic siRNA transfected into swine kidney cells (SK-6), could target nucleotides 1130-1148 in the nucleocapsid protein (C) of the CSFV with subsequent reduction in viral titer compared to either mock-treated or non-treated cells (77). This emphasizes the potential of siRNA to inhibit CSFV replication. Clearly, in vivo experiments need to be conducted to confirm this effect.

### 2.2.5. Rabies virus (RV)

Rabies is a zoonotic disease caused by RV, a member of the Rhabidoviridae family. The disease typically infects canines (78) and is usually transmitted by animal bites, causing a lethal encephalitis. The annual number of deaths due to rabies has been estimated to be approximately 59 000 (79). The control of RV in wild carnivores has moved from culling operations to parenteral and oral vaccination of susceptible species (80), but inhibiting viral replication with siRNA or miRNAs may be another promising approach. Cell lines have been used to assess the usefulness of siRNA in inhibiting RV replication either by using a pool of siRNAs (81) or by single and multiple artificial miRNA targeting RV nucleocapsid (N) (45). In these in vitro assays, there was a comparable virus reduction at 72 h post infection, especially when a single miRNA completely matched the target. Similar results were reported by others (e.g., (82)). In cultured cells and murine model, RV glycoproteins were proved to be essential for trans-synaptic viral spread between neurons (83). This observation encouraged other researchers to target the genes encoding such glycoproteins. Sonwane et al studied the ability of adenovirus-based siRNAs, delivered to BHK-21 cells, to inhibit RV replication and subsequently tested this approach in mice (84). In
this study, siRNA inhibited viral replication in cell lines and mice. In BHK-21 cells, siRNA targeting the RV polymerase gene (L gene) was found to be more effective than siRNA targeting the RV nucleoprotein (N gene) in inhibiting and reducing RV replication. Specifically, a 48.2% reduction of RV foci was seen in cells in which the L gene was targeted versus (vs.) a 41.8% reduction when the N gene was targeted. A significant, even greater, difference was observed at the mRNA level (17.8-fold vs. 5.7-fold reduction). In mice, inoculation of both siRNA vectors resulted in a 50% protection against a subsequent lethal RV injection. siRNAs simultaneously targeting the glycoprotein G and N genes led to an 87% reduction in viral release, demonstrating that siRNAs directed against different targets may act synergistically and increase efficacy of siRNA-based interventions against RV (85). Taken together, the above results do suggest that use of siRNAs constitutes a promising approach to interventions against RV.

2.2.6. Viral diseases of fish

Viral infection in fish aquaculture can be devastating and costly (86). Early reports of RNAi-based treatments described use of this technology in fish and shellfish in 2008 (87). In fish betanodavirus, there are two amino acid residues in the B2 protein (R53 and R60) which bind viral RNA to circumvent the RNAi pathway, underscoring the importance of the anti-viral role of the host RNAi machinery (88). Dang et al. showed an inhibitory effect of siRNA on seabream iridovirus, a marine fish virus (89). In this study, siRNA introduced into cells infected with red seabream iridovirus specifically and effectively bound to mRNA encoding the virus major capsid protein, leading to a reduction in the production of virus particles in the supernatant of virus-
infected cells, as compared to the cells receiving the control treatment. These results provide encouraging evidence that siRNA technology might be used to control fish viral diseases. More recently, a shRNA construct was found to inhibit the proliferation of viral hemorrhagic septicemia virus by targeting its G gene in a sequence-specific manner (90). Infection with herpesvirus 3 causes severe financial losses in the common carp and koi culture industries worldwide (91). Although most investigations have employed in vitro approaches, RNAi might be a promising tool to combat herpesvirus 3 in carp. For instance, a pool of siRNAs specific for DNA enzyme synthesis and capsid proteins of cyprinid herpesvirus 3 virus can be a potential inhibitor of virus replication in carp fibroblasts (92). Along the same line, Gotesman et al. demonstrated that siRNAs can inhibit the thymidine kinase and DNA polymerase genes of cyprinid herpesvirus 3, causing decreased release of viral particles from transfected common carp brain cells (93). Viral infection in shrimp constitutes a great problem, and excellent reviews have discussed the use of RNAi in controlling various viral infections in shrimp (e.g., (94-96)).

2.3. Potential use of RNAi to create genetically engineered virus-resistant animals

Genetic selection has been successful in mediating remarkable progress in livestock improvement. Genetic engineering of livestock is commonly used to produce pharmaceuticals or to enhance production characteristics of animals, but has also proven to be important in producing animals with infectious disease resistance. For example, cows have been genetically engineered to be resistant against
*Staphylococcus aureus* induced mastitis (97) and laboratory investigations have been conducted with regard to creating α-herpesvirus-resistant livestock (98). Furthermore, there are efforts to create livestock resistant against gastroenteritis coronavirus infection, but published studies are limited to work with mice (99). Against IAV infection, two potent lentivirus-based shRNAs targeting the nucleoprotein (NP) and polymerase acidic (PA) genes of IAV were used to generate IAV-resistant mice (100). However, a successful challenge experiment has not been reported in this system. Subsequent studies based on inhibiting genes of other pathogens have been conducted (101). With improved RNAi techniques, it is conceivable that genetically engineered disease-resistant animals, based on siRNA or shRNA technology, may someday become reality in veterinary infectious disease medicine. Even prion diseases have been the target of transgenic-animal technology featuring shRNAs. Golding and colleagues attempted the use of siRNA technology to generate prion-resistant goat and cattle (102). First, they designed a lentivirus-based shRNA tagged with green fluorescent protein (GFP), which was directed against caprine prion protein precursor (PrPc) mRNA and then transfected this vector into an adult goat fibroblast cell line. These cells were then used for somatic nuclear transfer to produce transgenic goat embryos for subsequent *in vitro* differentiation in various stages of pre-implantation development. They confirmed the silencing capacity of shRNA in brain tissue of the growing fetus compared to an age-matched normal fetus. The authors observed an approximate 90% reduction in the expression of PrPc. However, clinical efficacy in reducing the risk of a neurodegenerative disease was not determined and data regarding efficacy were not presented. This suggests that this technique had surpassed a major technical hurdle. Furthermore,
two studies described the efficacy of RNAi to silence FMDV in transgenic bovine fetal epithelium cells (BFEC), although rigorous negative controls were lacking, making it difficult to ascribe any effects to the transgenic manipulations. The first of these was conducted by Wang et al., who describe the construction of three recombinant lentiviral vectors containing shRNA against VP2 (RNAi-VP2), VP3 (RNAi-VP3), or VP4 (RNAi-VP4) of FMDV, and subsequent testing of their silencing power in both 293 and BHK-21 cells (103). The lenti-RNAi-VP4 vector was transfected into bovine fetal fibroblast cells. The stably transfected cells were transferred into enucleated oocytes, and the reconstructed embryos were then transferred to recipient cows. ShRNA expressed in transgenic fetuses significantly degraded viral RNA after inoculation with FMDV at a titer of 100 TCID_{50} and inhibited viral replication. Thus, primary transgenic bovine fetus tongue epithelium cells became much more resistant to FMDV challenge. In the second report, a shRNA-expressing lentiviral vector targeting VP1 of FMDV resulted in strong suppression of VP1 protein expression in 293T cells and also significantly inhibited viral replication in BHK-21 cells (104). The construct was then transfected into bovine fetal fibroblast cells. Cloning these somatic cells resulted in three month-old transgenic fetuses. FMDV RNA synthesis and viral replication were significantly reduced in primary tongue epithelial cells from the transgenic fetuses, suggesting that RNAi technology can be potentially used to generate transgenic cattle resistant against FMDV. Taken together, the studies summarized above support the idea that transgenic cloning may prove to be a useful tool to deliver anti-viral and anti-prion RNAi to the germ line of animals of veterinary importance, but substantial additional work remains to be done before this technology may demonstrate efficacy in veterinary practice.
3. Remaining challenges

Despite the excitement about utilizing non-coding RNAs to combat animal viral diseases, considerable challenges still need to be overcome before they can be used clinically. Animal breeders tend to rear their flocks in large groups under intensive or semi-intensive husbandry or on large farms. It would be wasteful in terms of money, time and labor to deliver these expensive molecules on an individual basis. In this case, most veterinarians prefer to use anti-viral therapies in a common source bio-vehicle, for instance food, water or air, to ensure quick accessibility. We think that using individual miRNA-based therapies will be more practical in special cases such as the following: race horses, the very expensive parent flocks of chickens and turkeys that are intended for production of specific pathogen free (SPF) eggs, purebred domestic animals kept as stock for distributing semen for artificial insemination, and cross breeding and improving certain animal traits for meat, milk or fat production. Controlling contagious viral diseases, for instance FMDV and IAV, necessitates a rapid intervention strategy to prevent virus spread from one farm to another and from animals to human. In this regard, RNAi that produces the inhibitory effect within one or two days in cell lines is considered to be insufficient, and a more rapidly operating approach is needed. Another technical challenge is that the excessive levels of the introduced miRNAs can saturate the internal host processing machine for other host small RNAs giving rise to toxicity, pathology and mortality to the animal under therapy (105). Therefore, the dose of the introduced RNAi-based therapy should be well controlled. The delivery of the RNAi molecule is a key roadblock in this whole process. This is because RNAi molecules are negatively
charged and do not penetrate the cell membrane effectively, a step that is necessary for subsequent silencing of mRNAs in the cytoplasm (106). Additionally, they may be quickly excreted, of low stability, non-tissue specific, and may have an inefficient intracellular release (107). Although the delivery of the silencing molecule may be mediated via vectors, suboptimal vector selection might reduce the silencing effect. Many delivery systems, such as nanoparticles, cationic lipids, calcium phosphate, antibodies, cholesterol and viral vectors have been tested (108). From another perspective, the use of a single RNAi silencing molecule with a low percent match with the target mRNA would lead to a poor target reduction. Possible solutions include either applying only one siRNA which is 100% identical to the sequences of interest or applying more than one siRNA sequence targeting different conserved regions of the target gene. In the case of IAV, spontaneous mutations were estimated to occur at a rate of approximately $1.5 \times 10^{-5}$ per nucleotide per infection cycle (50), suggesting that target sequence mismatches will arise inevitably. Another challenge is to develop a universal RNAi molecule against the same sequence in multiple influenza strains. Some viruses may evolve mechanisms to circumvent the targeting RNAi molecule, either by expressing virus-encoded suppressors or by mutation (109). In order to avoid this, scientists have tried to design RNAi molecules that simultaneously target several sequences within a viral gene (110). In practice, in the fish aquaculture system, RNAi-based therapy have demonstrated some limitations. As a rearing system in some fish farms, the rearing cages are kept floating in the sea or river water, the so-called open sea or river cage aquaculture. Under such system, introducing RNAi molecules into fish feed will allow settlement of the uneaten food, containing the therapy, to the bottom of the water body. This
would be ineffective and would also make the feed available to non-target organisms (111). Thus, an alternative improved approach would be to use RNAi in land-based ponds or tanks, owing to their direct accessibility to fish and the easy disposal of waste materials. The commercial field application of injectable therapy is neither practical nor realistic, especially with shrimp, which are reared in an intensive system. Despite its relatively high expense, soaking the shrimp in a solution containing the RNAi silencing molecule is a more practical way to ensure that an effective suppression of the gene is achieved (112). Unfortunately, there are no shrimp cell lines available for the research community, delaying a better understanding of the RNAi application in shrimp farms. Effective design of the RNAi molecule is also of special concern. Although various computational tools have been developed to systematically evaluate the targets for miRNAs and or siRNA (113-115), non-specific off-target effects need to be anticipated. The many parameters that influence specificity of miRNAs/siRNAs include the selected target region, size, the starting nucleotide, GC content, the thermodynamic properties of the introduced molecule, and the presence of internal repeats. Apart from an effective design, the use of accurate positive and negative controls is necessary to ensure the validity of RNAi data (116).

4. Future directions

From the evidence gathered thus far we have every reason to be optimistic about the future use of small non-coding RNAs in the diagnosis, monitoring and treatment of animal viral diseases. Zoonotic viruses continue to pose a public health threat to humans. There are miRNAs that are associated with zoonotic viral diseases that
were found to be conserved among the human and animal reservoirs and exhibit similar tissue tropism. It is important to investigate both the contribution of these miRNAs to the zoonotic nature of diseases and their potential roles as biomarkers or therapeutic tools for humans and animals. This is even more important for viral diseases affecting poultry populations that are reared under both intensive and semi-intensive systems, where the pathogens can be transmitted in a short time to populate the environment and infect susceptible hosts. Regarding the use of RNAi in combating viruses, the search for a target sequence conserved across strains is of highest priority in studies targeting animal viruses, in particular those featuring rapid genomic changes, such as IAV and other RNA viruses. However, using a pool of various siRNAs or a cocktail of siRNAs specific for virus and host genes might reduce escape of mutant viruses. In addition, it would be valuable to develop more rapidly acting RNAi technology to inhibit spread of highly contagious infections such as FMD. Prospectively, incorporating the RNAi molecule into animal feed or the water supply might be a practical choice for the treatment of animals reared in large numbers such as fish or poultry. Using this strategy, successful experiments have been recorded in shrimp infected with White Spot Syndrome Virus (WSSV) (117). In spite of the extensive efforts toward formulating a suitable vehicle, one that delivers the smallest RNAi quantity in a non-toxic way remains to be discovered. In this respect, the use of a natural exosome or a natural or synthetic high-density lipoprotein (HDLP) is a novel and promising approach. These are just a few areas of research that are likely to engage veterinary scientists and virologists for years ahead. These and other improvements should further facilitate the use of miRNA
and siRNA to prevent and control animal viruses at veterinary clinical sites and in the field.

5. Conclusions

Small non-coding RNAs have been known as crucial regulators of gene expression, and they have great potential for applications in the diagnosis, prevention and treatment of viral infectious diseases of veterinary importance. Research efforts are continuing to translate RNAi technology with its two arms, miRNAs and siRNA, to clinical applications in veterinary medicine (Fig 2). In this respect, the de-regulation of miRNAs upon infection, their stability and tissue specificity have made their study as biomarkers a fruitful area of research. SiRNA molecules together with miRNA mimics or agonists can be delivered to the infected animal as a treatment option. Although there are currently no genetically engineered virus-resistant animals, the likelihood of exploiting RNAi technology, including miRNAs, is growing and is expected to help attain this aim. Bringing these molecules to the market will remain to be challenging and many barriers still need to be overcome. In fact, in vitro models would enable more detailed studies on the clinical relevance of these molecules. However, experimental animal models and infections of natural hosts in laboratory investigations will afford more realistic insights into the best ways to utilize small non-coding RNAs to improve animal health. Importantly, developing animal-specific databases that contain experimentally validated small RNA molecules and related functional analysis will facilitate using these data for future research. The continual emergence of zoonotic viruses warrants effective collaborations between physicians and veterinarians in this issue. The available evidence suggests that the clinical use
of small non-coding RNAs in combatting animal viruses may be possible in the not too distant future.

**List of abbreviations**

AUC, area under the curve; APPs, acute phase proteins; BFEC, bovine fetal epithelium cells; CSFV, classical swine fever virus; FDA, Food and Drug Administration; FMDV, foot-and-mouth disease virus; FMD, foot- and -mouth disease; FFPE, formalin-fixed paraffin-embedded tissue; GFP, green fluorescent protein; HA, hemagglutinin; HDLP, high-density lipoprotein; IAV, influenza A virus; LNA, locked nucleic acids; miRNA, microRNAs; PPMOs, NP, nucleoprotein peptide-conjugated morpholino oligomers; PB-1, polymerase basic 1; PA, polymerase acidic; PrP<sub>c</sub>, prion protein of cell; RV, Rabies virus; ROC, receiver operating characteristic; RISC, RNA induced silencing complex; RNAi, RNA interference; shRNA, short hairpin RNA; SA, sialic acid; siRNA, small interfering RNA; sncRNAs, small non-coding RNAs; SPF, specific pathogen free; UTR, untranslated region; VEEV, Venezuelan equine encephalitis virus; WSSV, White Spot Syndrome Virus.

**Acknowledgments**

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**Competing interests**
The authors declare that they have no competing interests.

**Authors’ contributions**

M.S. did the literature search, wrote the initial draft of the manuscript and prepared the figures and tables. F.P. oversaw the project, edited the manuscript including the final version, and takes responsibility for the integrity of the data.

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### Table 1. Sequence conservation of selected mature miRNAs in humans and chicken\(^1\)

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Identity (%)</th>
<th>Human and chicken miRNA sequences</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>mir-29a</td>
<td>95.2</td>
<td>Human: UAGCACCAU, Chicken: UAGCACCAU</td>
<td>MIMAT0000086, MIMAT001096</td>
</tr>
<tr>
<td>mir-18a-5p</td>
<td>100.0</td>
<td>Human: UAAGGUGCAUCUAGUCGAGAUAG, Chicken: UAAGGUGCAUCUAGUCGAGAUAG</td>
<td>MIMAT0000072, MIMAT001113</td>
</tr>
<tr>
<td>mir-32-5p</td>
<td>100.0</td>
<td>Human: UAUGUGCAACUAUACUGUGCA, Chicken: UAUGUGCAACUAUACUGUGCA</td>
<td>MIMAT0000090, MIMAT001125</td>
</tr>
<tr>
<td>mir-223-3p</td>
<td>100.0</td>
<td>Human: UGUCAGUUUGUCAAAUAACCCCA, Chicken: UGUCAGUUUGUCAAAUAACCCCA</td>
<td>MIMAT000280, MIMAT001140</td>
</tr>
<tr>
<td>mir-34a-5p</td>
<td>100.0</td>
<td>Human: UGGCAGUGUCUUAGCUGGUU, Chicken: UGGCAGUGUCUUAGCUGGUU</td>
<td>MIMAT000255, MIMAT001173</td>
</tr>
<tr>
<td>mir-142-3p</td>
<td>100.0</td>
<td>Human: UGUAGGUGUUCCCUACUUAUGGA, Chicken: UGUAGGUGUUCCCUACUUAUGGA</td>
<td>MIMAT000434, MIMAT001194</td>
</tr>
<tr>
<td>miR-155-5p</td>
<td>100.0</td>
<td>Human: UUAUGCUAUAUCGUGAUAGGGGU, Chicken: UUAUGCUAUAUCGUGAUAGGGGU</td>
<td>MIMAT0000646, MIMAT001106</td>
</tr>
</tbody>
</table>

\(^1\) MiRNAs are listed in ascending numerical order. Non-conserved nucleotides are printed in red. Accession numbers of miRNAs sequence are according to MiRBase 21.
Figures

<table>
<thead>
<tr>
<th>Animal</th>
<th>MiR-146b-5p (mature sequences)</th>
<th>Accession numbers</th>
</tr>
</thead>
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<tr>
<td>Human</td>
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<td>MIMAT0002809</td>
</tr>
<tr>
<td>Mice</td>
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<td>MIMAT0003475</td>
</tr>
<tr>
<td>Chicken</td>
<td>UGAGAACUGAAUUCCAUAGGC--</td>
<td>MIMAT0003351</td>
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<tr>
<td>Dog</td>
<td>UGAGAACUGAAUUCCAUAGGC--</td>
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<td>Horse</td>
<td>UGAGAACUGAAUUCCAUAGGC--</td>
<td>MIMAT0012891</td>
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<tr>
<td>Cattle</td>
<td>UGAGAACUGAAUUCCAUAGGCUGU</td>
<td>MIMAT0009235</td>
</tr>
<tr>
<td>Pig</td>
<td>UGAGAACUGAAUUCCAUAGGC--</td>
<td>MIMAT0010190</td>
</tr>
<tr>
<td>Goat</td>
<td>UGAGAACUGAAUUCCAUAGGCUGU</td>
<td>MIMAT0035972</td>
</tr>
<tr>
<td>Atlantic salmon</td>
<td>UGAGAACUGAGUCCAUAGAUUGG--</td>
<td>MIMAT0032374</td>
</tr>
</tbody>
</table>

Figure 1. Sequence alignment of the mature form of miR-146b-5p among animals and humans. The open box illustrates the high degree of conservation of the seed region of miR-146b-5p. Accession numbers are according to miRBase 21 (1).
Figure 2. Diagrammatic illustration of potential uses of non-coding RNAs to combat animal viruses. MiRNAs can be used as biomarkers for pinpointing animal viral diseases. They can act as potential therapies and to create genetically virus-resistant animal breeds. Abbreviations: RT-qPCR, reverse transcriptase quantitative real-time PCR; RNAi, RNA interference; SiRNA, small interfering RNA; GO, gene ontology. Adapted from UGA Veterinary Diagnostic Laboratories, Werner et al. Current Opinion in Biotech. 2008, 19:50-64, and Livingston et al. Genome Biology 2005, 6:112.
3.3. Manuscript III

(In revision for Archives of Virology)

Title

Pandemic 2009 H1N1 variants and toll-like receptor (TLR) ligands induce cell- and virus-specific miRNA expression in A549 and dTHP-1 cells

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Abstract

MicroRNAs (miRNAs) have sparked considerable interest as key regulators of gene expression in different aspects of host-pathogens interactions. Profiling and functional studies provided evidence that miRNAs are expressed in lung tissue following influenza A virus (IAV) infection as well as after stimulation with various Toll-like receptor (TLR) ligands. However, little is known about the cell-specific expression of miRNAs under these conditions. In this study, we compared the expression levels of selected miRNAs in A549 (lung epithelial cell line) and dTHP-1 (monocytic/macrophage cell line) cells upon infection with the swine-origin pandemic influenza A/Giessen/06/2009 (H1N1 pdm) (S-OIV, wild, less virulent), its more virulent reassortant (S-OIV NS PR8) and after stimulation with TLR agonists (LPS and R848). The results showed that while LPS and R848 induced higher levels of miRNAs in dTHP-1 cells than in A549 cells, the two virus variants showed a reverse cellular pattern. Hsa-miR-223-3p showed the highest difference in expression between A549 and dTHP-1 cells when stimulated with TLR ligands and when infected with the S-OIV NS PR8 virus. Furthermore, infection of both cell lines with the predefined S-OIV variants demonstrated cell- and virus-specific miRNA expression signatures. Notably, the S-OIV NS PR8, but not the S-OIV wt triggered more expression of has-miR-223-3p, has-miR-155-5p, has-miR-155-3p and has-146b-1 in a cell-dependent manner. Considered together, our results indicate that IAV infection and LPS/R848 induce differential miRNA expression pattern in alveolar epithelial and macrophage cells, suggesting a cell-specific role for miRNA in IAV pathogenesis and possibly during infection with gram-negative bacteria.
**Introduction**

MicroRNAs (miRNAs) are non-coding RNAs ≈ 22 nucleotides (nts) in length, which can regulate gene expression in plants and animals post-transcriptionally [1]. The identification of *Lin-4* [2] and *let-7* [3] during a mutagenic screen in *Caenorhabditis elegans* (*C. elegans*) larvae drew, for the first time, the attention to the regulatory role of miRNAs in animal biology. The fact that one miRNA can regulate hundreds of mRNA genes and that one mRNA can be targeted by several miRNAs form the base for the complexity of the regulatory role of miRNAs. Host-encoded miRNAs have been reported to influence host-pathogen interactions [4], possibly through orchestrating immune responses [5,6]. Furthermore, *in vitro* studies suggested that enhanced virus replication might be, at least in part, caused by the activity of cellular miRNAs [7,8].

Viruses as well as inflammatory stimulants can engage multiple TLRs, the cellular sensors for pathogen associated molecular patterns (PAMPs) [9,10]. Among TLR ligands, lipopolysaccharide (LPS), which is a component of gram negative bacteria, can activate TLR4 [11], whereas imidazoquinoline compounds (e.g. R848) are known to stimulate TLR7 [12,13]. The recognition of PAMPs by TLRs initiates a down-stream signaling cascade [14]. Previous studies demonstrated that TLR signaling pathways could be modulated by miRNAs [15]. For instance, miR-146a/b can target interleukin-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor (TRAF6), which are known to be important components of the TLR4 signaling pathway [16]. LPS stimulation of human monocyte-derived dendritic cells up-regulates miR-155, which targets TGF-beta activated kinase 1 binding
protein (TAB2), an adaptor in TLR/IL-1 signaling cascade, thereby acting as a repressor of inflammatory cytokines [17].

Influenza A viruses (IAVs) belong to the *Orthomyxoviridae* family, which have a segmented negative sense RNA genome. IAVs continue to circulate in human populations giving rise to seasonal outbreaks and epidemics [18]. Profiling and functional studies have identified key roles of cellular miRNAs in many aspects of IAV-host interaction. Our previous literature search identified miRNAs that were commonly expressed after IAV infection in human, mice, chicken and pig, suggesting a cross-species conserved regulatory role for miRNAs (Samir et al., manuscript submitted). During the infection with IAV, we observed differences in lung miRNA expression patterns between PR8 H1N1-infected susceptible and resistant mouse strains (Preusse et al., in preparation for publication). Moreover, comparative studies indicated that miRNAs might contribute to IAV lethality [19,20], differential tissue tropism [21,22] and virus replication [23,24]. While these studies mostly involve lung as a predilection site for IAV replication, very little is known about the contribution of individual resident lung cells in miRNA-IAV interaction. Within lung matrix, type II epithelial cells and macrophages constitute important cells that defend against invading pathogens [25,26]. Although lung epithelium and alveolar macrophages have been reported to be targets for IAVs [27-29], both respond differently to IAV infection. Firstly, IAVs replicate more efficiently in lung epithelial cells than in alveolar macrophages [30,31]. Compared to seasonal IAV strains, the H5N1 strain and other highly pathogenic strains can replicate more efficiently and productively in macrophages [31]. Secondly, lung epithelial cells and macrophages elicit different chemokine and cytokine response to IAV infection. Lung epithelial cells produce
IL-8, RANTES, MCP-1 and low levels of IFNα/β; macrophages, instead, produce higher levels of IFN-α/β and other factors, including TNF-α, MIP-1-α/β, CXCL-1, IL-18, IL-6 and IL-1β (reviewed in [32]). Given this contrast in the response of lung epithelial cells and alveolar macrophages to IAV infection, coupled with the growing importance of miRNAs as key players in various lung pathological conditions [33], we hypothesized that cellular miRNAs might be expressed differentially in the two cell types and, thus, influence virus replication. Obtaining this knowledge will aid in understanding IAV pathogenesis, help uncovering the cell-specific expression of miRNA targets [34] and facilitate performing future functional studies at the cellular level.

In this study, we evaluated the cell- and virus-specific expression of six miRNAs which had previously been identified by our group as being differentially expressed in IAV-susceptible and -resistant inbred mouse strains (Preusse et al, manuscript in preparation). Additionally, these miRNAs have been implicated in other aspects of IAV-host interactions [35-37,20,38,39]. A549 and differentiated THP-1 (dTHP-1) cells were used as well-established model cell lines for lung epithelial cells and alveolar macrophages, respectively. TLR agonists, S-OIV (wild-type, less virulent) and S-OIV NS PR8 (reassortant, more virulent) pandemic 2009 H1N1 were used as stimulants. miRNA expression levels were relatively quantified using reverse transcriptase quantitative real-time PCR (RT-qPCR). The results of this study indicated that TLR agonists and IAVs induce a cell-specific expression of the individual targets, yet with a different pattern. Furthermore, miRNAs exhibited virus-specific expression signatures, which were themselves cell-specific.
Materials and Methods

Cell lines and cultures
Type II-like human lung epithelial cell line (A549) and human monocyte cell line cells (THP-1) were obtained from the German collection of microorganisms and cell cultures (DSMZ) and used in this study. A549 cells and THP-1 cells were maintained in 24 well plates containing DMEM and RPMI 1640 medium at 5 X 10^5/ml, respectively. Both media were supplemented with fetal calf serum (FCS) (10%), glutamax (1%) and sodium pyruvate (1%). The A549 cells were incubated in 5% CO2 at 37°C until adherence. The THP-1 cells were differentiated into alveolar macrophage-like cells by stimulating the cells with 200 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 2 days, then the cells were transferred to PMA-free fresh RPMI 1640 medium for additional 1 day [40].

TLR agonists stimulation and IAV infection
In order to define the dose at which A549 and dTHP-1 cells are responsive to LPS (TLR4 agonist, purified from Salmonella typhimurium, Sigma Aldrich) challenge, both cells were stimulated with various concentrations (100 ng, 200 ng, 500 ng, 1 µg, 100 µg and 200 µg/ml) of LPS and 6 h later, the relative expression of IL-8, IL-1β and IL-6 were measured. To examine the cell-specific expression of miRNAs, R848 (imidazoquinoline, TLR7 agonist, InvivoGen) at a concentration of 5 µg/ml was used as an analog for negative sense single strand RNA viruses (ssRNA) and LPS was used at a concentration of 500 ng/ml. For IAV infection, S-OIV (wt, A/Giessen/6/2009 (H1N1 pdm)) and S-OIV NS PR8 (a reassortant virus that was, by reverse genetics, produced by inserting the NS segment of influenza A/Puerto
Rico/8/1934 (H1N1, PR8) into the S-OIV wt), were used at multiplicity of infection (MOI) of 2. Control, TLR agonists-stimulated and IAV-infected A549 and dTHP-1 cells were harvested at 6, 12, 24, 48 hours (h) and cell pellets were directly used for RNA extraction.

**miRNA extraction and reverse transcription (RT-PCR)**
Total RNA, including small RNAs, was extracted (using miRNeasy kit (Qiagen) according to the instruction), and then DNase treated using RNase-Free DNase Set (Qiagen). RNA quality and quantity were evaluated with the Nanodrop measuring device (Thermo Scientific). The DNase-treated total RNA was then reverse transcribed using the miScript Reverse Transcription Kit (Qiagen) in a 20 µl reaction volume.

**Quantitative real time PCR (q-PCR)**
Quantitative real time PCR (qPCR) was performed using miScript SYBR Green PCR Kit (Qiagen). The primers used for miRNA relative quantification and that used in this experiment were obtained from Qiagen separately as part of the miScript PCR system. Primers used for relative quantification of mRNA genes are listed in Table 1. Human β actin and human RNU6b-2 were used to normalize mRNA and miRNA expression, respectively. Primer pairs for each gene were determined for specificity based on the melting curves. The efficiency of the qRT-PCR was assessed by determining the primer amplification efficiency. qPCR was carried out using the Light Cycler 480 real-time PCR instrument (Roch), software version 1.5. The cycling conditions started with a pre-incubation at 95°C for 15 minutes (min), followed by 45 cycles at 94°C for 15 seconds (s), 50-55°C (depending on the gene) for 30 s and
72°C for 30 s. Melting curves were created by increasing the temperature of the sample to 95°C. Final cooling was done at 40°C for 30 s. The relative quantification of miRNAs and mRNAs were calculated using the $2^{(-\Delta\Delta CT)}$ method [41].

**Statistical analysis**

To prove the responsiveness of A549 and dTHP-1 cells to LPS and R848, levels of *IL-8* and *IFN-α* in control and stimulated cells at each time points were compared by using repeated measures ANOVA test. The differences in miRNA levels between the A549 cells and dTHP-1 cells at each time point after LPS, R848 and S-OIVs stimulation were determined by independent t-test. *P* values of ≤0.05 were considered significant; *P* values of ≤0.0001 were considered as highly significant. All tests were two-tailed.

**Results**

**A549 and dTHP-1 cells respond differently to LPS and R848 stimulation**

In a previous study, LPS stimulated the release of *IL-6* and *IL-8* in pulmonary epithelial cells isolated from wild-type mice, but not from the ones isolated from TLR4-/- mice [42]. Nevertheless, data about the LPS concentration at which various lung cells become responsive demonstrate contrasting findings. In order to define this under our experimental condition, the expression levels of IL-8, IL-1β and IL-6 mRNA were measured at 6 h after stimulation of A549 and dTHP-1 cells with a gradient of LPS concentration. Our results showed a concentration-dependent induction of the three molecules in both cells, but with a variable pattern (Fig. 1). In A549 cells, the levels of the three cytokines increased gradually, reaching the peak when LPS used at 500 ng/ml, then gradually declined to their baseline level (Fig. 1a). Conversely,
LPS-stimulated dTHP-1 cells showed a consistent dose-dependent increase in the expression of IL-8 and IL-1β mRNA, which was maintained with increased LPS concentration (Fig. 1b). In both cells, IL-8 showed the highest induction level, whereas IL-6 showed the least one. We next examined if this is a time-dependent effect. As shown in Fig. S1a, further stimulation of both cells, with LPS (500 ng/ml) at various time points leads to a higher induction of IL-8 in dTHP-1 cells than in A549 cells at matched time points. We next measured the expression level of IFN-α as an indicator for the responsiveness of A549 and dTHP-1 cells to R848. It was clear that R848 evoked the production of IFN-α in a time-dependent manner in both cell lines (Fig. S1b).

LPS triggers cell-specific expression of miRNAs in A549 and dTHP-1 cells
To determine whether the expression of individual miRNAs will be variable between A549 and dTHP-1 cells, the cells were stimulated with LPS at a concentration of 500 ng/ml using the previously mentioned protocol, and then the relative expression of each miRNAs was measured 6, 12, 24 and 48 h post stimulation. As shown in Fig. 2, LPS stimulation resulted in a cell-dependent variation in miRNA expression with the majority of them being more highly expressed in dTHP-1 cells than in A549 cells. Of note, has-miR-223-3p and has-miR-155-5p were the only two miRNAs that showed the highest among-cells differences across all indicated time points (Fig. 2a and b). In contrast, has-miR-155-3p, has-miR-34c-1 and has-miR-146b-1 significantly exhibited this difference only at 6 h post stimulation, (Fig. 2c, e and f). Has-miR-449b-1 was the only one that did not show significant expression differences between the two cells in all time points except at 6 h post stimulation (Fig. 2d).
R848 induces cell-specific expression of miRNAs in A549 and dTHP-1 cells

In order to demonstrate if this a general cell-specific trend in miRNA expression, we investigated the expression level of the same miRNAs in A549 and dTHP-1 cells after stimulation with imidazoquinoline (R848) using a concentration of 5 µg/ml, which is known to be appropriate for cell stimulation (data not shown). Similar to LPS, R848 triggered more expression of has-miR-223-3p, has-miR-155-5p, has-miR-34c-1 and has-miR-146b-1 in dTHP-1 than in A549 cells (Fig. 3a, b, e and f). One exception was has-miR-155-3p (miR-155*), which showed a milder increase in A549 cells than in dTHP-1 cells (Fig. 3c). Has-miR-449b-1 exhibited similar cellular expression patterns as in LPS-stimulated cells (Fig. 3d). The timing patterns of miRNA expression were quite consistent with that when LPS was used in which has-miR-34c-1 and has-miR-146b-1 showed a significant variation only at 6 h post stimulation (Fig. 3e and f). As in LPS stimulation, expression of has-miR-223-3p and has-miR-155-5p showed the highest variation between A549 and dTHP-1 cells across all the indicated time points (Fig. 3a and b).

A549 and dTHP-1 cells express differential miRNA profile upon infection with various IAVs

To further dissect this phenomena using a pathogen that is known to infect the lung, we infected A549 and dTHP-1 cells with wild (S-OIV wt) and reassortant (S-OIV NS PR8) 2009 H1N1 pdm viruses using matched MOIs (MOI =2), followed by measuring miRNA expression levels across the indicated time points. Initially, we determined the replication kinetics of both viruses in these cell models. Fig. 4a and b illustrate the time-dependent replication competence of both viruses in A549 cells and dTHP-1 cells as evidenced by the level of viral haemagglutinin (HA) mRNA. In all time
points, both strains showed higher transcription efficiently in A549 cells than in dTHP-1 cells. The mRNA levels of S-OIV NS PR8 reassortant virus was higher than that of S-OIV wt in both cells lines (Fig. 4a and b). Considering these differences in replication kinetics, we asked whether infection with these viruses would induce cell- and virus-specific expression of cellular miRNAs. In terms of the cell-specific pattern, we noticed that, in contrary to the picture when TLR agonists were used, the infection with both viruses, regardless of the virus type, caused higher expression of miRNAs in A549 than in dTHP-1 cells (Fig. 5). It is worth noting that has-miR-223-3p showed the highest cell-dependent variation, with higher induction in A549 than in dTHP-1 cells. This was more pronounced when both cells infected with the S-OIV NS PR8 strain than when the cells infected with the S-OIV wt (Fig. 5a).

The cell-dependent differences in miRNA expression for all the studied miRNAs, except for has-miR-223-3p and has-miR-449b-1 (Fig. 5a and d) was observed beginning 12 h post infection, but not earlier, and then fluctuated at the following time points (Fig. 5). The results indicated that certain miRNAs exhibit similar expression patterns in the two cells irrespective of the virus strains. For instance, the expression levels of has-miR-155-3p and has-miR-146b-1 exhibited a gradual increase in A549 cells, which continued throughout the time course, whereas their expression showed a tendency to decrease in dTHP-1 cells (Fig. 5c and f). These results, at least for has-miR-155-3p, were consistent with those observed when both cells were primed with R848 (Fig. 3c).

We also identified a virus strain-dependent miRNA expression. Has-miR-223-3p showed the highest virus-dependent variation in which the S-OIV NS PR8 virus induced higher expression of this miRNA than did the S-OIV wt. Interestingly, this
difference was observed only in A549 cells (Fig. 6a). In contrast, no expression differences existed when dTHP-1 cells were infected with the two strains (Fig. 6a). Has-miR-223-3p exhibited down regulation in dTHP-1 cells regardless of the virus strain. Dissimilar to has-miR-223-3p, the S-OIV NS PR8 reassortant virus induced more expression of has-miR-155-5p, has-miR-155-3p and has-miR-146b-1 in dTHP-1 cells, but not A549 cells (Fig. 6b, c and f).

**Discussion**

Since their discovery in nematodes, miRNAs have shown to be critical regulators of multiple biological and pathological processes, mainly via binding to the 3’ untranslated (UTR) region of mRNA targets, which further results in either mRNA degradation or translation repression [43]. Evidence from prior studies clearly suggests roles for miRNAs in IAV-host interaction in aspects like apoptosis [44,45], virus-induced inflammation [46,24], viral virulence [19] and as a biomarker for disease prognosis [47]. Furthermore, miRNAs have been shown to impact TLR signaling pathways [15,48,39,49-51].

Upon IAV infection, lung epithelial cells and alveolar macrophages exhibited differences not only in their ability to support IAV replication [30,31], but also in the type and intensity of cytokines and chemokines produced after infection [31,32,52].

On the ground of our previous miRNAs profiling in lung harvested from H1N1-susceptible and -resistant mouse strains together with results from previous studies, we hypothesize that there might be a cell- and virus-dependent expression of cellular miRNAs, which might be correlated with the observed differential response of lung epithelial cells and alveolar macrophages to IAV infection.
In the current study, we investigated the cell- and virus-specific expression of selected miRNAs in A549 and dTHP-1 cells, which represent two main cellular components of human lung, and both have been known to be target cells for IAV replication. LPS (TLR4 agonist) was used because it is one of the best studied example of immunostimulatory bacterial components and can trigger systemic inflammation, therefore mimicking bacterial infection [53]. Additionally, R848 (TLR7 agonist) was chosen in order to identify if the observed miRNA expression is a general phenomenon associated with all negative sense RNA viruses or specific to IAVs. Wild type and a more virulent reassortant pandemic 2009 H1N1 reassortant were used to investigate the cell- and virus-specific miRNA expression.

There has been some debate about the responsiveness of different cell types to LPS priming. Therefore, we performed an initial experiment to address this. After 6 h of LPS stimulation, dTHP-1 cells responded more efficiently than A549, as evidenced by the gradual dose-dependent increase in the production of IL-8 and IL-1β mRNAs (Fig. 1). Measuring IL-8 mRNA in both cells after stimulation with various LPS concentrations re-confirmed the findings (Fig. S1a). An interpretation for this observed difference can be based on the difference in the surface expression of TLR4 [54] and/or the presence of absence of one or more components of the LPS sensor complex [55].

An important observation in our study is that, compared to A549 cells, dTHP-1 cells stimulated with LPS and R848 expressed higher levels of most of the studied miRNAs (for instance: has-miR-223-3p, has-miR-155-5p, miR-155-3p, miR-146b-1) (Table 2). Similar findings have also been reported previously by others. For instance, miR-146b was found to be expressed in LPS-stimulated monocytes and to
regulate pro-inflammatory mediators within the TLR4 pathway [39]. Similar findings were documented by Perry et al, who stated that stimulation of THP-1 cells with LPS resulted in a 3-fold increase in the expression of miR-146b at 6 hrs, which is even lower than in our case. These differences might be due to using different bacterial sources and doses of LPS; and in their study, these authors did not mention any prior activation of THP-1 by PMA, which might have an additional effect on miRNA expression. In the same study, and in agreement with our results, Perry et al showed that A549 cells and primary bronchial epithelial cells express more miR-146a than miR-146b, albeit using \( \text{IL-1}\beta \) as a stimulant [56]. Combining these data with ours suggests that LPS-stimulated lung epithelial cells are considered a poor producer of miRNAs such as miR-146b and miR-155, and possibly these miRNAs are expressed more highly in dTHP-1 cells following LPS challenge. The finding that dTHP-1 cells express high levels of miR-155-3p have been reported previously by Cornell et al, who showed that miR-155 is induced in murine bone marrow-derived macrophages after stimulation with various TLR ligands including LPS [36]. Along the same line, silencing technology combined with microarray analysis demonstrated that miR-155 is upregulated in monocyte-derived dendritic cells and is able to decrease expression of inflammatory cytokines [17]. It is worth mentioning that although miR-146 and miR-155 seem to be LPS-responsive genes that are co-activated through NF-\( \kappa \)B, they are responsive to different thresholds of LPS and can control different phases of TLR4 signaling [57]. The observation that dTHP-1 cells express higher level of has-miR-155-5p, -155-3p, -223-3p, -34c-1 and -146b-1 than A549 cells suggests a differential cellular sensitivity to TLR agonists. Furthermore, putting forward the assumption that these
miRNAs might play more important roles in macrophages than in lung epithelial cells. An interpretation of this phenomenon is not yet complete. The fact that different molecules within the LPS sensing complex (LPS binding protein (LBP), CD14, MD-2 and TLR4) [58,59] might be needed by different cells to fulfil an adequate response [60], together with the well-known differential cellular expression of TLR4 [61], suggests that these factors might account for the observed cell-specific miRNAs response in A549 versus dTHP-1 cells. As supported by the findings of Zarember and Godowski [62], it is plausible to speculate that using PMA in differentiating THP-1 cells might enhance miRNA expression.

Fig. 2 indicated that most of LPS-induced miRNA in dTHP-1 cells follow a certain timing pattern in which they peaked 6 h after stimulation, a finding that may be linked to the function of these miRNA. A similar finding was previously reported for miR-146 after LPS challenge of dTHP-1 cells (the level reached a plateau ≈ 8 h) [16]. Moschos et al. reported earlier peaking time (3 h) in mice aerosolized with LPS [63]. Discrepancies among these studies might be attributed to the source of LPS (Salmonella typhimurium versus Escherichia coli–derived LPS) as suggested by Koyama et al [64] as well as to the study model. Comparative studies and mathematical modelling demonstrated that various lung cells are considered targets of IAV [65,30] and that cell tropism could influence influenza severity [66]. Previous studies indicated that IAV replication competence [30,31] as well as the virus-triggered immune response [67] are cell-specific.

In the current study, we observed that the S-OIV NS PR8 reassortant replicated more efficiently than S-OIV wt in A549 and dTHP-1 cells (Fig. 4). This re-confirmed our previous observations using human and swine cell lines and human lung
explants (unpublished results). The demonstration that A549 cells support the replication of both viruses more than dTHP-1 cells is in agreement with previous studies, which confirm that A/HK/54/98, a seasonal H1N1 strain can replicate in alveolar epithelial cells (type I and II) more efficiently than in alveolar macrophages [31].

Compared to dTHP-1 cells, our results revealed that A549 cells infected with both viruses mounted greater expression of most of the studied miRNAs (Fig. 5). We suggest that the increased replication competence of both viruses in A549 cells compared to dTHP-1 cells might account for this. Indeed, immune histochemical analysis revealed that highly pathogenic H5N1, seasonal and pandemic viruses predominantly infect type II pneumocytes [30]. Pandemic and seasonal influenza viruses were also shown to mainly colonize type II pneumocytes [28,68], but not alveolar macrophages, where the infection might be aborted [31]. The study of Cline et al suggests that either using alternative receptor or a special feature of the HA glycoprotein grant H5N1 viruses the ability to overcome an early block of virus replication in macrophages [69].

Infection with H1N1 virus differs from stimulating the cells with TLR agonists because the virus has to modulate the host cell environment to establish the infection. This might account for the observed differences in the cell-specific up-regulation of miRNAs between TLR agonists and H1N1 viruses used in this study.

Our analysis revealed that infection with S-OIV NS PR8 triggered stronger up-regulation of has-miR-223-3p compared to S-OIV wt only in A549 cells. In contrast, the S-OIV wt caused its down regulation in both cell lines. This suggests an
association of miR-223-3p with virus virulence and proposes a different role for this miRNA in the two cells types.

*In vivo* mousece experiments involving the lethal reconstructed 1918 strain (r1918) and non-lethal A/Texas/36/91- infected mice suggested an association of this miRNA with increased virus virulence [70]. Similar experiments concluded the same results [19]. Mechanistically, it has been proposed that miR-223 tends to dampen the inflammation due to either an inhibitory effect of miR-223 on some of the pro-inflammatory pathway components, for instance *STAT3, IL-6* and *IL-β* [71] or via targeting the NOD-like receptor p3 gene (NLRP3) with subsequent inhibition of *IL-β* production [72].

We observed an induction of miR-155 with its two strands in A549 and dTHP-1 cells. However, the S-OIV wt induced higher expression of has-miR-155-3p (miR-155*) than the S-OIV NS PR8 only in A549 cells. These results suggest that the more virulent virus down regulates has-miR-155-3p for its benefit because miR-155 was found to be required for optimal primary CD8+ T cell responses [35] as well as also promoting type 1 IFN signaling through inhibition of suppressor of cytokine signaling (SOCS) [73].

In summary, our study has shown that miRNA expression exhibited cell-type specific signatures following stimulation with TLR agonists and infection with H1N1 pandemic 2009 viruses (*Table 2*). Furthermore, virus-specific miRNA expression was also observed especially in A549 cells. This study, therefore, lays the groundwork for exploring the role of miRNAs in certain host cell populations, which may improve our understanding the cell-based differences in replication competence of the pandemic 2009 H1N1 virus.
Acknowledgments

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Authors’ contributions

M.S. and F.P. conceived and designed the experiments. M.S., and A.H., performed the experiments. M.A. performed the statistical analysis. A.M. and S.P. contributed reagents and materials, in particular the virus strains. M.S. analyzed the data and prepared the initial draft of the manuscript. All authors revised and reviewed different drafts of the manuscript. M. S. and F. P. wrote the the final version and take responsibility for the integrity of the data.

Competing interests

The authors declare that they have no competing interests.

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(DAAD) and the Egyptian Ministry of Higher Education and Scientific Research (grant ID: A/11/92510) to M. Samir and by funds from iMed, the Helmholtz Association’s Initiative on Personalized Medicine to F. Pessler.

**Conflict of interest**

Mohamed Samir declares that he has no conflict of interest. Aamna Habib declares that she has no conflict of interest. Ahmed Mostafa declares that he has no conflict of interest. Manas Akmatov declares that he has no conflict of interest. Stephan Pleschka declares that he has no conflict of interest. Frank Pessler declares that he has no conflict of interest.

**Ethical approval**

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

**References**

44. Othumpangat S, Noti JD, Beezhold DH (2014) Lung epithelial cells resist influenza A infection by inducing the expression of cytochrome c oxidase Vlc which is modulated by miRNA 4276. Virology 468-470:256-264. doi:10.1016/j.virol.2014.08.007

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### Tables

**Table 1. Primer sequences for the target genes used in his study.** Fwd. and Rev. denote forward and reverse primers, respectively.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Sequence (5<code> to 3</code>)</th>
<th>Amplicon size (nt)</th>
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<td></td>
<td>Rev.</td>
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<td></td>
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<tr>
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<tr>
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<td>Rev.</td>
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<tr>
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<tr>
<td></td>
<td>Rev.</td>
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Table 2. Summary of cell-specific expression of miRNAs. A549 and d THP-1 cells were stimulated with TLR agonists (LPS and R848) and pandemic 2009 H1N1 (S-OIV wt and S-OIV NS PR8). For the corresponding miRNA, the (+) and (−) signs indicate up and down-regulation, respectively. The (+/-) sign indicates a fluctuation of expression. Double + or − signs indicate more pronounced up- or down-regulation, respectively.

<table>
<thead>
<tr>
<th>Stimulant</th>
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<th>R848</th>
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<th>S-OIV NS PR8</th>
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<tbody>
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<td>d THP-1</td>
<td>A549</td>
<td>d THP-1</td>
</tr>
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<td>Has-miR-223</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Has-miR-155-5p</td>
<td>Con¹</td>
<td>++</td>
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<td>Has-miR-155*</td>
<td>Con.</td>
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<td>++</td>
<td>+</td>
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<tr>
<td>Has-miR-449b</td>
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<td>+</td>
<td>+</td>
</tr>
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<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Has-miR-146b</td>
<td>Con.</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

¹ Con., constant
Figure 1. Dose-dependent induction of cytokines and chemokines in A549 and dTHP-1 cells after 6 h of LPS challenge. A549 (a) and dTHP-1 (b) cells were stimulated with LPS at a range of concentrations (200-500 ng). Fold changes in expression were calculated using the $2^{-\Delta\Delta CT}$ method after normalizing to human β-actin mRNA expression.
Figure 2. Differential expression of miRNAs in A549 and dTHP-1 cells after stimulation with LPS (500 ng/ml). Fold changes in expression were calculated using the $2^{(\Delta\Delta CT)}$ method after normalization to human RNU6b-2 expression. Data are presented as means of fold change ± SEM. Statistical significance was calculated by applying unpaired, two-tailed t-test using the Graphpad prism software V.5. Asterisks were assigned if the differences in the expressions of certain miRNA between both cells at each indicated time point were significant. * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$).
Figure 3. Differential expression of miRNAs in A549 and dTHP-1 cells after stimulation with R848 (5 µg/ml). Fold changes in expression were calculated using the $2^{\Delta\Delta CT}$ method after normalization to human RNU6b-2 expression. Data are presented as means of fold change +/- SEM. Statistical significance was calculated by applying unpaired, two-tailed t-test using Graphpad prism software V.5. Asterisks were assigned if the differences in the expressions of certain miRNA between both cells at each indicated time point were significant. * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$).
Figure 4. Relative quantification of hemagglutinin (HA) mRNA of S-OIV wt and S-OIV NS PR8 (reassortant) in A549 and dTHP-1 cells at various time points. Fold changes in expression were calculated using the $2^{-\Delta\Delta CT}$ method after normalization to human $\beta$-actin mRNA expression. Data are presented as means of fold change +/- SEM. Statistical significance was calculated by applying unpaired two-tailed t-test using Graphpad prism software V.5. Asterisks were assigned if the differences between the expressions of viral HA mRNA levels between both viruses at each indicated time point were significant. * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$).
Figure 5. Cell-specific expression of miRNAs upon infection with S-OIV wt and S-OIV NS PR8 at various time points. Fold changes in expression were calculated using the $2^{\Delta\Delta CT}$ method after normalization to human RNU6b-2 expression. Statistical significance was calculated by applying unpaired two-tailed t-test using Graphpad prism software V.5. Asterisks were assigned if the differences between the miRNA expression levels between both cells at each indicated time point were significance. * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$).
Figure 6. Relative quantification of miRNAs in A549 and dTHP-1 cells infected with S-OIV wt and S-OIV NS PR8. Fold changes in expression were calculated using the $2^{(\Delta \Delta CT)}$ method after normalization to human RNU6b-2 expression. Statistical significance was calculated by applying unpaired two-tailed t-test using Graphpad prism software V.5. Asterisks were assigned if the differences between the miRNA expression levels between both viruses at each indicated time point were significance. * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$).
Figure. S1. Time and cell-dependent induction of IL-8 (a) and IFN-α (b) mRNA in A549 and dTHP-1 cells. Both molecules are expressed in both cell types at various time points. Fold changes in expression were calculated using the $2^{(-\Delta\Delta CT)}$ method after normalization to human β-actin mRNA expression. Statistical analysis was done using repeated measures ANOVA tests. The relative expression for IL-8 or INF-α are shown as average of the fold change +/- SEM. Asterisks were assigned if the differences in the expression level of IL-8 or INF-α levels between the un-stimulated ans stimulated states at each indicated time points were significant. * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$).
3.4. Manuscript IV
(In preparation)

Title
A previously uncharacterized Egyptian strain of highly pathogenic avian influenza virus (H5N1) emerged in clade 2.2.1.2 and is highly pathogenic in an experimentally infected domestic duck breed

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Abstract

The highly pathogenic avian influenza (HPAI) H5N1 viruses continue to cause major problems in poultry and impact upon human health. Although H5N1 usually causes asymptomatic infection in ducks, recent isolates demonstrate unusual lethality. Here, we show that the Egyptian isolate A/chicken/Faquos/amn12/2011 belongs to clade 2.2.1.2 and spreads systemically in experimentally infected ducks. Neurological abnormalities and deaths became apparent within 48 hpi. Viral transcription (HA mRNA) was highest in lung, heart and kidney. It generally followed an exponential increase, peaking between 40 and 48 hpi, whereas only low levels were detected in brain. Lung, heart and pancreas were histologically most severely affected, especially at 40 and 48 hpi. Mild neuronal degeneration and gliosis were observed in brain. These results support the notion that isolates from clade 2.2.1.2 are lethal to domestic ducks and that their high pathogenicity may lead to increased incidence of local outbreaks at the farm or household level.

Keywords

Ducks, experimental infection, highly pathogenic avian influenza A virus (HPAI), histopathology, influenza A virus (H5N1), modeling, transcription

Highlights

- Upon infection with IAV (H5N1) virus, ducks often show mild clinical diseases.
- Recently, there have been reports of more virulent isolates, causing severe disease in ducks.
- In Egypt, such isolates have emerged within IAV (H5N1) clade 2.2.1.2.
- We analyzed an uncharacterized Egyptian IAV (H5N1) isolate, (A/chicken/Faquos/amn12/2011).
- It clusters within clade 2.2.1.2., causes severe disease in ducks, and spreads systemically.
Introduction

The highly pathogenic avian influenza viruses (HPAI) of the H5N1 lineage are of particular concern because they cause systemic illness and high mortality in birds and are capable of infecting mammals. The first isolation of H5N1 virus in 1997 from a 3-year-old boy and, later, from 18 individuals in Hong Kong demonstrated the potential of this strain to cause severe disease in humans (de Jong et al., 1997; Subbarao et al., 1998). HPAI H5N1 viruses belong to the Orthomyxoviridae family and possess negative sense segmented RNA genomes. Although a wide range of bird species can be infected with HPAI H5N1 (Alexander, 2000), inter-species variation in terms of disease susceptibility and outcomes have been reported (Cornelissen et al., 2013). Previous studies involving natural and experimental infection indicated that chicken and turkey exhibited systemic disease followed by high mortality, whereas ducks developed minimal or no clinical signs despite infection (Cornelissen et al., 2013; Kayali et al., 2011; Kuchipudi et al., 2014). This may be due to higher levels of inflammatory cytokines and higher viral loads in chicken and turkey compared to ducks (Burggraaf et al., 2014) and/or inter-species variation in the induction of virus-sensing molecules in the host cells (Cornelissen et al., 2012). Webster et al suggested that ducks represent a silent reservoir for H5N1 viruses since they can shed high viral titers in pharyngeal and fecal excretion in the absence of debilitating disease (Webster et al., 1992). This was further supported by data from previous outbreaks that indicate the potential of wild ducks (mallard) to be long-distance vectors for H5N1 viruses (Gilbert et al., 2006). Besides being resistant, ducks are cosmopolitan and occupy most aquatic habitats. Therefore, the duck is considered an important component in the ecology and propagation of HPAI H5N1 viruses (Kim et al., 2009).

In late 2002, many H5N1 isolates became unusually lethal in ducks (Sturm-Ramirez et al., 2004). Reports of fatal outbreaks in wild birds in western China (Chen et al., 2005; Liu et al., 2005) and Korea (Kwon et al., 2005; Rhyoo et al., 2015) reinforced this notion. In parallel, in vivo experiments reflected the same pattern for post-2002 H5N1 isolates (Kishida et al., 2005; Londt et al., 2008; Yamamoto et al., 2007). A
similar pattern has been observed in Egyptian poultry after 2002 (OIE, 2008). Two Egyptian H5N1 viruses isolated in 2007/2008, belonging to clade 2.2.1, were reported to be highly pathogenic in Peking ducks (Anas platyrhynchos) (Wasilenko et al., 2011). More recently, Hassan et al. identified HPAI H5N1 (clade 2.2.1) in asymptomatic ducks even in the summer season when the virus was not supposed to circulate (Hassan et al., 2013). This sudden shift in HPAI H5N1 virus virulence in ducks might be attributed either to the long persistence of the viruses inside their natural host after development of humeral immunity (Wibawa et al., 2014) and/or the extensive use of unapproved vaccines (Lee et al., 2004). These factors tend to impose immune pressure on the virus and drive mutations in the viral genome (Ludwig et al., 1995), leading to the appearance of novel isolates.

In Egypt, various H5N1 clades have been circulating in domestic poultry. Clade 2.2, which appeared in Egypt in February 2006 (Saad et al., 2007), quickly diversified into a classic clade (clade 2.2.1) (Abdelwhab et al., 2010) and a variant clade, which was later designated clade 2.2.1.1/2.2.1.1a (WHO/OIE/FAOH. N. Evolution Working Group WHO, 2012). Recent phylogenetic and viral protein analysis confirmed that clade 2.2.1 has evolved into a novel distinct clade (El-Shesheny et al., 2014), which was delineated later by the HO/FAO/OIE evolution work group as clade 2.2.1.2. However, a recent study involving 29 H5N1 viruses that were isolated after October 2014 reported the emergence of another novel cluster within clade 2.2.1.2 (Arafa et al., 2015).

When characterizing a new IAV isolate it is important to assess the kinetics of viral spread and to correlate these data with clinical and histopathological indices of disease severity. Although influenza A virus (IAV) kinetics have been investigated in various studies (Beauchemin and Handel, 2011; Hernandez-Vargas et al., 2014), the models generally did not explore viral kinetics in multiple compartments that may have different virus replication patterns and hence reflect the spread of the virus in various body tissues. Several mathematical models can be applied for this purpose, e.g. the target cell limited model and the logistic growth model (Baccam et al., 2006). However, when data on viral replication are restricted to HA mRNA expression,
model parameter estimation is difficult to perform, and polynomial regression and correlation studies are more suitable to assist the interpretation of the experimental data, for instance by modeling of kinetic curves (Ramsay et al., 2007).

Considering the continuous evolution of HPAI H5N1 viruses, the presence of multiple clades circulating in Egyptian poultry, and the previously described increase in virulence of H5N1 isolates to the duck (which was believed to be relatively resistant to H5N1 infection), it is important to investigate newly emerging H5N1 isolates in terms of phylogeny, replication dynamics, and clinical and pathological features in this natural host. We therefore performed a detailed analysis of these parameters in a previously uncharacterized Egyptian HPAI H5N1 virus (A/chicken/Faquos/amn12/2011) that was isolated in 2011 following a fulminant outbreak in a broiler chicken flock in El-Sharkia province, Egypt. We used a native duck breed derived from the mallard duck (Anas platyrhynchos) as an experimental model since it is one of the most common locally produced and raised crossbreed in Egypt and thus represents a potentially important host in future HPAI H5N1 outbreaks in Egypt.

Our results indicate that the new isolate belongs to the newly identified clade 2.2.1.2., spreads systemically to multiple organs with organ-to-organ variations in virus kinetics, growth rate, and pathological lesions, and can be lethal to this host. The results lend further support to the aforementioned observations of an increased pathogenicity of emerging HPAI H5N1 isolates to the duck. They suggest that the duck may constitute an increasingly important source of local outbreaks, which may have implications for our understanding and management of current and future HPAI H5N1 outbreaks not only in Egypt, but also in remote endemic places.
Materials and Methods

Virus propagation and titration

We used the HPAI strain (A/chicken/Faquos/amn12/2011(H5N1)), accession number JQ627585.1, which was kindly provided by Vet. Abdo Nagy (Virology Department, Faculty of Veterinary Medicine, Zagazig University). It was isolated following a fulminant outbreak in household broiler chicken flocks in Faquos territory, El-Sharkia province, Egypt. Procedures for virus propagation, titration and re-isolation were carried out as described previously (Salem, 2014). Briefly, 200 µl of the virus were inoculated into 11 day-old specific pathogen-free (SPF) embryonated chicken eggs (SPF-ECEs), and equal numbers of SPF eggs were inoculated with an equal volume of PBS and were used as control group. All SPF eggs were incubated at 37˚C and 60%-70% humidity and monitored daily for viability. Non-viable eggs were collected and refrigerated at 4˚C. The allantoic fluids of all SPF eggs were assessed for viral hemagglutination (HA) activity. The propagated virus was identified in the harvested allantoic fluid by hemagglutination inhibition (HAI) test as described previously (Salem, 2014). Virus titers were calculated according to the Reed and Muench method (REED and MUENCH, 1938).

Phylogenetic analysis and amino acid characterization

For phylogenetic analysis, a partial sequence of the hemagglutinin (HA) gene of the new isolate was compared to sequences of the HA gene of another 37 H5N1 isolates that belong to clades 2.2, 2.2.1 (classic), 2.2.1.1 (variant) and 2.2.1.2 (Table S1 and Fig. 1) and were isolated between 2006-2011 from different localities in Egypt. We also included the parental H5N1 virus that was isolated in 1996 in Guangdong province from infected geese. Evolutionary analyses were conducted using the Bioedit sequence alignment editor and the MEGA 5 software tools (Hall, 1999; Tamura et al., 2011). The evolutionary history of the virus was inferred using the neighbor-joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch lengths = 0.44213287 is shown (Fig. 1). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances
were computed using the p-distance method (S, 2000) and are in the units of the number of base differences per site. All positions containing gaps and missing data were eliminated. There were 379 positions in the final dataset. In order to confirm the results obtained by the phylogenetic analysis at the protein level, a stretch of amino acids (aa 110-165) of the new isolate was compared to that of 27 other H5N1 isolates spanning the same clades that comprised the phylogenetic analysis (Table S2 and Fig. 2).

**Experimental infection**
A commercial two-week-old native duck breed derived from the mallard duck (*Anas platyrhynchos*) was used in the experiment. The ducks were housed in a sterile self-contained isolation unit under continuous lighting for 5 days. Prior to virus inoculation, hemagglutination inhibition (HAI) and ELISA tests were conducted to ensure the absence of previous H5N1 infection (Salem, 2014). Ducks (average weight 400 g) were separated into control (n = 10) and infection groups (n = 50). The control and the infected ducks were inoculated intranasally with 50 µl of PBS and 50 µl of $10^{6.7}$ embryonated egg infective dose 50 (EID$_{50}$), respectively. At least three ducks per time point from the virus-inoculated group were euthanized at 8, 16, 24, 32, 40, 48, 72, 96 and 120 hours post infection (hpi). Three ducks from the control group were euthanized for comparison. The animals were weighed at t = 0 h and all subsequent time points. Tissue samples from lung, trachea, brain, heart, pancreas, spleen, kidney, and intestine were collected from infected and control ducks and stored at - 80°C to be used for RNA extraction; another piece of the same tissue was immersed for a maximum period of 48 hours (h) in 10% v/v buffered formalin for histopathological and immunohistochemical analyses. The RNA-based analyses were not performed on intestine (due to poor RNA quality) and cecal tonsil, bursa of Fabricius, and liver (to limit the scope of the study). The ducks were observed daily for clinical signs. Those with signs of severe disease (e.g., loss of balance, refusal to feed or drink, paralysis, recumbent posture) were euthanized and subjected to postmortem examination, but they were not included in the RT-qPCR and histological analyses. All experimental work was performed in compliance with the
local rules on animal welfare and was approved ahead by the Ethics Committee for Animal Studies at the Faculty of Veterinary Medicine, Zagazig University, Egypt.

**RNA extraction and reverse transcription**
From each organ, approx. 30 mg of tissue was homogenized in a mixture of 600 µl RLT buffer and 6 µl β-mercaptoethanol (β-ME) using beads in a rotor-based homogenizer. Total RNA was extracted using the miRNeasy kit (Qiagen) and was then DNase-treated using the RNase-Free DNase Set (Qiagen). RNA quality and quantity were determined with the Nanodrop S1000 spectrophotometer (Thermo Scientific). The DNase-treated RNA was reverse transcribed using the miScript Reverse Transcription Kit (Qiagen) in a volume of 20 µl.

**Quantitative reverse transcriptase real-time PCR (RT-qPCR)**
To measure H5N1 HA mRNA expression, RT-qPCR was performed using the miScript SYBR Green PCR Kit (Qiagen). Duck β-actin was used as a reference mRNA. The following primers were used (H5N1 HA: forward CAGCATGTCCATACCAGGGAA, reverse CCACTTTGCCCCTTTACCTT; β-actin: forward CCATTGAACACGGTATTGTCACC, reverse GCTACATACATGGCTGGGGT). Primer sets were checked for specificity based on the melting curves. The efficiency of the RT-qPCR runs was assessed by determining the primer amplification efficiency and correlation coefficient on a scale of two-fold diluted cDNA samples from kidney, lung, brain and heart. A Light Cycler 480 real-time PCR instrument (Roche, software version 1.5) was used for RT-qPCR. The cycling conditions started with pre-incubation at 95°C for 15 min, followed by 40 amplification cycles at 94°C for 15 seconds (s), 55°C for 30 s and 72°C for 30 s. Melting curves were generated by heating the samples to 95°C. Fold change (FC) in expression was calculated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

**Histopathological lesions in infected organs**
Formalin-fixed tissues from at least 3 ducks per time point were processed according to standard procedures and embedded in paraffin. For histopathological examination, triplicates of paraffin-embedded tissues were sectioned (thickness of
the sections 3 µm) and stained with hematoxylin/eosin (H&E). In order to assess the degree of the histopathological lesions, each H&E-stained slide was analyzed with respect to inflammatory and degenerative changes. The degree of damage was scored as mild, moderate or severe.

**Mathematical modeling and statistical analysis**

The RT-qPCR data were used to apply local polynomial regression (LOESS), a flexible tool that does not require the specification of a model function to the data (Jianqing Fan, 1996). In order to describe the exponential growth rate of the virus in the studied organs, we made the assumption that the virus grows exponentially with rate $g$, i.e., $y_t = ae^{gt}$, where $y$ is the viral concentration measured, $t$ is the corresponding time points, and $a$ is the intercept of the statistical model (Smith et al., 2010). The Pearson correlation coefficient was used to assess correlations in viral transcription across the organs. To determine significant differences between uninfected and infected groups in terms of HA mRNA fold change of expression, statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test using the FC values at each time point. $P$ values of $< 0.05$ were considered significant. Statistical analyses were done with the Graph pad prism V.5 software (Graph Pad Software, Inc., San Diego, California).

**Results**

**Phylogenetic analysis and amino acid characterization**

To explain the genetic relatedness of the studied virus to other strains that have circulated in Egypt, we constructed a phylogenetic tree based on comparing a partial sequence of the HA gene of the new isolate to partial and full HA sequences of 37 other strains (Table S1). As shown in Fig. 1, the phylogenetic tree indicated that the A/chicken/Faquos/amn12/2011 isolate clustered with the recently emerged H5N1 clade 2.2.1.2 and is phylogenetically close to clade 2.2.1, but more distant from the variant clade 2.2.1.1. It showed a close relation with Egyptian viruses isolated between 2011 and 2014. In order to understand the protein characteristics of A/chicken/Faquos/amn12/2011, we aligned a sequence of 55 amino acids of its HA
protein to that of a panel of HPAI H5N1 clades that have been circulating among Egyptian poultry (Fig. 2). The analysis indicated that the new isolate shares a common amino acid signature with viruses belonging to the new clade (2.2.1.2), and that these residues differ from those of other H5N1 virus clades (Fig. 2 and Table S2). This signature includes H110, D120, S123, Δ129, R140, S141, F144, A156, K162 and N165, although one strain (A/Duck/Egypt/14154FAOS/2014) has the amino acid residue S at position 129. Compared to clade 2.2, residues such as H110, S123, R140, S141, F144, A156 and N165 were conserved, whereas some mutations including S120D, S129Δ, I151T and R162K were observed. The presence of D120 discriminated the clade 2.2.1.2, including our isolate, from all other clades. It was also clear that differences exist in the various amino acid residues between our virus and those clustering in clades 2.2.1.1 and 2.2.1.1a. Examples of these differences are R110H, S120D, P123S, S/Δ129Δ, G140R, P141S, Y144F, and H165N. However, some amino acid residues were infrequently conserved between the two clades (e.g. I/T151T, A/T156T and K162).

Clinical observations and findings of gross pathology
The PBS inoculated ducks appeared healthy throughout the period of the experiment, and pathological lesions were not seen upon necropsy. Fig. 3 summarizes the timeline of clinical signs and gross pathology observed on the infected ducks. It was not possible to calculate the natural mortality rate of the infected ducks in this experiment, as several ducks needed to be euthanized due to signs of severe disease (see Methods). During the first 40 hpi, the infected ducks exhibited general signs of illness including ruffled feathers, recumbence and decrease in food and water intake. At 48 hpi, labored breathing, neurological manifestations (tremors, circling and loss of balance) and death of the first duck were recorded. Dissection of the ducks at this stage revealed several areas of congestion in most of the internal organs. Some ducks developed paralysis. The intestine was filled with greenish content. At 96 hpi, the ducks appeared dehydrated. The intestines were empty, and splenomegaly associated with congestion in most of the internal organs was observed. An overall 35% of infected animals were found dead across all time points. There were no significant differences in weight between the
infected and the control ducks across the time course, which was likely due to widespread fluid retention in the infected animals due to congestion of liver and lung and congestion coupled with enlargement of the spleen.

**Histopathological lesions in selected organs**

Upon histological examination, infected lungs showed a lympho-histiocytic, interstitial pneumonia and various degrees of dispersed necro-suppurative foci starting by 8 hpi. There was mild to moderate multifocal necrosis of epithelial cells. Additionally, hyperplasia of the airway-associated lymphoid tissue and moderate to severe hyperemia were noticed. Generally, lung lesions were most prominent at 48 and 72 hpi. In the infected trachea, only mild to moderate lympho-histiocytic inflammation could be observed. We also detected single necrotic epithelial cells at 120 hpi. Interestingly, the heart of infected ducks was severely affected at various time points. Moderate to severe lympho-histiocytic epi- and myocarditis were noted at 8, 24, 40 and 48 hpi. In some animals, a mild to moderate myocardial degeneration was also detected at 8, 24, 40, 48 and 72 hpi. The pancreatic tissue showed mild to moderate heterophilic to lympho-histiocytic inflammation mixed with multifocal necrotic areas. These changes started as early as 8 hpi and persisted until 96 dpi. The most severe lesions were found at 40, 48 and 72 hpi. In the brain, the lesions were less severe than in other organs, as evidenced by the presence of mild degenerative lesions consisting of mild neuronal degeneration and gliosis, which were observed in one animal at 48 hpi. In the intestine, only a mild to moderate lympho-plasmacytic to heterophilic inflammation was detected. Lesions were more pronounced at 72 and 96 hpi. The cecal tonsil showed only mild to moderate heterophilic inflammation, which could also be found in control tissue. Additionally, few apoptotic lymphoid cells were present from 24 hpi on. The bursa of Fabricius of infected animals displayed mild infiltration with heterophilic granulocytes, but a similar picture was also seen in control animals.

**Quantification of viral transcription (HA mRNA) in selected organs**

Viral HA mRNA in selected organs was detected by RT-qPCR. As shown in Fig. 4, HA mRNA was detected in all organs examined. However, kinetics of viral
transcription varied depending on the organ and the time post infection. The virus exhibited similar kinetics in lung, trachea, heart, kidney, pancreas and spleen, where HA mRNA was first detected at 8 hpi, peaked by 48 hpi, and then decreased gradually. In most of the organs, HA mRNA expression had begun to decrease by 96 hpi. Interestingly, a short viral rebound could be observed in most of the organs by 120 hpi. In brain, the virus follows different kinetics in that HA mRNA could also be detected by 8 hpi, but then rose to a sustained plateau for most of the remainder of the time course. While highest viral transcription was recorded in lung, kidney, spleen, and heart, the lowest was observed in brain (Fig. 4 and 5). Of note, some variations in fold change (FC) were also observed in the pancreas, spleen and kidney (Fig. 4 and 5). As expected, HA mRNA was not detected in the PBS-inoculated control animals.

**Mathematical modeling of HA transcription in selected organs**

Transcription of the HA segment apparently followed similar kinetics in most organs, in that there was a rapid, exponential increase that peaked between 40 - 48 hpi in all organs except the brain (Fig. 5). Notably, LOESS did identify significant differences in viral exponential transcription rate, \( g \), among the organs: during the first 48 hpi, it was highest in heart followed by spleen, lung and trachea (range of \( g \), 0.1 - 0.2), intermediate in pancreas (\( g \), 0.05) and kidney (\( g \), 0.09), and lowest in brain (\( g \), 0.03) (Fig. 5, dashed red lines). With regard to correlations of HA transcription among these organs, high temporal correlations were observed between lung versus (vs.) trachea, spleen, and kidney; between trachea vs. brain, heart, and kidney; and between spleen and kidney. Low correlations were found between lung vs. brain and pancreas; and between pancreas vs. kidney (Fig. 6).

**Discussion**

Since the emergence of HPAI H5N1 in Egypt in 2006, this virus has continued to cause outbreaks in domestic poultry followed by sporadic human cases (Kayali et al., 2011). The recent emergence of the new Egyptian H5N1 clade 2.2.1.2 in domestic birds (Arafa et al., 2015; El-Shesheny et al., 2014; Smith et al., 2015),
coupled with an increase in virulence in domestic avian hosts, motivated us to investigate phylogenetic relationships, replication dynamics and virus-induced tissue pathology caused by a previously uncharacterized Egyptian H5N1 isolate. A native duck breed was used as an experimental model.

**Phylogenetic clustering of the new isolate**

Based on a partial nucleic sequence of the HA segment, our isolate clearly clustered with other H5N1 strains which had previously been shown to belong to clade 2.2.1.2. ([Fig. 1](#)) ([El-Shesheny et al., 2014; WHO/OIE/FAO. N. Evolution Working Group WHO, 2012]). Furthermore, the identified amino acid signature of the corresponding partial amino acid sequence ([Fig. 2 and Table S2](#)) was also consistent with a phylogenetic origin within this clade (El-Shesheny et al., 2014). According to our amino acid sequence alignment, the presence of the mutation S120D solely in our isolate along with those of the clade 2.2.1.2 points out that this residue may represent a central feature of a signature unique to this clade. It is worth noting that the mutation T156A, which was observed in our analysis and was also reported by El-Shesheny et al., might lead to a change in the glycosylation site at positions 154-156, which may further increase the likelihood of transmission of clade 2.2.1.2 isolates to humans ([Herfst et al., 2012](#)). Collectively, the analysis highlights the importance and need for continuous surveillance for HPAI H5N1 isolates in order to correctly assign them to specific clades and to predict their potential impact on poultry and human health.

**Correlates of clinical severity of infection with the new isolate.** We showed that the domestic duck breed used is highly susceptible to infection with the HPAI A/chicken/Faquos/amn12/2011(H5N1) isolate, as evidenced by the characteristic clinical signs and high mortality. The virus disseminated systemically and reached all internal organs tested, and even the brain, albeit at lower levels. The replication kinetics, HA exponential transcription rate, and the virus-induced pathological lesions varied in a time and tissue-dependent manner throughout the course of the experiment. The early onset of clinical signs in our study (48 – 72 hpi) agrees with previous reports of HPAI H5N1 infection of susceptible mallard and peking ducks.
Our results also provide evidence for CNS involvement during infection with A/chicken/Faquos/amn12/2011(H5N1), even though viral transcription was markedly lower in brain than in the other organs tested. Considering the relatively low degree of viral transcription in brain, the neurological manifestations were remarkably strong. This may be due to long-distance effects from infection of other organs such as release of cytokines, organ dysfunction, or metabolic disarrangements. Nonetheless, the findings clearly show that this new strain can cause major neurological involvement, supporting the notion of the pathogenicity shift of recent HPAI H5N1 isolates with respect to the duck.

**Histological correlates of systemic spread of infection with the new isolate**
The histological analyses revealed that the most severely affected organs were lungs, pancreas and heart. Similar results have been described by others, albeit using different duck breeds (pure-bred mallard, Peking) and inoculation routes (intranasal, intraocular and oral) (Keawcharoen et al., 2008; Londt et al., 2008; Wasilenko et al., 2011). Data from previous studies revealed that multiple organs might be severely affected with the HPAI H5N1 virus, likely because of its high virulence (Londt et al., 2008; Vascellari et al., 2007). Of note, this multi-organ involvement of HPAI (H5N1) infection is not seen in infection with low pathogenic avian influenza (LPAI) viruses (Daoust et al., 2011). This differential involvement of internal organs suggests that, in addition to the commonly taken cloacal and nasal swabs, veterinarians should investigate multiple organs when investigating a potential HPAI H5N1 outbreak.

**Potential causes of severe disease and/or death**
The results presented here suggest that the death of inoculated ducks might be due to either myocardial damage or neurological dysfunction. This agrees with the study conducted by Kishida et al, who reported that a more virulent H5N1 strain, DK/Yokohama/03, could be detected at higher titer in brain and showed more evidence of neurological signs than a less virulent strain, Ck/Yamaguchi/04 (Kishida et al., 2005). Keawcharoen et al. found that the wild mallard did not show overt
clinical disease and no evidence of brain invasion, whereas other wild ducks such as tufted ducks and pochards, exhibited a multifocal viral encephalitis that co-localized with foci of influenza virus nucleoprotein (NP) (Keawcharoen et al., 2008). This reflects the different roles played by the wild mallard, a potential long distance vector, and the domestic duck, like the one used in our study, which may play more important roles in the local spread of HPAI H5N1 outbreaks. The systemic spread of A/Chicken/Faquos/amn12/2011(H5N1) most probably occurred following a well-known H5N1 infection scenario in which IL-6, which is released from infected macrophages and epithelial cells, triggers the production of acute phase proteins, which further increase vascular permeability (Swayne et al., 2007). There is evidence that the track taken by the virus to reach various duck organs might account for the organ-specific virus replication kinetics. For instance, after intranasal inoculation, as in our experiment, it is possible that the virus reaches the brain mainly through olfactory and trigeminal nerves (Bodewes et al., 2011). In contrast, the virus might reach other internal organs via the blood stream (Yen et al., 2009). Considering that in natural infection avian hosts are exposed to HPAI H5N1 through more than one portal of entry (e.g., nasal and oral), and that in natural infection host immunity may be compromised by a variety of factors including poor hygiene and co-infections, one may postulate that in natural infection viremia may be an important factor contributing to systemic spread, including to the CNS. In contrast, when only intranasal infection is used in experimental infection, as was done in our study, viremic spread to the CNS may play a lesser role.

Kinetics of viral transcription and systemic spread
The LOESS analysis demonstrated that viral exponential transcription rates varied markedly among organs and that HA mRNA expression declined notably after 48 hpi (Fig. 4 and 5). This inter-organ variability in virus growth rates might be attributed to differences in the availability and spatial distribution of the corresponding receptors (Kuchipudi et al., 2009), variation in the induction of immune-related components (Wei et al., 2013) and differences in apoptosis induction (Cornelissen et al., 2013). The decline in viral transcription in some organs suggests an induction of immune response and depletion in virus target cells (Pawelek et al., 2012). Similar
findings have been reported in lung and trachea of ducks experimentally infected with other strains of H5N1 viruses (Kishida et al., 2005; Tang et al., 2009). Interestingly, in certain organs such as lung, trachea and heart, a short second peak of HA transcription was observed at 120 hpi. While there is no direct explanation of this phenomenon in avian hosts, similar phenomena have been described for H4N8 and H4N6 viruses in mice (Bui et al., 2012) and after a single passage of H5N1 clade 2.2 in mice and in cell lines (Bogs et al., 2011; Mase et al., 2006). Bogs et al. reported that a reversion mutation in the PB2 protein (E627K) might lead to this late increase in virus transcription. Studying the replication kinetics of H3N8 in ponies revealed similar results (Pawelek et al., 2012). These authors explained this phenomenon by the gradual loss of IFN-induced antiviral effects 48 hpi, resulting in a reversion of the host cell from a refractory to a susceptible state. This explanation is supported by the fact that virus-resistant cells cannot maintain resistance without continuous IFN signaling (Pawelek et al., 2012). It remains to be determined why this virus rebound was observed only in some organs. However, it is tempting to speculate that under natural conditions, where the bird is dehydrated and its immunity is suppressed, exposure to several attacks of the same H5N1 virus might produce similar second virus peaks involving more organs.

The fluctuations in viral transcription that were seen in pancreas, kidney and spleen in our study (Fig. 4 and Fig. 5) might be a result of host-virus interaction and the host genetic background (Londt et al., 2008). The correlation matrix displayed in Fig. 6 illustrates that viral activity in kidney correlated highly with that in other organs, indicating that the kidney might be a favorable site for replication of this isolate.

**Limitations of the present study**

This study is limited by several factors. Firstly, to avoid post-mortem artefacts, all ducks found dead at the scheduled time point were excluded from RT-qPCR and histopathological analyses. Thus, we may have under-estimated the true extent of viral spread and of the histopathological changes. Secondly, HA mRNA expression could not be assessed in intestine due to low quality of the RNA isolated from this organ. Thirdly, due to resource limitations at the time of the infection experiment,
viral titers in the various organs by inoculation of chicken eggs could not be determined. This also prevented the determination of viremia.

Conclusions

The current study indicates that H5N1 viruses of the clade 2.2.1.2 were circulating in Egyptian domestic poultry in 2011, the year in which A/chicken/Faquos/amn12/2011 was first isolated. The data also provide evidence that this new isolate can spread systemically in experimentally infected ducks, causing severe clinical manifestations and pathological lesions, thus reflecting the important role of domestic ducks as a reservoir of these viruses at the local level. From an epidemiological point of view, one can hypothesize that the recently observed more virulent HPAI H5N1 isolates, including this new strain, may turn the domestic duck into a disseminator of local outbreaks. In contrast, wild ducks, which are still relatively H5N1-resistant, are more likely to play roles as long-distance vectors, even in transcontinental spread.

Because viral transcription varied depending on the organ and the sampling time, the findings of this study have implication for the surveillance and control programs of H5N1 viruses. It seems important to include samples from internal organs, or pooling samples from different tissues, in routine and targeted survey programs to optimize sensitivity of IAV detection (Keawcharoen et al., 2008). Continuous and close investigation of domestic duck populations in Egypt for the recent clades of the HPAI H5N1 viruses, in particular 2.2.1.2, is highly recommended as it might provide early warning of outbreaks with a high risk to domestic duck populations, other land-based poultry susceptible to HPAI H5N1 infection such as chicken and turkey (which often mingle freely with ducks) and, possibly, close human contacts. It remains to be determined whether members of this clade will also spread to other countries.
Acknowledgments

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Author contributions

M. Hamed and A. A. H. Ali designed the experiment; M. Samir, M. Hamed and A. Hamed conducted the experiments; M. Samir, M. Hamed, V. K. Nguyen and E. H. Hernandez-Vargas analysed the data. W. Baumgärtner and F. Seehusen provided the histological assessments. A. A. H. Ali and F. Pessler oversaw the project. M. Samir wrote the initial draft of the manuscript. All authors edited the manuscript. M. Samir and F. Pessler prepared the final version of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest relating to conduct of the study and publication of this manuscript.
References


Tables

Table S1. Viral clades used to generate the phylogenetic analysis

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<th>Virus Strains</th>
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¹ HA mRNA accession numbers (according to PubMed) are shown in the right hand column. The strain that is the subject of this study (A/Chicken/Faquos/amn12/2011) is printed in red.

² Accession numbers are according to PubMed.
Table S2. Comparison of selected amino acid residues in the HA protein in the viruses belonging to clade 2.2.1.2 and clades 2.2, 2.2.1, 2.2.1.1 and 2.2.1.1.

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¹The right hand column lists the amino acids conserved among the analyzed clade 2.2.1.2 strains, including A/chicken/Faquos/amn12/2011. Abbreviations: Δ denotes a deletion mutation. The capital letters refer to the one-letter amino acid abbreviations.
Figure 1. Phylogenetic relation of A/chicken/Faquos/amn12/2011 (red rectangle) to the circulating Egyptian H5N1 clades, based on a partial nucleic acid sequence (1-384) of the HA segment. This previously uncharacterized isolate clusters with clade 2.2.1.2. The analysis was done using the BioEdit and MEGA 5 software tools.
**Figure 2. Alignment of amino acid residues (110-165) of the HA protein of Egyptian H5N1 clades.** The name and the amino acid sequences of A/Chicken/Faquos/amn12/2011 are printed in red and show an amino acid signature identical to that of the viruses belonging to clade 2.2.1.2. Protein IDs and amino acid sequences are according to PubMed. A dash (-) in the alignment denotes a deletion mutation. The alignment was done with the MEGA 5 software.
Figure 3. Summary of clinical signs and gross pathology lesions noted on infected ducks throughout the experiment. Death of the first duck was observed at 48 hpi. Clinical signs were recorded for 3 ducks per time point.
Figure 4. Relative quantification of HA mRNA of A/chicken/Faquos/amn12/2011(H5N1) in various duck organs using RT-qPCR. To compare replicate means, one way ANOVA followed by Tukey’s multiple comparison test was conducted. The data are presented as mean FC (n = 3 animals/time point) on the log10 scale +/- SEM. P values ≤ 0.05 were considered significant. The asterisks indicate significant differences between viral HA mRNA between each time point at the non-infected state. The analysis was done with GraphPad Prism software, V.5. * (P ≤ 0.05), ** (P ≤ 0.01), *** (P ≤ 0.001).
Figure 5. Viral HA mRNA exponential transcription rates of A/chicken/Faquos/amn12/2011 (H5N1) in various organs, as modelled with LOESS as described in Methods. The black solid lines indicate the LOESS fitting of the kinetics of viral HA mRNA transcription. Grey bands, standard error. Red dashed lines fitted exponential transcription rate (g) in the first 48 hpi. The data are based on FC in infected animals (n = 3 animals/time point) with respect to non-infected (t = 0 h) animals.
Figure 6. Matrix showing the degree of correlation in A/chicken/Faquos/amn12/2011(H5N1) HA mRNA transcription among selected organs across the time course. Each black dot corresponds to the HA mRNA level (log_{10}) in one duck in any possible pair of organs. The corresponding Pearson correlation coefficients (shown above) were computed with the R program V. 3.0.2.
3.5. Manuscript V
(in preparation)

Title:
Highly pathogenic influenza A virus (H5N1) induces tissue-specific differential expression of small non-coding RNAs in experimentally infected duck

Authors
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Abstract

Ducks infected with the highly pathogenic avian influenza (HPAI) viruses of H5N1 subtypes often exhibit mild or no clinical disease. However, recent HPAI H5N1 isolates have shown to be lethal in this species. Our previous characterization of (A/chicken/Faquos/amn12/2011 (H5N1)) in a domestic duck breed (derived from Anas platyrhynchos) revealed that the degrees of viral transcription and virus-induced tissue lesions were much lower in brain than in other organs, notably the lung. As small non-coding RNAs (sncRNAs) including miRNAs have been implicated in the host response to H5N1 virus infection, we sought to compare their expression patterns and predicted functionality between H5N1-infected duck lung and brain using small RNA sequencing (RNA-seq) in a time series spanning 120 hpi. Compared to the control animals, more differentially expressed (DE) miRNAs were identified in lung (n= 157) than in brain (n= 23). Interestingly, small nucleolar RNAs (sncoRNAs) represented the most strong DE non-miRNA sncRNAs class. Overall, miRNA reprogramming evolved earlier and to a larger extent in lung than in brain. In both organs, the highest magnitude of miRNA reprogramming took place during the late phase of infection (72-120 hpi) after the peak of viral transcription. Among the 13 miRNAs that were common DE in both organs, miR-205 was the most highly up-regulated one. Certain miRNAs were DE exclusively in lung (e.g. miR-34c, miR-10, miR-499 and miR-146) or brain (miR-138, miR-183 and miR-1416), suggesting roles for these miRNAs in tissue tropism of H5N1 virus or organ-specific defense mechanisms. The marked miRNA reprogramming in the lung was associated with the presence of more enrichment of mRNA targets and influenza A virus (IAV)-associated KEGG pathways than in brain. This study is the first to underscore the importance of miRNAs as regulatory host factors that impact the tissue-specific predilection of HPAI H5N1 viruses in duck.

Key words: brain, duck, H5N1 virus, influenza virus infection, lung, miRNA, non-coding RNA, small RNA sequencing.
Introduction

In addition to protein-coding genes, multiple small non-coding RNAs (sncRNAs) are transcribed from the genome and may perform regulatory functions. MicroRNAs (miRNAs) are short non-coding RNAs ≈ 22 nucleotides (nts) in length. Although they were firstly discovered in the nematode Caenorhabditis elegans\textsuperscript{1,2}, it has been recognized that their regulatory activities and functions affect nearly all known biological processes\textsuperscript{3,4}. The intricate involvement of miRNAs in gene networks comes from the fact that one miRNA can regulate hundreds of mRNA genes and that several miRNAs can target the same mRNA\textsuperscript{5}. With these features, several studies have reported roles for miRNAs in host-pathogen cross-talk\textsuperscript{6}, in particular during viral infection\textsuperscript{7}. The highly pathogenic avian influenza (HPAI) viruses of the subtype H5N1 belong to influenza A viruses (IAVs), which are classified under the family orthomyxoviridae\textsuperscript{8}. Since their emergence, the viruses continue to circulate globally leading to devastating economic losses in the poultry trade and posing risks of human infection\textsuperscript{9,10}. While wild aquatic birds are considered to be natural reservoirs of HPAI H5N1 viruses and contributing factors in their transcontinental spread\textsuperscript{11}, domestic poultry, such as chicken and ducks, can also contract the infection, leading to outbreaks and spread at the local level\textsuperscript{12,13}. However, HPAI H5N1 isolates have different ability to induce clinical disease in poultry species. Upon infection with HPAI H5N1, chicken and turkey develop severe disease with high mortality, whereas the ducks are usually considered to be resistant hosts despite shedding high quantities of the virus in their excretions\textsuperscript{14}. Recently, more aggressive HPAI H5N1 strains have been emerging in Egypt\textsuperscript{15} and other countries\textsuperscript{16,17} that display unusual high virulence in wild and domestic ducks, as evidenced by shorter time-to-death, high overall mortality, and major histopathological alterations in internal organs consistent with multi-organ failure.

We have recently performed a detailed phylogenetic, histopathological and transcriptional analysis of such a strain, named (A/chicken/Faquos/amn12/2011(H5N1)) upon experimental infection of a domestic duck bread (Anas platyrhynchos). We found that this isolate belongs to the clade
2.2.1.2, which emerged recently in Egypt and might include other isolates that are highly pathogenic to ducks. In that study, the brain appeared to represent a unique ecological niche for the virus in that, even though the animals developed substantial neurological deficits, overall viral transcription and virus-induced histopathological lesions were much lower than in the other organs, notably the lung (Samir et al. manuscript submitted). This observation suggested that, among organs, there may be not only differences in the kinetics of viral entry and spread, but also in the host responses. As miRNAs have been implicated strongly in the host response of various species, including humans, to IAV infection\textsuperscript{18-21}, and as our own results with inbred mouse strains of differential susceptibility to IAV (Preusse et al. manuscript in preparation) showed major and consistent differences in miRNomes, we reasoned that miRNA and other sncRNAs might serve as restriction factors that limit viral spread and tissue destruction to a much stronger extent in brain than in the more severely affected organs such as the lung.

In a first step, the objective of the present study therefore was to identify miRNA and other sncRNA molecules that are differentially expressed (DE) between brain and lung during infection with this newly characterized HPAI (H5N1) strain and to predict their functions and regulatory network in these organs. To attain this goal, we used small RNA sequencing (RNA-seq) to profile miRNA expression in RNA isolated from lung and brain of infected and control ducks. The OASIS tool, an online tool for the analysis of small RNA-seq data\textsuperscript{22}, was then employed to predict the known and novel small RNA types and to retrieve their DE patterns. Clustering and functional predictions of the DE miRNAs were done using a combination of bioinformatics tools and statistics.

The results showed that in both organs, miRNA reprogramming occurred after the peak of the viral transcription. In lung, this evolved earlier and to a higher magnitude than in brain. This was concurrent with a greater enrichment of predicted mRNA targets and associated pathways in lung than in brain. Upon caspase 3 (Casp3) staining, we observed a markedly higher degree of apoptosis in lung than in brain, which was consistent with the results of the pathway analysis. Furthermore, we identified organ-specific miRNAs that have unique clustering patterns in each organ.
Materials and Methods

Virus propagation and titration
The A/chicken/Faquos/amn12/2011 (H5N1) isolate (PubMed accession number: JQ627585.1) was kindly provided by Vet. Abdo Nagy (Virology Department, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt) and was used in this experiment. This isolate stemmed from a fulminant outbreak in household broiler chicken flocks in El-Sharkia province, Egypt. Viral propagation and titration were done as previously described. Briefly, 200 µl of the virus was inoculated into 11 day-old specific pathogen-free (SPF) embryonated chicken eggs (SPF-ECEs), and equal numbers of SPF-ECEs were used as control groups. All SPF-ECEs were incubated and monitored daily for viability. The propagated virus was identified in the harvested allantoic fluid by hemagglutination inhibition test (HAI) as described previously. Virus titers were calculated according to the Reed and Muench method.

Experimental infection
Animal Infection was done as described previously (Samir et al. manuscript submitted). Briefly, a commercial two week-old native duck breed derived from Anas platyrhynchos was used in the experiment. Prior to virus inoculation, HAI and enzyme-linked immunosorbent assay (ELISA) were conducted to ensure the absence of previous H5N1 infection. Ducks were separated into control (n = 10) and infected groups (n = 50). The control and the infected ducks were inoculated intranasally with 50-µl of PBS and 50-µl of 10^6.7 embryonated egg infective dose 50 (EID50), respectively. At least three ducks from the virus-inoculated group were euthanized at 8, 16, 24, 32, 40, 48, 72, 96 and 120 hours post infection (hpi). Three ducks from the control group were euthanized for comparison. Tissue from lung, trachea, brain, heart, pancreas, spleen and kidney was collected from infected and control ducks, stored at -80ºC to be used for RNA extraction, and another piece of the same tissue was processed for histological analyses. The remaining ducks were observed daily for clinical signs. Those which showed sings of severe illness (e.g. tremors, head shaking and imbalance) were euthanized and subjected to...
postmortem examination. All experimental work was performed in compliance with the local rules on animal welfare and was approved ahead by the Ethics Committee for Animal Studies at the Faculty of Veterinary Medicine, Zagazig University, Egypt.

**RNA extraction and reverse transcription (RT)**

From each organ, 30 gm tissue was homogenized in a mixture of 600-µl RLT buffer and 6-µl β-mercaptoethanol (β-ME) using beads in a rotor-based homogenizer. Total RNA was extracted with the miRNeasy kit (Qiagen), and was then DNase treated using the RNase-Free DNase Set (Qiagen). RNA quality and quantity were determined with the Nanodrop S1000 spectrophotometer (Thermo Scientific). The DNase-treated RNA was reverse transcribed using the miScript Reverse Transcription Kit (Qiagen) in a volume of 20-µl.

**Relative quantification of H5N1 HA mRNA by reverse transcriptase quantitative real-time PCR (RT-q-PCR)**

Reverse transcriptase real-time PCR (RT-qPCR) with the miScript SYBR Green PCR Kit (Qiagen) was used for relative quantification of HA MRNA. Duck β-actin mRNA was used as internal reference. The following primers were used (H5N1 HA: forward CAGCATGTCCATACCAGGGAA, reverse CCACCTTTGCCCCTTACCTT; duck β-actin: forward CCATTGAACACGGTATTGTCACC, reverse GCTACATACATGGCTGGGGT). Primer specificity was assessed by determining the primer amplification efficiency and correlation coefficient using two-fold diluted cDNA samples from kidney, lung, brain and heart, and by inspecting the melting curves. A light cycler 480 real-time PCR instrument (Roche, software version 1.5) was used. RT-qPCR started with pre-incubation at 95°C for 15 min, which was followed by 40 amplification cycles at 94°C for 15 seconds (s), 55°C for 30 s and 72°C for 30 s. Melting curves were generated by heating the samples to 95°C. Fold change (FC) in expression was calculated using the $2^{-\Delta\Delta CT}$ method.²⁵
Evaluating the degree of apoptosis in duck lung and brain

Serial 4 µm-thick sections were stained with rabbit polyclonal antibody directed against cleaved Casp-3 (dilution 1:300, Cell Signal Technology, ASP 175, Chicago, IL, US). Biotin-labeled goat anti-rabbit IgG (Thermo scientific, TP-060-BN) was used as secondary antibody. Positive caspase 3-stained tissue and negative control tissue stained only with the secondery antibody were included in each IHC run. For semi-quantitative scoring, lung and brain tissues from at least 3 control and infected animals were screened for Casp3 positive cells at each time point. For scoring, the slides were fully examined under magnification x40 based on 2 criteria: intensity of staining (0, no staining; 1, low intensity; 2, intermediate intensity; 3, high intensity) and the percentage (%) of the involved area out of the total slide (1 = 1-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100%).

Library preparation and small RNA sequencing

Small RNA-seq was carried out as described previously. Sequencing reactions were performed using the TruSeqTM Small RNA-seq Kit, Version January 2011 (Illumina, Inc., San Diego, CA, USA). Approximately 1250 ng of input RNA was used and the synthesized cDNA was amplified through 15 PCR cycles. Complementary DNA (cDNA) library fragments with a length of about 150 nucleotides (nts) were separated by gel electrophoresis and then isolated from the corresponding excised gel slice. Tissues from 3 animals per condition were analyzed. Use of Illumina molecular barcode allowed sequencing of eight samples per lane. To reduce batch effects, samples from the same time point were never run on the same lane. Sequencing was done using the HiSeq 2500 (Illumina Inc.) sequencer. The TruSeqTM Small RNA-seq Kit is specific for miRNAs and other small RNAs (sRNAs) that have a 3’OH group resulting from enzymatic cleavage by RNA processing enzymes.

Small RNA identification, prediction of the duck miRNome, and differential expression (DE) analysis

Small RNA prediction and identification were conducted using the OASIS tool. First, the sRNAs detection module was used to align the sequencing reads to the
duck genome in order to annotate known and predicted novel sncRNAs including miRNAs. The resultant output files were used to check the quality of each sample and to determine the sncRNA read counts for each sample. Next, the FASTAQ files were used as inputs in the DE module of OASIS. This allows calculating the DE of each sncRNAs\textsuperscript{22}. All primer and adaptor sequences were removed with the cutadapt software (version 1.8.1)\textsuperscript{27}. Quality control was done using the fastqc software (version 0.11.2) In order to check variability among replicates, principle component analysis (PCA) plots were produced and the outliers were excluded before conducting the DE analysis. Since the duck genome is not annotated for miRNAs and the duck miRNome is not included in the most recent version of miRBase (version 21)\textsuperscript{28}, predicted duck miRNAs were annotated using the Mirdeep2 software (version .2)\textsuperscript{29} and by similarity with miRNAs annotated for other species in miRBase version 21\textsuperscript{28}. Other small RNAs including Piwi-interacting RNA (piRNAs), small nucleolar RNA (snoRNAs), small nuclear RNA (snRNAs) and ribosomal RNA (rRNAs), were retrieved from the Ensembl database 2014\textsuperscript{30}. All libraries were mapped against the duck genome version ap1 using Spliced Transcripts Alignment to a Reference (STAR) software. The applied parameters were: out filter Mismatch Nover Lmax 0.05 (0 mismatches for reads with length 15-19, 1 mismatch for reads with length 20-32), out filter Match N min 15, out Filter Score Min Over Lread 0, out filter Match N min Over Lread 0, align Intron Max 1. After mapping, in order to count the number of reads on each predicted and annotated miRNA, we used the Feature-Counts software (version 1.5.0)\textsuperscript{31} with the default parameters. DE small RNAs were evaluated with the DeSeq2 R package (version 1.10.1)\textsuperscript{32} and the final DE analysis was conducted using OASIS\textsuperscript{22}. MiRNAs with adjusted p-values below 0.05 and a FC of ≥1 and ≤1 were considered up- or down regulated, respectively.

**Cluster analysis of DE miRNAs in lung and brain**

Co-regulation of subgroups of DE miRNAs was assessed with PCA and hierarchical cluster analysis. The PCA and the hierarchial clustering was performed using the Matlab statistics tool box. In the PCA, the log\textsubscript{2} FC of each
miRNA was used as input. Hierarchical cluster analysis was done based on the Euclidean distances among all predicted miRNAs based on their log2 FC values.

**Prediction of mRNA targets of the DE miRNAs**

In order to identify the mRNA targets of the DE miRNAs, the sequences of the 3’ untranslated regions (UTRs) of the annotated duck mRNAs were retrieved from the duck ensemble database using biomaRt queries (version 2.27.2) (http://www.biomart.org/). For calibration purpose, the frequencies of dinucleotides were calculated using all 3’ UTR sequences. Using the frequencies of dinucleotides, RNA calibrate (parameters "-d 3 UTR_duck.freq -k 5000 -l 500,300") was executed to estimate the position and shape parameters of the 3’ UTRs. The resulting values were 2.20 for position and 0.17 for shape and were used as input for the RNA hybrid target predictor software (-d 2.20, 0.17). All targets with P values below 0.001 were selected.

**Functional analysis of DE miRNA regulatory circuits**

To explore the function of predicted targets of the DE miRNAs, Gene Ontology (GO) enrichment and Kyoto Encyklopedia of Genes and Genomes (KEGG) analysis were performed. In these analyses, the list of predicted gene target of DE miRNAs were used as inputs for ClueGo V2.1.7, a Cytoscape (V 3.2.0) plug-in, using their default parameter and duck (Anas platyrhynchos) as a background species. The enriched GO and KEGG terms were selected with a cutoff level of P ≤0.05. IAV-related GO terms and the KEGG-enriched pathways were ranked according to the number of mRNA genes and their percentage (calculated by the equation: number of identified gene/total number of mRNA genes present in the respective GO term or KEGG pathway).

**Results**

**H5N1 virus replicate differentially in lung and brain**

As shown in Fig. 1, the relative quantification of HA mRNA from two independent experiments revealed that viral transcription was higher in lung than in brain,
particularly between 32-48 hpi. The decline in viral transcription started earlier in brain (at 40 hpi) than in lung (48 hpi). After 96 hpi, we observed a short rebound in viral transcription in lung, but not in brain.

**Apoptosis was higher in H5N1-infected duck lung than in H5N1-infected brain**

To investigate whether apoptosis is induced differentially in lung and brain, expression of Casp3, an enzyme that is highly associated with apoptosis, was determined immunohistochemically (**Fig. 2A and B**). The semi-quantitative scoring revealed that in lung, Caspase 3 positive cells appeared as early as 8 hpi, peaked at 40 hpi and gradually declined thereafter. In contrast, in brain Casp3 positive cells were seen only in one duck, which had few casp3-positive cells at 48 hpi (**Fig. 2C**).

**Duck lung contains a larger repertoire of sncRNAs than duck brain**

In order to determine the sncRNAs population in duck lung and brain, small RNA-seq was employed. The analysis of the data with the OASIS tool revealed that the number of identified expressed sncRNA classes varied among the sequenced libraries (**Fig. S1A**). We obtained higher numbers of sequenced reads in lung than in brain (**Table 1**). miRNAs (known and novel) represented the most abundant detected type, accounting for an average of 52.7% of all sncRNAs followed by snoRNA (43%), snRNA (3.1%) and rRNA (1.2%). In both organs, the percentages of expressed and DE known miRNAs that have previously been annotated for other species were higher than that of the novel putative ones. Interestingly, the brain had higher percentages of novel putative expressed and DE miRNAs than lung (**Table 2**). Of note, brain infected for 24 h contained the highest number of expressed miRNA (known and novel). The lowest coverage of miRNA expression was observed in H5N1-infected brain at 16 and 32 hpi (**Fig. S1A**). The overall number of expressed sncRNAs was higher in the non-infected lung than in the non-infected brain. However, the number of expressed miRNAs showed the reverse pattern. Using the miRDeep2 algorithm, we could identify higher number of expressed known miRNAs in lung than in brain; whereas the number of the expressed novel miRNAs was higher in brain than in lung (**Table 2**).
Differential expression of sncRNAs during H5N1 infection in duck lung and brain

In a next step, the log_{2} FC values of all detected sncRNAs between infected and non-infected organs were calculated. Interestingly, the majority of the DE sncRNAs corresponded to snoRNAs, followed by miRNAs, snRNAs and rRNAs (Fig. S1B). To determine the overall differences in DE of miRNAs and non-miRNAs sncRNAs between infected duck lung and brain, the total numbers of DE miRNAs and non-miRNA sncRNAs were compared between both organs across all time points. The data indicated that the number of all DE sncRNAs was higher in lung than in brain (Fig. 3A and 4A). In both organs, the majority of DE miRNAs was known for other species (Table 2). Of note, irrespective of organ, the DE patterns of miRNAs and the non-miRNAs sncRNAs were different in that there were more up-regulated than down-regulated miRNAs and the reverse pattern was identified for the non-miRNA sncRNAs (Fig. 3B and 4B). The distribution of non-miRNAs sncRNAs was consistent in lung and brain with the snoRNA accounting for the majority in both organs, although they did constitute a larger fraction in lung (88%) than in brain (61%) (Fig. 4C).

Trends in miRNA reprogramming in lung and brain at different infection phases

To obtain an overview about the differences between lung and brain in terms of global trend of miRNAs reprogramming, we compared the average number of DE miRNAs between lung and brain across all time points. As shown in the scatter plot (Fig. S2), more DE miRNAs were found in lung compared to brain. In both organs, up-regulated miRNAs were more than the down regulated ones. This was consistent with the number and pattern of DE miRNAs in lung and brain (Fig. 3A and B).

In order to investigate the global miRNA reprogramming at different infection phases, the whole infection course was divided based on the viral transcription data (Fig. 1) into early (8-24 hpi), middle (40-48 hpi) and late (72-120 hpi) infection phases. The time point 32 hpi was excluded because we could not find any significant differences in the level of viral transcription between this time point and
16 or 24 hpi. As shown in Fig. 5, H5N1 infection caused an overall higher degree of miRNAs reprogramming in lung than in brain. In both organs, the highest degree of miRNA reprogramming was observed at late infection phase (72-120 hpi). Furthermore, we found that miRNA reprogramming started and peaked earlier in lung (96 hpi) than in brain (120 hpi) (Fig. 5, 6A and 7A). In infected lung, there was a characteristic DE pattern in which, during the early infection phase, more miRNA were up-regulated. During the middle phase, these up-regulated miRNAs returned to normal, resulting in almost equal numbers of up- and down regulated miRNAs. During the late infection phase, the number of up-regulated miRNAs increased again (Fig. 5).

**Common and unique DE miRNAs in H5N1-infected lung and brain at different phases of infection**

In total, we could identify 80 (62 known and 18 novel) and 18 (12 known and 6 novel) DE miRNAs in lung and brain, respectively. The analysis also revealed that 67 and 5 miRNAs were exclusively DE in lung and brain, respectively (Fig. 3C, 6A and 7A). Additionally, 13 miRNAs were DE in both organs at least at one time point (Fig. 3C, 3D and S3A), indicating the presence of a common miRNA signature associated with H5N1 virus infection. Of note, most of these common miRNAs were DE at many time points in lung compared to one or two time points in brain (Fig. 3D and S3A). The data also showed inter-organ variations in the degree of DE of some miRNAs. For instance, the two members of miR-205 family were the highest up-regulated miRNAs in lung at 96 hpi, while they did not show any DE at the same time point in brain (Fig. 3D). On the contrary, miR-215 was strongly down regulated in the brain at 48 hpi, whereas no change in expression was detected for this miRNA at similar time point in lung. This clearly reflects the divergence in the DE patterns of miRNAs between infected lung and brain.

It is worth noting that the majority of the organ-specific DE miRNAs were identified in late infection phase (72-120 hpi), accounting for 83.5% and 60% of the total unique DE miRNAs in lung and brain, respectively. Lower percentages of the unique DE miRNAs in both organs were found in the other infection phases (Fig. S4A). In an attempt to explore the potentially most important miRNAs in each
organ, we examined whether some of the unique DE miRNAs were also DE at all infection phases. Interestingly, applying this analysis in lung identified 2 known DE miRNAs (miR-130a and miR-125b) and additional 3 putative DE miRNAs (Fig. S4A), suggesting a potential role for these miRNAs in the host response to H5N1 infection irrespective of the time. Similarly, one known (miR-183) and one putative miRNAs were identified in brain (Fig. S4B).

Patterns of miRNA clustering in duck lung and brain
To further understand the potential relationships between the DE miRNAs, we performed PCA and hierarchical clustering analysis based on the log2 FC values of the DE miRNAs. The PCA was restricted to the first two components, as the addition of a third component did not lead to significant additional classification. The analyses revealed that the DE miRNA in the two organs tend to form two main clusters irrespective of the time point (data not shown). This also holds true for the miRNA that are DE in both organs. There were more sub-clusters in lung than in brain. The clustering based on the time points revealed that the time point 120 hpi in duck brain forms a separate cluster with respect to other time points. On the other hand, the miRNAs that were DE in duck lung at 96 h clustered separately from the other time points. Similar clustering patterns with regard to time were observed for the miRNA DE in both lung and brain. Investigating the clustering patterns for each individual miRNA showed that one brain specific miRNA (miR-183) clustered apart from the remaining 4 brain specific miRNAs (Fig 7B). Interestingly and as shown in Fig S3B, clustering of the commonly DE miRNAs in both organs separately and in combination demonstrated that miR-205_KB742896, miR-205_KB742722, miR-194 and miR-215 formed separate clusters with regard to the other miRNAs. Moreover, one miRNA (miR-203), although commonly DE in both organs, formed a separate cluster only in brain.

Target prediction of the DE miRNAs in lung versus brain
The regulatory functions of miRNAs during infections mainly rely on targeting host and/pathogen-derived genes. Therefore, predicting the target genes for the DE miRNAs is central to determine their regulatory roles in HPAI H5N1 infection in
duck, and likely other hosts. As shown in Fig. 8, the majority of target mRNAs were shared between the two organs (n=1318). More predicted targets were found in lung (n=481) compared to brain (n=78). Interestingly, in both organs, the unique mRNA targets were found to be mostly enriched in late infection phase (Fig. S4B). We also identified 16 and 1 mRNA targets that were unique in lung and brain, respectively, and also targeted by DE miRNA at all infection phases (Fig. S4B and Table 3).

**Gene ontology and KEGG pathway enrichment analyses**

To analyze the predicted function of the targeted mRNA genes, GO terms within the immunological and biological processes for the targeted mRNAs were annotated as described in the Materials and Methods. Overall, 4 and 11 GO terms were identified in lung and brain, respectively (data not shown). Positive regulation of JUN kinase activity was annotated in both infected lung and brain, yet with higher percentages of associated genes in lung than in brain. In brain, regulation of neutrophil migration ranked the highest GO term with a percentage of 42.9%. Of note, other immune-related GO terms were also enriched in brain, but not in the lung. The obtained GO terms varied at different phases of infection in both organs (Table S3). Further characterization of the KEGG pathways showed that 79 and 70 enriched KEGG pathways were identified in lung and brain, respectively. Their numbers varied among different infection phases in lung and brain (Table S4). Sixty-one KEGG pathways were commonly enriched in both organs throughout the time course. In order to check if there are differences in the degree of enrichment of these common pathways between lung and brain, the percentages of the associated genes were compared between both organs for 14 KEGG pathways that are known to be regulated during IAV infection. As expected, the overall percentages of associated genes were somehow higher in lung (13.3%) than in brain (10.5%) (Fig. 9). Interestingly, certain common pathways such as apoptosis, cytokine-cytokine receptor interaction, phosphatidylinositol signaling, FoxO signaling and Wnt signaling were enriched earlier in lung than in brain (Table S4). We also identified 21 and 9 pathways that were exclusively enriched in lung
and brain, respectively. Importantly, IAV infection and NOD-like receptor signaling were enriched at an early infection phase in lung, but not in brain (Fig. S5).

Discussion

Our previous investigations suggested that in an experimentally infected domestic duck, the HPAI (H5N1) isolate A/chicken/Faquos/amn12/2011 exhibited organ-dependent variation with regard to transcription kinetics, growth rate and the degree of virus-induced tissue lesions (Samir et al. Manuscript submitted, manuscript IV). The main objective of the present study was to identify differences in miRNA responses between lung and brain to predict shared and distinct functional features of regulatory miRNA circuits predicted to be active in the two organs before and during infection.

Coupling H5N1 virus transcription kinetics and virus-induced apoptosis in duck lung and brain

Viral transcription, using HA mRNA as a proxy, and virus-induced apoptosis were higher in lung than in brain (Fig. 1 and 2). This suggests a relationship between viral infection and apoptosis as proposed previously. Interestingly, the absence of apoptosis in duck brain in our analyses (Fig. 2A, B and C) corroborates the results reported by Cornelissen et al in ducks experimentally infected with HPAI H7N1. Furthermore, the rapid and early induction of apoptosis in duck lung has been reported previously in duck embryos and primary lung cells infected with H3N2, H1N1 and H5N1 viruses. Based on these results, we could hypothesize that in H5N1-infected duck, apoptosis is induced in lung early on as part of the anti-viral host response. In contrast, in brain the absence of apoptosis might permit continuing viral replication at a relatively constant level, which would be expected to contribute to the observed neurological signs.

Expression of miRNAs and other sncRNAs in duck lung and brain

Our results clearly showed that the expressed miRNAs account for the majority of the sncRNAs in lung and brain (Fig S1A). This was comparable to the results
obtained by Preusse, who stated that 88% of the small RNA reads in H1N1 PR8-infected mouse lung corresponded to miRNAs\textsuperscript{41}. Li et al\textsuperscript{42} obtained average miRNA percentages of 42%-94% in SPF and H5N1-infected thymus, bursa and spleen of chicken and ducks. A percentage at the lower end of this spectrum (45%) was obtained in a study that involved 20 mouse lung samples infected with IAV\textsuperscript{43}. The percentages of identified miRNAs might differ among studies depending on the host, tissue and the sequencing method. It has been shown previously that the miRNA pools in humans and mice vary in a tissue dependent manner\textsuperscript{44,45}. Furthermore, Li et al showed that the miRNA repertoires in the immune organs of SPF chicken and ducks did differ despite being highly conserved\textsuperscript{42}. Therefore, it was expected that the number of expressed sncRNAs, notably miRNAs, would vary between duck lung and brain under non-infected condition, as shown in Fig. S1A. Even though both miRNAs and other sncRNAs were enriched in lung and brain, we observed differences in their expression (Fig S1A) and the degree and patterns of their DE (Fig S1B). These data highlight the need for detailed investigations of the relation between miRNA pool and population of other sncRNAs also in other organs of duck upon infection with H5N1 virus.

\textit{Potential importance of snoRNAs in HPAI H5N1 virus infection in duck}

The current study unraveled the existence of a large population of snoRNAs in the duck genome (Fig S1A). Indeed, the features of chicken and duck snoRNAs are poorly understood and underestimated because their discovery mostly involved similarity searches in mammalian counterparts, which are already far away on the evolutionary tree\textsuperscript{46}. SnoRNAs can modify chromatin structure, splicing and mediate oxidative stress\textsuperscript{47}. They can also modify other small RNAs, such as rRNA\textsuperscript{48}, snRNA\textsuperscript{49} and tRNA\textsuperscript{50}. Of special note, some snoRNA types could generate miRNA-like small RNAs of \textasciitilde 17-19 nt in length, which could further regulate gene and protein expression\textsuperscript{51,52}. It is worth noting that certain snoRNAs can regulate pre-mRNAs of serotonin receptor 2C\textsuperscript{53} and fibroblast growth factor receptor 3\textsuperscript{54}, which were known to have anti-viral activities\textsuperscript{55}. The observation that the NS1 segment of some IAV strains is also able to block the splicing complex prior to the formation of mature mRNA\textsuperscript{56} suggests possible immune-escape strategies of IAV.
and propose a yet undefined role of snoRNAs as regulatory factors during IAV-host interaction.

Differential reprogramming of miRNAs in duck lung and brain

The current study revealed that H5N1 infection triggered an overall higher degree of reprogramming of the sncRNAs, notably miRNA, in lung than in brain (Fig. 3A and 4A), indicating an organ-specific response. In fact, it is not uncommon that different organs have a divergent miRNA response to IAV. Previous studies suggested that H5N3-infected chicken lung and trachea exhibited differential miRNA responses. In these studies, miR-206 was more highly expressed in infected than in non-infected lungs, whereas the reverse was reported for trachea. More recently, Zhao et al. reported that upon H3N2 virus infection, 28 miRNAs were expressed at lower levels in canine trachea than in lung. Thus, miRNAs likely modulate the host response to IAV infection in a tissue-dependent manner and this might pertain to the preferential ability of the virus to invade and replicate in different tissues.

Data from previous studies demonstrated a time-dependent regulation of mRNAs during IAV infection. Similarly, we identified a time dependency in miRNA reprogramming (Fig. 5) in that the greatest changes occurred during the late phase of infection (72-120 hpi). Also, the majority of the unique DE miRNAs and their predicted mRNA targets in each organ were enriched to a greater extent in late infection (Fig. S4A and B). Similar findings were previously reported for murine pulmonary miRNAs after infection with H1N1 PR8 virus irrespective of the mice strain used. Collectively, this indicates that the bulk of miRNA reprogramming after IAV infection takes place mostly during late infection phase and thus may be more of a consequence than a cause of infection-associated changes in host cell metabolism. Comparing lung and brain, our data revealed earlier and quicker miRNA reprogramming and stronger evidence of apoptosis in the lung. Taken together, these data suggest that the tissue response to infection, including miRNome reprogramming, is more rapid and vigorous in lung. From the point of view of evolution of the interaction between IAV and their hosts, it is quite plausible that more vigorous responses in frequently affected organs such as the lung have provided
selective advantages. Nonetheless, further experimental evidence is necessary in order to distinguish between processes driven by viral replication vs. those driven by the potential intrinsic to the host and/or tissue involved.

**Potential biomarkers: common and unique miRNA signatures in H5N1-infected duck lung and brain**

The presence of organ-specific miRNA signatures is of great relevance to the diagnosis and prognosis of H5N1 virus infection, particularly in cases where viral infection of specific organ such as the brain may be closely related to clinical outcome. In the current study, It is interesting to note that miR-183, which was down regulated only in brain at most of the time points (Fig. 7A) was also found by others to be down regulated in the brain of mice upon rabies virus infection\(^{59}\), arguing that the down regulation of this miRNAs may be a general feature of neurotropic viruses. We also identified two lung-specific miRNAs, miR-125b and miR-130a (Fig. 6A). miR-125b, which was up-regulated in our study, was found by others to be down-regulated in response to H1N1 pandemic 2009 infection of piglet alveolar macrophages and in human patients\(^{18,60}\). This difference in DE might be attributed to the host and/or virus strain studied. miR-205, which was the most highly up-regulated miRNA in our study (Fig. S3A), was up-regulated in thymus, bursa and spleen of HPAI H5N1-infected chicken and ducks\(^{42}\). The unique miRNA signatures identified in the current study emphasize the potential utility of miRNAs as candidate biomarkers for duck lung and brain response to H5N1 infection. Clearly, these candidate markers merit further investigation to assess their clinical relevance, both in animal and human hosts.

**Clustering patterns of the DE miRNAs**

The cluster analysis assisted in predicting the invisible interrelations between variables, particularly in big datasets. Despite the inconsistencies that we observed in the patterns of DE miRNAs in lung and brain, certain miRNAs were prone to form clusters while others remained separated. In the current study, the high intensity of miRNA reprogramming in the lung was associated with high
degrees of sub-clustering when compared to brain (data not shown). Although this needs further empirical validation, it proposes that the miRNA response in lung might ramify into different regulatory circuits more readily than in brain, where miRNA activity may be more concerted. Individually, the brain-specific miR-183 clustered away from other brain DE miRNAs (Fig. 7B). This mirrors its particular importance in the brain response to H5N1 infection. Along similar lines, miR-205, miR-215 and miR-194, although they were DE in both organs, they formed a separate cluster in lung (Fig. S3B), suggesting a unique function mediated by these miRNAs in H5N1-infected lung. The clustering of miRNAs suggests that these miRNAs either co-regulate or coordinately regulate similar or multiple biological processes\textsuperscript{61}, imposing another layer of complexity to the predicted miRNA-mRNA networks. Indeed, approximately half of the annotated miRNAs are co-localized on the genome and co-expressed with their co-localized host genes\textsuperscript{62}. Therefore, it is reasonable to assume that the co-deregulation of these miRNAs is a result of coordinated expression of their host mRNA genes. Parallel profiling of the mRNAs that are genomically associated with each miRNA cluster would be needed to test this model.

*Putative regulatory functions of selected DE miRNAs*

In the current study, we identified a higher number of predicted target mRNAs in lung than in brain (Fig. 8), indicating a more complex interaction in lung upon H5N1 infection. The identification of organ-specific miRNA regulatory network has implications to the potential use of miRNAs as biomarkers, particularly when these miRNAs are released from their tissue of origin into the circulation\textsuperscript{63}. In the absence of experimental validation, the identified miRNA-mRNA interactions might help to predict whether miRNA induction is part of the tissue-intrinsic anti-viral response or a virus-derived effect. For example, in our study, miR-145 and miR-125b, which were exclusive DE in lung, were predicted to target the genes encoding zink finger proteins ZFYVE19 and ZBTB8OS, which are type-1 IFN mediators in seasonal influenza\textsuperscript{64} and sindbis virus\textsuperscript{65}. Since these Zink finger proteins were also exclusively targeted in lung, one could hypothesized that the induction of these miRNAs-mRNA interaction is lung-specific and might be driven by H5N1 virus to assist blocking host
immunity. On the other hand, miR-194, miR-20b, miR-106 and miR-146, which were also lung-specific, tend to target protein tyrosin phosphatase (PTPN2), a suppressor of interferon response\textsuperscript{66,67} suggesting that this may be part of the intrinsic host response geared toward initiating IFN-based anti-viral activities. Clearly, further experimental evidence is needed to address these hypotheses. Although there were predicted pathways that were enriched in both organs, the overall percentage of associated predicted mRNAs was somewhat higher in lung (13.3\%) than in brain (10.5\%) (Fig. 9), argues that more miRNAs, and hence mRNAs, are implicated in regulating the biological network in H5N1-infected lung compared to infected brain. Interestingly, most of the identified pathways such as Notch signaling, endocytosis and autophagy have previously been associated with IAV infection\textsuperscript{68-71}. Of note, apoptosis signaling was enriched earlier in lung than in brain and as expected, with a higher percentage of associated genes (Fig. 9). Coupling these findings with the semi-quantitative scoring of Casp 3 in both organs (Fig. 2) clearly suggests that miRNA modulation during H5N1 virus infection is associated with an early induction of apoptosis in lung, possibly as a defense mechanism to restrict viral spread\textsuperscript{72} whereas apoptosis in brain is low and occur late in infection, likely to support viral infection\textsuperscript{73}. The identification of IAV life cycle, Toll-like receptor signaling and NOD-like receptor pathways only in lung indicates that the organ-specific miRNA responses to H5N1 infection are more complex in lung than in brain.

It is worth mentioning that while miR-223 and miR-155 have been known to be implicated in different aspects of IAV pathogenesis such as virulence\textsuperscript{74}, inflammasome regulation\textsuperscript{75}, adaptive immune response\textsuperscript{76}, interferon signaling\textsuperscript{77}, and in controlling mouse susceptibility to H1N1 PR8 infection\textsuperscript{41}, in the current study, these miRNAs were either not DE at all (miR-223) or DE in a non-significant manner (miR-155). This clearly substantiates the above-mentioned insights that the miRNA response to IAV infection may differ substantially depending on the host species and viral strain used. Further in-depth investigation into roles of these miRNAs in avian-to-human transmission of H5N1 virus are warranted.
Limitations of the presented study
The low number of biological replicates per time point, which was necessary in order to find a compromise between sampling sufficient number of time points and achieving an acceptable statistical power per treatment, obviously limits our ability to generalize from the data obtained. Future work should be directed at using higher numbers of biological replicates, but focusing on selected time points, as suggested by the current data. In addition, including one or more time points beyond 120 hpi would be indicated, as both viral transcription and miRNome reprogramming were still ongoing at 120 hpi. This will help exploring the long-term ramifications of the infection of duck organs, particularly the brain.

Conclusions
The miRNAs that were identified in this study provided novel insights into host- and tissue-specific responses of miRNA populations in an avian host of HPAI H5N1 virus, the duck. Additionally, the data are providing new evidence that promises to unravel potential tissue-specific biological networks that contribute to the anti-viral defences and, ultimately, end-organ damage and clinical and epidemiological outcomes. The identified miRNAs clearly merit further investigation as to their potential as diagnostic and prognostic biomarkers in IAV infection in animals and, by extension, humans.

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Author contributions
Still in preparation
Conflict of interest

The authors declare that they do not have conflicts of interest.

References


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Tables

Table 1. Average numbers and percentages of reads generated by small RNA-seq of infected and non-infected lung and brain of dcuks. The numbers and percentages in the table depict the averages in all libraries.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Total number of sequencing reads</th>
<th>% of trimmed reads</th>
<th>Number (%) of unique mapped reads</th>
<th>Average length reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>30360321</td>
<td>99.32%</td>
<td>10871008 (36%)</td>
<td>21</td>
</tr>
<tr>
<td>Brain</td>
<td>28558210</td>
<td>98.93%</td>
<td>7338988(26%)</td>
<td>21</td>
</tr>
</tbody>
</table>

1 Reads that are mapped to one of the small RNA databases (miRNAs, snoRNA, snRNA, rRNAs), miscellaneous RNA or the respective genome (reads shorter than 20 nt with 0 mismatches, longer than 20 nt with 1 mismatch).

Table 2. Numbers of identified DE known and novel miRNAs in infected and non-infected lung and brain. The numbers depict a summation of the corresponding miRNAs type in all libraries.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Expressed miRNAs¹</th>
<th>DE miRNAs²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Known (predicted)</td>
<td>Known (predicted)</td>
</tr>
<tr>
<td></td>
<td>Novel (predicted)</td>
<td>Novel (predicted)</td>
</tr>
<tr>
<td>Lung</td>
<td>1854 (81.1%)</td>
<td>113 (71.9%)</td>
</tr>
<tr>
<td></td>
<td>430 (18.8%)</td>
<td>44 (28%)</td>
</tr>
<tr>
<td>Brain</td>
<td>1721 (73.4%)</td>
<td>14 (60.8%)</td>
</tr>
<tr>
<td></td>
<td>621 (26.5%)</td>
<td>9 (39.1%)</td>
</tr>
</tbody>
</table>

1 MiRNAs, which are present with at least five reads.
2 MiRNAs with log₂ FC values > 0 (up-regulated) or < 0 (down regulated) and adjusted P values < 0.05.
Table 3. Selected predicted mRNA targets of DE miRNAs in lung and brain.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Official gene name¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lung (n=16)</strong></td>
<td></td>
</tr>
<tr>
<td>AHI1</td>
<td>Abelson helper integration site 1</td>
</tr>
<tr>
<td>KDELC2</td>
<td>KDEL (Lys-Asp-Glu-Leu) containing 2</td>
</tr>
<tr>
<td>KDF1</td>
<td>Keratinocyte differentiation factor 1</td>
</tr>
<tr>
<td>MTCH1</td>
<td>Mitochondrial carrier 1</td>
</tr>
<tr>
<td>PGPEP1</td>
<td>Pyroglutamyl-peptidase I</td>
</tr>
<tr>
<td>SLC39A13</td>
<td>Solute carrier family 39 (zinc transporter), member 13</td>
</tr>
<tr>
<td>ST6GAL1</td>
<td>ST6 beta-galactosamido alpha-2,6-sialytranferase 1</td>
</tr>
<tr>
<td>ZFYVE19</td>
<td>Zinc finger, FYVE domain containing 19</td>
</tr>
<tr>
<td>CAPN6</td>
<td>Calpain-5; Uncharacterized protein</td>
</tr>
<tr>
<td>ELOVL7</td>
<td>ELOVL fatty acid elongase 7</td>
</tr>
<tr>
<td>PTPN2</td>
<td>Protein tyrosine phosphatase, non-receptor type 2</td>
</tr>
<tr>
<td>RPL30</td>
<td>Ribosomal protein L30</td>
</tr>
<tr>
<td>SLC38A6</td>
<td>Solute carrier family 38, member 6</td>
</tr>
<tr>
<td>SNAP47</td>
<td>Synaptosomally-assiated protein, 47kDa</td>
</tr>
<tr>
<td>TNR</td>
<td>Tenascin R</td>
</tr>
<tr>
<td>ZBTB8OS</td>
<td>Zinc finger and BTB domain containing 8 opposite strand</td>
</tr>
<tr>
<td><strong>Brain (n=1)</strong></td>
<td></td>
</tr>
<tr>
<td>TMEM254</td>
<td>Transmembrane protein 254</td>
</tr>
</tbody>
</table>

¹ mRNA targets presented in this table are unique for each organ and were found to be predicted by the DE miRNA in all phases of infection. Target prediction of DE miRNAs was done using biomaRt queries and RNAhybrid target predictor software. All targets with $P$ value ≤ 0.001 were selected.
Table S1. Average numbers and percentages of reads generated by small RNA-seq of infected and non-infected lung and brain of ducks. The numbers and percentages in the table depict the averages in all libraries.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% of trimmed reads</th>
<th>Initial Nr. of reads</th>
<th>Nr. of mapped reads(^1)</th>
<th>Nr. of unique mapped reads(^2)</th>
<th>% of unique mapped reads</th>
<th>Average reads length</th>
<th>Nr. of Excluded reads(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung (Ctr)</td>
<td>98.77</td>
<td>30718289</td>
<td>18882148</td>
<td>9966136</td>
<td>32.49</td>
<td>21.71</td>
<td>8916012</td>
</tr>
<tr>
<td>Lung (8)</td>
<td>99.27</td>
<td>29089637</td>
<td>20568956</td>
<td>12124095</td>
<td>41.45</td>
<td>21.53</td>
<td>8444861</td>
</tr>
<tr>
<td>Lung (16)</td>
<td>98.80</td>
<td>29466874</td>
<td>19610782</td>
<td>10012703</td>
<td>34.85</td>
<td>21.80</td>
<td>9598080</td>
</tr>
<tr>
<td>Lung (24)</td>
<td>99.13</td>
<td>24618442</td>
<td>17584105</td>
<td>9502130</td>
<td>38.57</td>
<td>21.41</td>
<td>8081974</td>
</tr>
<tr>
<td>Lung (32)</td>
<td>99.33</td>
<td>28604074</td>
<td>19046869</td>
<td>9987993</td>
<td>35.03</td>
<td>21.59</td>
<td>9058876</td>
</tr>
<tr>
<td>Lung (40)</td>
<td>99.47</td>
<td>29580228</td>
<td>21850978</td>
<td>11860414</td>
<td>40.05</td>
<td>21.55</td>
<td>9990564</td>
</tr>
<tr>
<td>Lung (48)</td>
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<td>33046665</td>
<td>22426845</td>
<td>13086647</td>
<td>40.90</td>
<td>21.00</td>
<td>9340199</td>
</tr>
<tr>
<td>Lung (72)</td>
<td>99.73</td>
<td>32687165</td>
<td>22075663</td>
<td>10632255</td>
<td>32.98</td>
<td>21.25</td>
<td>11443409</td>
</tr>
<tr>
<td>Lung (96)</td>
<td>99.70</td>
<td>31408310</td>
<td>23517300</td>
<td>11780284</td>
<td>34.14</td>
<td>21.45</td>
<td>11737016</td>
</tr>
<tr>
<td>Lung (120)</td>
<td>99.43</td>
<td>34383523</td>
<td>23517300</td>
<td>11780284</td>
<td>34.14</td>
<td>21.45</td>
<td>11737016</td>
</tr>
<tr>
<td>Brain (Ctr)</td>
<td>98.97</td>
<td>30435301</td>
<td>19721374</td>
<td>8932504</td>
<td>29.22</td>
<td>21.84</td>
<td>10788870</td>
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<tr>
<td>Brain (8)</td>
<td>96.97</td>
<td>25869198</td>
<td>15665265</td>
<td>6613118</td>
<td>25.60</td>
<td>21.34</td>
<td>9052147</td>
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<td>Brain (16)</td>
<td>99.30</td>
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<td>24.13</td>
<td>21.10</td>
<td>8576703</td>
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<td>27.17</td>
<td>21.22</td>
<td>12467764</td>
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<td>Brain (32)</td>
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<td>7482033</td>
<td>24.59</td>
<td>20.75</td>
<td>11013966</td>
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<td>Brain (40)</td>
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<td>28468823</td>
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<td>6550077</td>
<td>22.80</td>
<td>20.64</td>
<td>9740865</td>
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<td>Brain (48)</td>
<td>99.33</td>
<td>28493564</td>
<td>17395014</td>
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<td>21.09</td>
<td>10162176</td>
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<tr>
<td>Brain (72)</td>
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<td>16739112</td>
<td>7063884</td>
<td>24.80</td>
<td>21.14</td>
<td>9675228</td>
</tr>
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<td>Brain (96)</td>
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<td>7487437</td>
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<td>20.56</td>
<td>10475259</td>
</tr>
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<td>Brain (120)</td>
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<td>16789927</td>
<td>6585217</td>
<td>25.83</td>
<td>20.71</td>
<td>10204710</td>
</tr>
</tbody>
</table>

\(^1\)Numbers of reads that remained after applying length filtering.
\(^2\)Reads that are mapped to one of the small RNA databases (miRNAs, snRNA, snRNA, rRNAs), miscellaneous RNA or the respective genome (reads shorter than 20 nt with 0 mismatches, longer than 20 nt with 1 mismatch).
\(^3\)Reads that are too short (< 15 nt) or mapped to multiple sites.
Table S2. Number of identified DE known and novel putative miRNAs in lung and brain.\(^1\)

<table>
<thead>
<tr>
<th>Library</th>
<th>Known</th>
<th>Novel (predicted)</th>
<th>Total miRNAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung- 8 hpi</td>
<td>17</td>
<td>5</td>
<td>22</td>
</tr>
<tr>
<td>Lung- 16 hpi</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Lung- 24 hpi</td>
<td>10</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td>Lung- 32 hpi</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Lung- 40 hpi</td>
<td>7</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>Lung- 48 hpi</td>
<td>6</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Lung- 72 hpi</td>
<td>25</td>
<td>10</td>
<td>35</td>
</tr>
<tr>
<td>Lung- 96 hpi</td>
<td>30</td>
<td>12</td>
<td>42</td>
</tr>
<tr>
<td>Lung- 120 hpi</td>
<td>15</td>
<td>5</td>
<td>20</td>
</tr>
</tbody>
</table>

**Total (lung)**   | 113   | 44                | 157          |

<table>
<thead>
<tr>
<th>Library</th>
<th>Known</th>
<th>Novel (predicted)</th>
<th>Total miRNAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain- 8 hpi</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Brain- 16 hpi</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Brain- 24 hpi</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Brain- 32 hpi</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Brain- 40 hpi</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Brain- 48 hpi</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Brain- 72 hpi</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Brain- 96 hpi</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Brain- 120 hpi</td>
<td>8</td>
<td>6</td>
<td>14</td>
</tr>
</tbody>
</table>

**Total (brain)** | 14    | 9                 | 23           |

\(^1\) MiRNAs with log\(_2\) FC values > 0 (up-regulated) or < 0 (down regulated) and adjusted \(P\) values < 0.05.

Table S3. Significant enriched GO terms in lung and brain. The analysis was done using the GO terms function in ClueGo V2.1.7, a Cytoscape software (V 3.2.1) plug-in.

<table>
<thead>
<tr>
<th>GO IDs</th>
<th>GO terms (Immunological and biological processes)</th>
<th>Nr. Genes(^1)</th>
<th>% Associated gene(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0008054</td>
<td>Negative regulation of cyclin-dependent protein serine/threonine kinase by cyclin degradation</td>
<td>3</td>
<td>75</td>
</tr>
<tr>
<td>GO:0060261</td>
<td>Positive regulation of transcription initiation from RNA polymerase II promoter</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>GO:1902622</td>
<td>Regulation of neutrophil migration</td>
<td>6</td>
<td>42.9</td>
</tr>
<tr>
<td>GO:0039529</td>
<td>RIG-I signaling pathway</td>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td>GO:0060338</td>
<td>Regulation of type I interferon-mediated signaling pathway</td>
<td>3</td>
<td>33.3</td>
</tr>
<tr>
<td>GO:1901522</td>
<td>Positive regulation of transcription from RNA polymerase II promoter involved in cellular response to chemical stimulus</td>
<td>5</td>
<td>31.25</td>
</tr>
<tr>
<td>GO:0051571</td>
<td>Positive regulation of histone H3-K4 methylation</td>
<td>4</td>
<td>30.77</td>
</tr>
<tr>
<td>GO:0043011</td>
<td>Myeloid dendritic cell differentiation</td>
<td>3</td>
<td>30.0</td>
</tr>
<tr>
<td>GO:0001779</td>
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</tr>
<tr>
<td>GO:0030219</td>
<td>Megakaryocyte differentiation</td>
<td>6</td>
<td>23.1</td>
</tr>
</tbody>
</table>

**Lung (middle phase, 40-48 hpi)**

| GO:0000185 | Activation of MAPKKK activity | 3 | 75 |
| GO:0070234 | Positive regulation of T cell apoptotic process | 3 | 37.5 |
| GO:0006367 | Modulation by host of viral transcription | 3 | 17.6 |
| GO:0042093 | T-helper cell differentiation | 4 | 16.7 |
| GO:0001569 | Patterning of blood vessels | 5 | 16.1 |
| GO:0006919 | Activation of cysteine-type endopeptidase activity involved in apoptotic process | 7 | 15.9 |
| GO:0042100 | B cell proliferation | 6 | 12.8 |
| GO:0015908 | Fatty acid transport | 4 | 10.8 |
| GO:0045765 | Regulation of angiogenesis | 10 | 9.1 |

**Lung (late phase, 72-120 hpi)**

| GO:0051571 | Positive regulation of histone H3-K4 methylation | 3 | 23.1 |
| GO:0043280 | Positive regulation of MAP kinase activity | 19 | 18.4 |
| GO:0008054 | Negative regulation of cyclin-dependent protein serine/threonine kinase by cyclin degradation | 3 | 75 |
| GO:0039535 | Regulation of RIG-I signaling pathway | 3 | 42.9 |
| GO:1903670 | Regulation of sprouting angiogenesis | 4 | 36.4 |
| GO:0034243 | Regulation of transcription elongation from RNA polymerase II promoter | 6 | 31.6 |
| GO:0060218 | Hematopoietic stem cell differentiation | 3 | 27.3 |
| GO:0034121 | Regulation of toll-like receptor signaling pathway | 5 | 23.8 |
| GO:0001784 | Peptidyl-diphthamide biosynthetic process from peptidyl-histidine | 1 | 33 |
| GO:0190089 | Response to nerve growth factor | 1 | 17 |
| GO:0042168 | Heme metabolic process | 1 | 7 |
| GO:0030308 | Negative regulation of cell growth | 4 | 5 |
| GO:0050879 | Multicellular organismal movement | 1 | 5 |
| GO:1901532 | Regulation of hematopoietic progenitor cell differentiation | 1 | 4 |

**Brain (middle phase, 40-48 hpi)**

| GO:0051284 | Positive regulation of sequestering of calcium ion | 1 | 100 |
| GO:0071109 | Superior temporal gyrus development | 1 | 100 |
| GO:1902262 | Apoptotic process involved in patterning of blood vessels | 4 | 80 |
| GO:0001784 | Peptidyl-diphthamide biosynthetic process from peptidyl-histidine | 1 | 33 |
| GO:0042168 | Heme metabolic process | 1 | 7 |
| GO:0030308 | Negative regulation of cell growth | 4 | 5 |
| GO:0050879 | Multicellular organismal movement | 1 | 5 |

**Brain (late phase, 72-120 hpi)**

| GO:0000185 | Activation of MAPKKK activity | 3 | 75 |
| GO:1902622 | Regulation of neutrophil migration | 6 | 43 |
| GO:0039528 | Cytoplasmic pattern recognition receptor signaling pathway in response to virus | 4 | 36.4 |
| GO:0090023 | Positive regulation of neutrophil chemotaxis | 4 | 36.4 |
| GO:1903580 | Positive regulation of ATP metabolic process | 3 | 33.3 |
| GO:0031054 | Pre-miRNA processing | 3 | 30 |
| GO:0032785 | Negative regulation of DNA-templated transcription, elongation | 3 | 30 |
| GO:0043923 | Positive regulation by host of viral transcription | 3 | 30 |
| GO:0006919 | Activation of cysteine-type endopeptidase activity involved in apoptotic process | 11 | 25 |
| GO:0002042 | Cell migration involved in sprouting angiogenesis | 4 | 23.5 |
| GO:000387 | Spliceosomal snRNP assembly | 4 | 21.1 |
| GO:0045581 | Negative regulation of T cell differentiation | 4 | 21.1 |
| GO:000187 | Activation of MAPK activity | 13 | 21.0 |
| GO:006096 | Glycolytic process | 7 | 19.4 |
| GO:0002293 | Alpha-beta T cell differentiation involved in immune response | 5 | 19.2 |
| GO:0034121 | Regulation of toll-like receptor signaling pathway | 4 | 19.0 |
| GO:0045824 | Negative regulation of innate immune response | 3 | 19 |
| GO:0016525 | Negative regulation of angiogenesis | 8 | 17.8 |
| GO:0042130 | Negative regulation of T cell proliferation | 5 | 17.2 |
| GO:0021795 | Cerebral cortex cell migration | 6 | 17.1 |
| GO:0000380 | Alternative mRNA splicing, via spliceosome | 4 | 16.7 |
| GO:0032088 | Negative regulation of NF-kappaB transcription factor activity | 6 | 15.4 |
| GO:0035065 | Regulation of histone acetylation | 4 | 14.8 |
| GO:0042102 | Positive regulation of T cell proliferation | 6 | 14.0 |
| GO:0002285 | Lymphocyte activation involved in immune response | 10 | 14 |
| GO:0002253 | Activation of immune response | 20 | 14 |
| GO:0032873 | Negative regulation of stress-activated MAPK cascade | 3 | 13.0 |
| GO:0045766 | Positive regulation of angiogenesis | 7 | 12.7 |
| GO:0061098 | Positive regulation of protein tyrosine kinase activity | 3 | 12.5 |
| GO:0030097 | Hemopoiesis | 56 | 12 |
| GO:0045637 | Regulation of myeloid cell differentiation | 13 | 11 |
| GO:007091 | Metaphase/anaphase transition of mitotic cell cycle | 3 | 10 |
| GO:002275 | Myeloid cell activation involved in immune response | 3 | 9 |
| GO:0070374 | Positive regulation of ERK1 and ERK2 cascade | 6 | 7.5 |
| GO:0051091 | Positive regulation of sequence-specific DNA binding transcription factor activity | 8 | 7.3 |
| GO:0043154 | Negative regulation of cysteine-type endopeptidase activity involved in apoptotic process | 3 | 7.3 |
| GO:00445638 | Negative regulation of myeloid cell differentiation | 3 | 6 |
| GO:0030817 | Regulation of cAMP biosynthetic process | 3 | 6.4 |

1 Number of the predicted genes found in each GO term.
2 Percentage of associated genes (number of predicted genes / overall number of genes present in the corresponding GO term).
Table. S4. Significant enriched KEGG pathways in lung and brain at different infection phases.

The analysis was done using the KEGG function function in ClueGo V2.1.7, a Cytoscape software (V 3.2.1) plug-in.

<table>
<thead>
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<th>KEGG pathway ID</th>
<th>KEGG pathways</th>
<th>Nr. Genes</th>
<th>% Associated Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>KEGG:00130</td>
<td>Ubiquinone and other terpenoid-quinone biosynthesis</td>
<td>3</td>
<td>33.3</td>
</tr>
<tr>
<td>KEGG:03020</td>
<td>RNA polymerase</td>
<td>8</td>
<td>33.3</td>
</tr>
<tr>
<td>KEGG:00220</td>
<td>Arginine biosynthesis</td>
<td>5</td>
<td>31.3</td>
</tr>
<tr>
<td>KEGG:00630</td>
<td>Glyoxylate and dicarboxylate metabolism</td>
<td>7</td>
<td>29.2</td>
</tr>
<tr>
<td>KEGG:04130</td>
<td>SNARE interactions in vesicular transport</td>
<td>6</td>
<td>23.1</td>
</tr>
<tr>
<td>KEGG:00270</td>
<td>Cysteine and methionine metabolism</td>
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<td>22.6</td>
</tr>
<tr>
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<td>Proteasome</td>
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<td>Basal transcription factors</td>
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<td>Fructose and mannose metabolism</td>
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<td>KEGG:00100</td>
<td>Steroid biosynthesis</td>
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<td>16.7</td>
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<tr>
<td>KEGG:00860</td>
<td>Porphyrin and chlorophyll metabolism</td>
<td>4</td>
<td>16.7</td>
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<td>KEGG:03440</td>
<td>Homologous recombination</td>
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<td>16.7</td>
</tr>
<tr>
<td>KEGG:04146</td>
<td>Peroxisome</td>
<td>12</td>
<td>16.7</td>
</tr>
<tr>
<td>KEGG:00512</td>
<td>Mucin type O-Glycan biosynthesis</td>
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<td>15.6</td>
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<tr>
<td>KEGG:04142</td>
<td>Lysosome</td>
<td>16</td>
<td>15.5</td>
</tr>
<tr>
<td>KEGG:04012</td>
<td>ErbB signaling pathway</td>
<td>11</td>
<td>15.3</td>
</tr>
<tr>
<td>KEGG:00410</td>
<td>beta-Alanine metabolism</td>
<td>4</td>
<td>13.8</td>
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<td>KEGG:03320</td>
<td>PPAR signaling pathway</td>
<td>8</td>
<td>13.8</td>
</tr>
<tr>
<td>KEGG:00040</td>
<td>Pentose and glucuronate interconversions</td>
<td>3</td>
<td>13.6</td>
</tr>
<tr>
<td>KEGG:02010</td>
<td>ABC transporters</td>
<td>5</td>
<td>13.5</td>
</tr>
<tr>
<td>KEGG:04110</td>
<td>Cell cycle</td>
<td>15</td>
<td>13.4</td>
</tr>
<tr>
<td>KEGG:00760</td>
<td>Nicotinate and nicotinamide metabolism</td>
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<td>13.3</td>
</tr>
<tr>
<td>KEGG:04514</td>
<td>Cell adhesion molecules (CAMs)</td>
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<td>13.3</td>
</tr>
<tr>
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<td>Spliceosome</td>
<td>13</td>
<td>13.1</td>
</tr>
<tr>
<td>KEGG:04141</td>
<td>Protein processing in endoplasmic reticulum</td>
<td>19</td>
<td>13.1</td>
</tr>
<tr>
<td>KEGG:00510</td>
<td>N-Glycan biosynthesis</td>
<td>6</td>
<td>13.0</td>
</tr>
<tr>
<td>KEGG:00260</td>
<td>Glycine, serine and threonine metabolism</td>
<td>4</td>
<td>12.9</td>
</tr>
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**Lung (late phase, 72-120 hpi)**

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KEGG:00280  Valine, leucine and isoleucine degradation  3  6.7
KEGG:04630  Jak-STAT signaling pathway  5  4.5
KEGG:04080  Neuroactive ligand-receptor interaction  11  4.1

1 Number of the predicted genes found in each KEGG pathway.
2 Percentage of associated genes (number of predicted genes / overall number of genes present in the corresponding KEGG pathway).

Figures

![HA mRNA](image)

**Figure 1. Relative quantification of HA mRNA in H5N1-infected duck lung and brain.** The FC in expression was calculated using the $2^{-\Delta\Delta CT}$ method considering duck β-actin mRNA as internal reference and plotted on the log$_{10}$ scale. The data are presented as mean±SEM of the FC of the HA mRNA from two independent experiment, each with 3 animals/time point. The statistical significance of the differences between both organs with regard to the FC values was calculated using unpaired, two-tailed t-test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. The analysis was performed using Graphpad prism V.5 software.
Figure 2. Apoptosis degree in duck lung versus brain. A. Infected duck lung at 24 hpi showing a caspase 3 positive stained cell (black arrow). B. Infected duck brain at 24 hpi showing absence of caspase 3 stained cells. D. Semi-quantitative scoring of apoptosis as determined by caspase 3 positive stained cells in lung and brain. Slides of caspase 3 stained tissues from 3 animals/time points were blindly scored for the intensity of positive stained cells and the percentage of the caspase 3 positively stained area. Data are plotted as a mean +/- SEM of values from 3 animals. Statistical significance for differences between means was calculated using an unpaired, two-tailed t-test. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001. The analysis was performed using Graphpad prism V.5 software.
Figure 3. Differential expression analyses of miRNAs in lung versus brain. 

A. Number of known and novel putative DE miRNAs in lung and brain at all time points. 

B. Patterns of miRNAs DE in lung and brain at all time points. 

C. Numbers of DE miRNAs that are common to both organs (n=13) and those that are unique to lung (n=67) and brain (n=5) at all time points. 

D. Heat maps showing the patterns and degree of DE of the common DE miRNA in lung and brain (n=13) throughout the time course. miRNAs that have log₂ FC values > 0 (up-regulated, red colored cells in the heat maps) or < 0 (down regulated, blue colored cell in the heat maps) and adjusted P values < 0.05 are considered DE. miRNAs starting with KB indicate the identified novel putative miRNAs.
Figure 4. Number, distribution and DE patterns of non-miRNAs sncRNAs in lung and brain at all time points. A. Number of DE non-miRNA sncRNAs in lung and brain. B. Patterns of DE non-miRNAs sncRNAs in lung and brain. (C) Percentages of each class of DE non-miRNAs sncRNAs in the two organs. The percentages represent the average number of the respective sncRNA class in all libraries in each organ. Non-miRNAs sncRNAs with log2 FC values > 0 (up-regulated) or < 0 (down regulated) and adjusted P values < 0.05 are considered DE.
Figure 5. Changes in the trend of reprogramming of known and novel DE miRNAs in different phases of H5N1 virus infection. Each dot represents one miRNA. The x-axis refers to the average number of each miRNA in the non-infected state. The y-axis refers to the average number of each miRNA in the infected state. The whole infection course was divided into early (8-24 hpi), middle (40-48 hpi) and late (72-120 hpi) phases. miRNAs with log₂ FC values > 0 (up-regulated, red dots) or < 0 (down regulated, blue dots) and adjusted P values < 0.05 are considered DE.
Figure 6. Patterns of DE unique miRNAs in lung (n=67) and their clustering patterns. A. Heat map showing the patterns of DE and log₂ FC values of the lung-specific DE miRNAs (n=67) throughout the time course. miRNAs with log₂ FC values > 0 (upregulated, red colored cells in the heat map) or < 0 (down regulated, blue colored cell in the heat map) and adjusted P values < 0.05 are considered DE. miRNAs that start with KB indicate the identified novel (putative) miRNAs. B. PCA of the unique DE miRNAs in lung (n=67) based on the log₂ FC value of each DE miRNAs. Each color in the color code refers to one miRNA cluster. The analysis was done using the Matlab statistics tool box.
(A)

Log2 FC

miRNAs ID
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miR-1416_KB742918
novelmirna_KB742876
KB742904_1_86929_86952
miR-183_KB742427

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Figure 7. Patterns of DE unique miRNAs in brain (n=5) and their clustering patterns. A. Heat map showing the patterns of DE and log$_2$ FC values of the brain unique miRNAs (n=5) throughout the time course. miRNAs that have log$_2$ FC values > 0 (up-regulated, red colored cells in the heat map) or < 0 (down regulated, blue colored cell in the heat map) and adjusted $P$ values < 0.05 are considered DE. miRNAs that start with KB indicate the identified novel (putative) miRNAs. B. PCA of the unique DE miRNAs in brain (n=5) based on the log$_2$ FC value of each DE miRNA. Each color in the color code refers to one miRNA cluster. The analysis was done using the Matlab statistics tool box.
Figure 8. Organ-specific target mRNA for the DE miRNA. BiomaRt queries were used to retrieve all the 3’ UTRs of duck mRNAs from the Ensemble database. RNAhybrid target predictor software was used to predict the mRNA targets. Target mRNAs with adjusted $P$ values < 0.001 were selected for this analysis. The majority of mRNA targets were shared between the two organs, whereas more organ-specific mRNA targets were predicted in lung than in brain.
Figure 9. KEGG pathway enrichment analysis. The figure show selected KEGG pathways that are commonly enriched in both organs and were found to be associated with IAV. The numbers inside the pie charts refer to the percentages of the associated genes (determined by dividing the number of the predicted target genes by the overall number of genes present in the corresponding pathway). For each organ, the numbers below the pie chart indicate the average number of the percentages of associated genes combining all the pathways. The analysis was done using the KEEG pathway function in ClueGo V 2.1.7, a Cytoscape (V 3.2.0) plug-in.
(A)

Lung (SPF)
- Lung-8 hpi
- Lung-16 hpi
- Lung-24 hpi
- Lung-32 hpi
- Lung-40 hpi
- Lung-48 hpi
- Lung-72 hpi
- Lung-96 hpi
- Lung-120 hpi

Brain (SPF)
- Brain-8 hpi
- Brain-16 hpi
- Brain-24 hpi
- Brain-32 hpi
- Brain-40 hpi
- Brain-48 hpi
- Brain-72 hpi
- Brain-96 hpi
- Brain-120 hpi

miRNAS (Known and novel) (52.7%)
snoRNAs (43%)
snRNA (3.1%)
rRNA (1.2%)
Figure S1. Distribution of expressed and DE small RNA types in all libraries. A. Bar plot indicating the numbers (N) and percentages (%) of expressed small RNAs (>100 counts per million, CPM). CPM was calculated by applying the equation: N of particular miRNA / the total N of reads per library X 10^6. The percentages of each small RNA species depict the average of all libraries. B. Bar plot indicating the number and percentage of DE small RNAs classes. The percentages of each small RNA type depict the average in all libraries. miRNAs that have Log_2 FC > 0 (up-regulated) or <0 (down regulated) and adjusted P values < 0.05 are considered DE.
Figure S2. Changes in the trend of reprogramming of DE miRNAs (known and novel) in lung and brain. Each dot represents one miRNAs. The x-axis refers to the average number of each miRNAs in the non-infected state. The y-axis refers to the average number of each miRNAs in the infection state. miRNAs that have log₂ FC values > 0 (up-regulated, red dots) or < 0 (down regulated, blue dots) and adjusted P values < 0.05 are considered DE.
(A)

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Hours post infection

Color key (Log2 FC)

-3 -2 -1 1 2 3
Figure S3. Patterns of common DE miRNAs in lung and brain (n=13) and their clustering patterns. A. Heat map show the patterns and log2 FC values of common DE miRNAs (n=13) throughout the time course of the experiment. miRNAs that have log₂ FC values > 0 (up-regulated, red colored cells in the heat map) or < 0 (down regulated, blue colored cell in the heat map) and adjusted P values < 0.05 are considered DE. miRNAs start with KB indicate the identified novel (predicted) miRNAs. B. PCA of the common DE miRNAs in lung and brain (n=13) based on the log₂ FC value of each DE miRNAs. Each color in the color code refers to each miRNA cluster. For the same miRNA, “b” at the end of the miRNA name indicate the clustering of the brain-specific miRNA.
Figure S4. DE miRNAs and their target mRNAs are enriched at late infection phase. Venn diagrams show the infection phase-dependent enrichment of the organ-specific DE miRNAs (A) and their predicted target mRNAs (B). H5N1 Infection were divided into early, 8-24 hpi; middle, 40-48 hpi and late, 72-120 hpi phases. The organ-specific DE miRNAs and predicted mRNA targets were more enriched at late phase of infection (their organ Venn diagrams were created using “Venny 1.0”, an online tool (http://bioinfogp.cnb.csic.es/tools/venny/).
Figure S5. KEGG pathway enrichment analysis. The figures show the organ-specific enriched KEGG pathways in lung (A) and brain (B). The numbers inside the pie charts refer to the percentages of the associated genes (determined by dividing the number of the predicted target genes by the overall number of genes present in the corresponding pathway). The analysis was done using the KEEG pathway function in ClueGo V 2.1.7, a Cytoscape (V 3.2.1) plug-in. “early” or “late” beside the name of certain pathways indicate the respective pathways were enriched at early infection phase (8-24 hpi) or late infection phase (72-120 hpi). The pathways with no defined infection phase are the ones that are enriched during all infection phases.
3.6. Own contributions to the manuscripts

- **Manuscript I**
  I conceived the idea of the review, conducted the literature search and the hand-curated data extraction, and wrote the initial draft of the manuscript. I also prepared the figures and tables and participated in editing the final version of the manuscript.

- **Manuscript II**
  I conceived the idea of the review. I performed the literature search and wrote the initial draft of the manuscript. Additionally, I prepared the figures and tables. I also participated in the edition of the final version of the manuscript.

- **Manuscript III**
  I took part in designing and conceiving the experiment. I performed the experiments and analyzed the data. I prepared the initial draft of the manuscript including the tables and the figures. I participated in editing the final version of this manuscript.

- **Manuscript IV**
  I participated in performing the experiment. I analyzed the data related to the phylogenetic analysis, protein alignment as well as the viral quantification data (RT-qPCR). I wrote the initial draft of the manuscript and prepared most of the figures and tables (≈ 70%). I participated in preparing the final version of this manuscript.

- **Manuscript V**
  I designed and participated in conducting the experiment. Before conducting the small RNA sequencing, I extracted total RNA and analyzed its quality. I also measured the viral mRNAs in duck lung and brain. I performed the semi-quantitative scoring of lung and brain for apoptosis. With regard to the bioinformatics analysis, I used the OASIS tool (see Materials and Methods of Manuscript V) to perform the differential expression analysis of the small RNAs. I also performed the gene ontology and KEGG pathways annotations using the Cytoscape software (see
I partially wrote the initial draft of the manuscript and prepared most of the figures and tables (≈ 80%).

4. Unpublished Data

4.1. Differential miRNA expression in genetically diverse H1N1-infected mouse strains

Early evidence from 1918 demonstrated that although many people were infected with the pandemic strain H1N1 (1918), only 8% of those infected died and the remaining survived the infection with clinical signs ranging from mild to severe [108], indicating an inter-individual variation in the susceptibility to H1N1 infection. Comparing the high number of people exposed to H5N1 virus in 1997 to the low number of confirmed cases reinforced this notion [109] and further supported the idea that this genetic effect is virus-independent. The susceptibility to IAV infection is a result of combination of virus-associated factors such as virus virulence and evolution [110] and host genetics [111]. The mouse model has been widely exploited to study genetic variation in the host response to infection. This is because of the easiness of producing in-bred mouse strains with a well-defined genetic background and the possibility to produce multiple mouse lineages that lack or express certain genes. Previous reports demonstrated that the DBA/2J mouse strain is susceptible to infection with the PR8 H1N1 virus, whereas C57BL/6J mice are relatively resistant. Compared to C57BL/6J, the higher susceptibility of DBA/2J to PR8 infection mice presented as rapid weight loss, a much lower mouse lethal dose 50 (MLD$_{50}$), and a high viral load and cytokine levels in lung, which appears severely damaged upon histopathological assessment [112]. In a comparable experiment using a more virulent virus strain (H5N1), DBA/2J mice were identified as most susceptible strain, as compared to 20 other mouse strains including C57BL/6J. This increased susceptibility correlated with an increased production of inflammatory mediators (CCL2, CXCL2, TNF-α, IFN-α, IFNβ and CSF3) and a higher viral load [113]. In this context, the majority of studies have focused on the role of host genes
in host-associated susceptibility to IAV infection. However, relatively few investigations have focused on the role of cellular miRNAs. Recently, profiling miRNA expression in PR8 H1N1-infected DBA/2J and C57BL/6J mice strains revealed earlier and more intense miRNA reprogramming in DBA/2J (48 hpi) than in C57BL/6J (120 hpi) mice. In this study, 20 miRNAs were found to be regulated upon PR8 infection and were differentially expressed between both mice strains in a significant manner. These include miR-467, miR-449, miR-34 families, miR-223, miR-146b-3p and miR-155-3p [114].

Real time PCR has been traditionally used to validate miRNA expression data obtained by high throughput profiling approaches such as RNA-seq [115], microarrays [83, 116] and TaqMan PCR arrays [85]. Here, real-time PCR provides the opportunity for technical validation of miRNA expression with an independent test. In this part of my thesis, out of the 20 miRNAs identified in the above study, I selected 10 miRNAs (mmu-miR-142-1-5p, mmu-miR-21-2-5p, mmu-miR-21-1-3p, mmu-miR-223-2-3p, mmu-miR-223-1-5p, mmu-miR-146b-1-5p, mmu-miR-211-1-5p, mmu-miR-147-2-3p, mmu-miR-99b-2-5p and mmu-miR-150-1-5p) and validated their relative expression that was generated by RNAseq using RT-qPCR. Next, I calculated the degree of agreement between results of both approaches. This allowed determining by how much the results of deep sequencing differed from that obtained by RT-qPCR. Additionally, I analyzed the expression levels of genes that have been shown to be targets for some of the differentially expressed miRNAs.

**Aims**

The aim of this experiment was to:

1. Validate the results of RNA-seq with regard to the differential miRNA expression in PR8 H1N1-infected mouse strains using RT-qPCR and calculate the degree of agreement between the two methods.

2. Determine the differences in the expression of two genes, (phosphoinositide 3-kinase (PI3K) and protein kinase B (PKP or Akt) in the two mouse strains upon infection with PR8 H1N1 virus.
Materials and Methods

In this experiment, I used the RNA that was provided by Dr. Matthias Preuße (Helmholtz Center for Infection Research) as a starting material. As described in [114], Matthias’s study involved intranasal infection of DBA/2J (susceptible) and C57BL/6J (resistant) mice with 2x10^3 focus forming units (FFU) of the mouse adapted IAV strain A/Puerto Rico/8/34 (PR8). Lungs from control and treated mice were homogenized and total RNA, including miRNAs, was isolated using the miRNeasy kit (Qiagen) according to the manufacturer’s recommendations.

In my experiment, RNA quality and quantity were determined with the Nanodrop S1000 spectrophotometer (Thermo Scientific). The RNA was reverse transcribed using the miScript reverse transcription kit according to the manufacturer’s recommendations. The complementary DNA (cDNA) was used to relatively quantify miRNAs using the LightCycler® 480 PCR instrument (Roche), software version 1.5., in 96 well plates in 20-μl reaction volumes. Murine RNU6b-2 and murine β-actin mRNAs were used as reference genes for miRNA and mRNA expression, respectively. In each qPCR run, non-cDNA controls were included and melting curves were inspected to exclude primer dimer formation. In addition, non-reverse transcribed RNA (RT minus control) was included to rule out the possibility of genomic DNA contamination. The relative expression levels of the 10 selected miRNAs as well as the mRNA of PI3K and Akt were measured at 24 and 48 hpi and the fold change (FC) in expression was calculated using the 2^(-ΔΔCT) method [117]. The miRNA primers were ordered as a part of miScript Primer Assays from Qiagen Inc. Other primers used in this experiment are listed in Table 3.

In order to determine the involvement of the 10 studied miRNAs in IAV pathogenesis, the DNA intelligent analysis (DIANA) tool, a web server [118], was used to screen for pathways that contain target genes for these miRNAs.

Agreement between the results of RNA-seq and RT-qPCR

For each miRNA, the degree of agreement between the two methods at each time point was calculated based on two criteria: the direction of miRNA de-regulation and
the degree of FC. The direction of deregulation was compared considering a cutoff level of FC ≥1 (for RT-qPCR approach) and FC ≥1.5 (for RNA-seq). For calculating the agreement based on the FC values, the method of Bland and Altman was applied [119]. In this method, for each miRNA, the average values of both tests are plotted on the x-axis and the difference between the values given by each test (RNA-seq and RT-qPCR) was plotted on the y-axis. Furthermore, limits of agreement were applied in all conditions.

Statistical analysis
In order to determine if the differences in the values of miRNAs, PI3k and Akt between the two mice strains are statistically significant, T-test (two-tailed and unpaired with unequal variances) was applied for each pair of comparisons. \( P \) values ≤ 0.05 were considered significant.

Results and Discussion

1. Relative quantification of miRNAs in DBA/2J and C57BL/6J mice strain

Analyzing the differences in miRNA expression between the two mice strains revealed that 4 out of the 10 studied miRNAs were more up-regulated in C57BL/6J compared to the DBA/2J mice. These include miR-142-1-5p, miR-146b-1-5p, miR-211-1-5p and miR-99b-2-5p (Fig. 1A). Another 4 miRNAs showed the reverse expression pattern. These include miR-150-1-5p, miR-147-2-3p, miR-223-2-3p and miR-223-1-5p (Fig.1B). Interestingly, the differences between mice in these miRNA were greater at 48 hpi. This agrees with the data obtained from profiling miRNA expression in the DBA/2J strain using RNAseq [114]. Since HA mRNA of the virus peaked also at 48 hpi, this suggests that the host-dependent difference in miRNA regulation was, in part, virus-derived. Additionally, after 48 h, the reprogramming remained whereas HA mRNA level declined indicating that at later phases, viral-host interaction became the driving force for miRNA reprogramming [114]. MiR-21-2-5p and miR-21-1-3p showed a temporary up-or down regulation in both mice (Fig. 1C). Taken together, these data suggest the involvement of miRNAs in host susceptibility.
to H1N1 virus and thus value their utility as biomarkers for the host response to infection.

1.1. Calculating the agreement in miRNA expression changes between the RT-qPCR and the small RNA deep sequencing data

To validate the results obtained by RNA-seq in terms of measuring the relative expression of miRNAs, RT-qPCR-based relative quantification was performed for 10 selected differentially expressed miRNAs (mmu-miR-142-1-5p, mmu-miR-21-2-5p, mmu-miR-21-1-3p, mmu-miR-223-2-3p, mmu-miR-223-1-5p, mmu-miR-146b-1-5p, mmu-miR-211-1-5p, mmu-miR-147-2-3p, mmu-miR-99b-2-5p and mmu-miR-150-1-5p). Obtaining this information is valuable to validate the results of RNA-seq and further confirm the differential expression of candidate miRNAs between the two mouse strains. With regard to the direction of miRNA deregulation, the results showed that in DBA/2J mice, the RT-qPCR results agreed with the RNA-seq results in 20% (2 out of 10 miRNAs) and 90% (9 out of 10 miRNAs) at 24 and 48 hpi, respectively (Fig. 2). In C57BL/6J mice, the agreement between the results of the two assays was 0 (0 out of 10 miRNAs) and 80% (8 out of 10 miRNAs) at 24 and 48 hpi, respectively. Therefore, the patterns of miRNAs differential expression in both tests agreed well at 48 hpi in both mouse strains. However, the agreement was low at 24 hpi.

In the next step, I examined the agreement between RT-qPCR and RNA-seq based on the relative expression values of the 10 miRNAs (FC). This analysis demonstrated that there was a high degree of agreement in both mouse strains at 24 hpi and, in addition, in C57BL/6J mice at 48 hpi (Fig. 3). In contrast, there was low agreement between the two tests in the DBA/2J strain at 48 hpi. In both mouse strains, there were two outlier (miRNAs) at 24 hpi, as evidenced by their presence outside the limits of agreement. Collectively, these data suggest that, under our experimental conditions, RT-qPCR was a valid technique to confirm the results obtained by RNA-seq.
2. Pathway enrichment of the miRNAs

By using these 10 miRNAs as input into the DIANA tool, the majority of them were found to target genes in pathways related to IAV infection to varying degrees (Fig. 4). Examples of these pathways are T-cell receptor signaling, endocytosis, cytokine-cytokine receptor interaction, MAPK signaling, TGF-beta signaling pathway, and the PI3-Akt signaling pathway. The PI3K pathway is mainly involved in apoptosis. Binding of the IAV NS1 gene to the P85β regulatory subunit activates PI3K leading to the phosphorylation of the downstream effector molecule Akt, which then phosphorylates both caspase 9 and GSK-3β, thereby suppressing apoptosis and prolonging viral infection [120]. Besides the PI3K pathway, MAP kinases have been reported to promote IAV ribonucleoprotein capsid trafficking and virus production [121] and to regulate production of RANTES [122]. These data suggest that miRNAs might play a role in host genetic susceptibility to IAV infection by influencing pathways that are important in regulating the host response to IAV infection. These pathways are mostly related to the immune response.

3. Differential expression of PI3K and Akt mRNAs between DBA/2J and C57BL/6J mice strain

The results also indicated that PI3K and Akt mRNAs are differentially expressed in the two mouse strains (Fig. 5). The level of PI3K mRNA showed a trend toward higher expression in C57BL/6J than in DBA/2J mice at 24 and 48 hpi. However, Akt mRNA levels were significantly higher in C57BL/6J than in DBA/2J mice only at 24 hrs. These results suggest that both genes are related to each other as components of the PI3K-Akt signaling pathway, and that expression of these mRNAs is regulated during IAV infection.
Table 3. Sequences of forward and reverse primers of the mRNA genes used in the study.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequences 5' → 3' orientation</th>
<th>Amplicon length (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine PI3K</td>
<td>Forward ATCGGCCCACTAGTCAGTCT</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td>Reverse GCATCCCAAGGATCCAGTCT</td>
<td></td>
</tr>
<tr>
<td>Murine AKt</td>
<td>Forward ACTCATCCAGACCCACGAC</td>
<td>176</td>
</tr>
<tr>
<td></td>
<td>Reverse CCGGTACACCACGTCTTCT</td>
<td></td>
</tr>
<tr>
<td>Murine β-actin</td>
<td>Forward CCACCATGTACCCAGGCATT</td>
<td>253</td>
</tr>
<tr>
<td></td>
<td>Reverse AGGGTGTAACACGCACTCA</td>
<td></td>
</tr>
<tr>
<td>Hemagglutinin (HA) of S-OIVs.</td>
<td>Forward CTCGTGCTATGGGGCATTCA</td>
<td>308</td>
</tr>
<tr>
<td></td>
<td>Reverse TTGCAATCGTGACTGGTGT</td>
<td></td>
</tr>
<tr>
<td>Interferon-α (IFN-α)</td>
<td>Forward TTCAGGGGCATCAGTCCCTA</td>
<td>269</td>
</tr>
<tr>
<td></td>
<td>Reverse CCGTCCATTCCTTGATTTGTT</td>
<td></td>
</tr>
<tr>
<td>Interferon-β (IFN-β)</td>
<td>Forward CAGCAATTTTCAGTGCAGAAGC</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Reverse TCATCCTGTCCCTTGAGGACGT</td>
<td></td>
</tr>
<tr>
<td>CXCL10</td>
<td>Forward AGGAACCTCCAGTCTAGCA</td>
<td>203</td>
</tr>
<tr>
<td></td>
<td>Reverse CAACACGTGGACAAATTGG</td>
<td></td>
</tr>
<tr>
<td>Human β-actin</td>
<td>Forward AGAGCTACGAGCTGCCTGAC</td>
<td>184</td>
</tr>
<tr>
<td></td>
<td>Reverse AGCAGCTGTGGCGCTACAG</td>
<td></td>
</tr>
</tbody>
</table>

1 The primers for this gene were used before in [123].
Figure 1A. Relative expression of miRNAs in DBA/2J and C57BL/6 mice at 24 and 48 hpi. FC in expression were calculated using the $2^{(-\Delta\Delta CT)}$ method after normalization to murine RNU6b-2 expression and is plotted on a log$_{10}$ scale. Data are shown as average of the FC (n=3 animals) +/- SEM. Statistical significance was calculated by t-test (unpaired and two-tailed). The asterisks indicate significant differences in miRNAs expression between the two mice strain at each time point. The analysis was done with GraphPad Prism software, V.5. * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$).
Figure 1B. Relative expression of miRNAs in DBA/2J and C57BL/6 mice at 24 and 48 hpi. FC in expression were calculated using the $2^{(-\Delta\Delta CT)}$ method after normalization to murine RNU6b-2 expression and is plotted on a log$_{10}$ scale. Data are shown as average of the FC (n=3 animals) +/- SEM. Statistical significance was calculated by t-test (unpaired and two-tailed). The asterisks indicate significant differences in miRNAs expression between the two mice strain at each time point. The analysis was done with GraphPad Prism software, V.5. * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$).
Figure 1C. Relative expression of miRNAs in DBA/2J and C57BL/6J mice at 24 and 48 hrs post infection. FC in expression were calculated using the $2^{-\Delta\Delta CT}$ method after normalization to murine RNU6b-2 expression and is plotted on a log$_{10}$ scale. Data are shown as average of the FC (n=3 animals) +/- SEM. The asterisks indicate significant differences in miRNAs expression between the two mice strain at each time point. The analysis was done with GraphPad Prism software, V.5. * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$).

Figure 2. Side-by-side comparison between the relative expression values of selected miRNAs by RNA-seq and RT-qPCR techniques. The values in the table are presented as FC differences between infected and non-infected mice at each time point. FC was set to a cutoff of FC > 1.5 (RNA-seq) and FC > 1 (RT-qPCR). Red-colored cell indicate up-regulation and blue-colored cells indicate down regulation.
Figure 3. Measurements of degree of agreement between RNA-seq and RT-qPCR approaches in relative quantification of host miRNAs. The scale on the x-axis denotes the average of the values given by both approaches for each miRNA. The y-axis depicts the differences between the values given by both tests for each miRNA. Each dot represents one miRNA. The limits of agreement are represented by the upper and lower horizontal lines. The dashed line represents the highest degree of agreement between both approach.
Figure 4. Heat map showing the enriched pathways that contain miRNA target genes of the 10 selected miRNAs. DIANA microT-CDS computes miRNA predicted interaction in the 3’ UTR and the coding sequences (CDs) of the target transcript. The heat map was created with DIANA tools (mirpath).

Figure 5. Relative quantification of PI3K and Akt mRNA in DBA/2J and C57BL/6J mice at 24 and 48 hpi. FC in expression were calculated using the $2^{-\Delta\Delta CT}$ method after normalizing to β-actin mRNA expression and is plotted on a log_{10} scale. Data are shown as average of the FC (n=3 animals) +/- SEM. The asterisks indicate significant differences in mRNA expression between the two mice strain at each time point. The analysis was done with GraphPad Prism software, V.5. * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$).
4.2. Establishment of human lung tissue explant (HLTE) to study miRNA expression in response to 2009 swine-origin influenza viruses (S-OIVs) of differential virulence

Pandemic swine origin 2009 IAVs H1N1 (pdm009) were first identified in April 2009 in Mexico and the United States [124]. The virus is a completely new reassortant version of H1N1 in which the HA, NS and NP genes originated from the classic swine lineage (which entered the pig population from an avian host in 1918 [125] and the NA and M genes were derived from the Eurasian swine lineages of IAV, which entered the swine population in 1979 from avian host [126]. The PB2 and PA gene segments are in the swine triple reassortant lineage. Viruses that seeded this lineage, originally of avian origin, entered pigs in North America around 1998 [127]. The PB1 gene segment originated from the swine triple reassortant lineage. This PB1 segment was transmitted to swine from humans around 1998, and was itself seeded to humans from birds around 1968 [128]. Compared to seasonal IAVs, S-OIVs replicated more efficiently in the ferret respiratory tract [129]. They also have the ability to infect and replicate ex vivo in human conjunctival tissue, likely due to their ability to bind to the α-2,3 sialic acid receptor, which is not the case in seasonal IAVs [130]. Pandemic S-OIVs induce an earlier immune response than seasonal H1N1 viruses. However, at later infection stages, S-OIVs cause a suppression of host immunity and a reduction in the expression of immune mediators, indicating that the weak host immune responses induced by the S-OIVs could be the reason for the high transmissibility and mortality caused by these viruses [123].

So far, the role of host-encoded miRNAs in IAV virulence has been mostly studied in mouse and macaque models and in cell lines [97-100, 131, 132]. The results of these studies revealed that the virulence of highly pathogenic IAVs and the mammalian adaptation of avian IAVs might be mediated, in part, by the alteration in cellular levels of miRNA. Nevertheless, these models have limitations such as lack of cell-cell interaction and the presence of the non-human background. Therefore, there is a need to develop a system that can convey the realistic picture of human lung so that the adequacy and transferability to humans can be attained.
In this part of my thesis, I established the human lung tissue explant (HLTE) model, with the ultimate goal to study the potential of cellular miRNAs as biomarkers to discriminate between pandemic S-OIVs of variable virulence. The advantage of this model is that it mimics human lung where complex interactions between specialized cell types and the extracellular component exist. This is essential for the host response and pathogenesis of IAVs. Furthermore, protease activity, which is needed for the cleavage of HA of low pathogenic IAV strains, is known to be preserved in HLTEs. In addition, the HLTEs can remain viable for up to 96 h as proved by constant low level of the released lactate dehydrogenase enzyme [37]. The HLTE model can support the replication of various IAVs, albeit to varying degrees. It has been also shown that the H7N9 strain, although being an avian virus, can readily infect and replicate in HLTE without prior adaptation [133]. Collectively, these studies suggest the suitability of the HLTE as a model to study various aspects of IAV-host cross talk.

**Aims**

The aim of this part of my thesis was to:

1. Establish the HLTE as a model system to investigate IAV replication.
2. Use the HLTE model to study differences between two IAV variants in terms of virus replication, virus-induced immune response and tissue lesions.
3. Characterize the miRNA responses in HLTE infected with IAV strains with variable virulence.

**Materials and Methods**

1. **Establishment of IAV infection in HLTE model**

This experiment was done in collaboration with the research group of Prof. Michael Steinert, Institute of Microbiology, TU Braunschweig, and Dr. Richter, Dept. of Cardiothoracic and Vascular Surgery (Klinik für Thorax-, Herz- und Gefäßchirurgie) of the Klinikum Braunschweig. The study was approved by the ethics committee of Klinikum Braunschweig, and informed consent was obtained from all patients.
Tissues from the tumor-free margin were excised from patients undergoing lung resection for lung cancer. Next, the excised tissue was investigated for quality by a pathologist at the Dept. of Pathology of the Klinikum Braunschweig. Normal appearing tissue of good quality was sent to Prof. Steinert's laboratory, where the infection experiments were done. In these experiments, I used the same viruses that were used in Manuscript III, i.e. the wild-type S-OIV H1N1 and its reassortant carrying the NS segment from the more virulent PR8 isolate. These viruses were provided by Prof. Stephan Pleschka and Dr. Ahmed Mostafa, Institute of Medical Virology, Justus Liebig University Giessen, Giessen, Germany.

In order to check the viral dose needed to infect the HLTE, 3 doses (10^5, 4X10^5 and 10^6 FFU) of the S-OIV wt were used to infect the human lung tissue. For virus infection, small pieces of the excised lung tissue (height ≈ 3 mm; diameter ≈ 5 mm), each weighing ≈ 30 mg, isolated from 5 donors, were incubated for 1 h in virus infection medium (RPMI medium containing 1% glutamax (100x, Gibco life technology) and 1% sodium pyruvate, 100Mm, Gibco life technology). To remove the unabsorbed virus particles, the tissues were washed 3 times in phosphate buffered saline (PBS) and then transferred to tissue maintenance medium (RPMI medium containing 10% fetal calf serum, 1% glutamax and 1% sodium pyruvate, 100 Mm). Non-infected and infected tissues were harvested 8, 24, 48 and 72 hpi and were used in the following experiments.

1.1. Relative quantification of viral HA mRNA using RT-qPCR

Total RNA, including small RNAs, was extracted using the miRNAeasy kit (Qiagen) according to the manufacturer's instructions, and then DNase treated using the RNase-Free DNase Set (Qiagen). RNA quality and quantity were determined with the Nanodrop S1000 spectrophotometer (Thermo Scientific). The DNase-treated total RNA was then reverse transcribed using the miScript Reverse Transcription Kit (Qiagen) in a 20-µl reaction mix.

qPCR was performed using miScript SYBR Green PCR Kit (Qiagen). The primers used in this experiment are listed in table 3. Human β-actin mRNA was used for normalization. In each run a non-cDNA control and - RT control were added to assess the formation of primer dimers and contamination with genomic DNA.
respectively. Specificity of primer pairs for each target was determined based on analyzing the melting curves. RT-qPCR was carried out using the LightCycler® 480 instrument, Roche, software version 1.5., in 96 well plates. The cycling conditions started with a pre-incubation at 95°C for 15 min, followed by 45 cycles at 94°C for 15 seconds (s), 54°C for 30 s and 72°C for 30 s. Melting curves were created by increasing the temperature of the sample to 95°C. Final cooling was done at 40°C for 30 s.

1.2. Induction of immune-related genes upon infection of HLTE with S-OIV wt at a dose of 10^6 FFU  
The cDNA that was mentioned in section 1.1 was used to investigate whether the infection of the HLTE with S-OIV wt could induce an inflammatory response. Relative levels of the IFN-α, IFN-β and CXCL10 mRNAs were quantified using RT-qPCR. The RT-qPCR was run using the same conditions as in section 1.1. The primer used to detect the mRNA targets are listed in table 3.

1.3. Histopathological examination and immunoistochemical (IHC) staining of virus NP  
Slides from infected and non-infected HLTE were stained for both histopathological evaluation and immunohistochemical detection of viral NP at the mouse pathology unit, Helmholtz Center for Infection Research (HZI), Braunschweig, Germany. Briefly, pieces of tissue from 3 donors were processed according to standard practice and embedded in paraffin. For histopathological examination, the paraffin-embedded tissues were sectioned into 3 µm-thick sections and stained with hematoxylin/eosin (H&E) according to standard laboratory procedures. For IHC, the embedded tissue was cut into 4-µm thick sections, re waxed in xylazine baths for 10 min and then rehydrated. Antigen retrieval was carried out by immersing the slides in citrate buffer followed by pressure-cooking in 100-µl distilled water. The slides were then cooled on ice and endogenous peroxidase activity was blocked by adding hydrogen peroxide (3%) for 10 min. Next, the slides were incubated with primary Ab (H1N1 goat-anti-NP-protein antibody, ViroStst 1301) overnight at 4°C. The complex containing the H1N1 NP protein and the primary antibody was labeled.
by staining the slides with a biotin-labeled rabbit anti-goat secondary antibody, dilution 1:250 (Kirkegaard & Perry Laboratories, KPL, 16-13-06). Streptavidin bound to biotin-labeled horseradish peroxidase (HRP) enzyme was then added and diaminobenzidine was used as a substrate. Hemalaun was used as a counter stain to visualize tissue architecture. Positive and negative control sections were included in each IHC run.

2. Differential transcription of S-OIV wt and S-OIV NS PR8 in the HLTE model

In order to investigate differences in transcription capacity, and indirectly viral replication, of S-OIV wt and S-OIV NS PR8 in the HLTE, the previously mentioned infection protocol was done using both viruses at an infection dose of $10^6$ FFU. The following experiments were performed.

2.1. Relative quantification of viral HA mRNA using RT-qPCR

The relative quantification of HA mRNAs of both viruses was done as described in section 1.1.

2.2. Relative quantification of IFN-α mRNA using RT-qPCR

The same cDNA that was used for viral HA mRNA relative quantification was used to investigate the ability of both viruses to induce differential expression of immune-related genes. The RT-qPCR-based relative quantification of IFN-α mRNA was done as described in section 2.1, except that annealing temperatures varied. Primers used for the detection of IFN-α mRNA are listed in table 3.

2.3. Relative quantification of IFN-α protein using Enzyme-Linked Immunosorbant Assay (ELISA)

This part was done in collaboration with members from the research group of Prof. Ulrich Kalinke, Institute for Experimental Infection Research, TWINCORE, Hannover, Germany.

The aim of this experiment was to investigate the difference between S-OIV wt and S-OIV NS PR8 viruses in terms of the ability to induce secretion of the IFN-α protein.
In this experiment, the protein level of human IFN-α was measured in the supernatants of S-OIV wt and S-OIV NS PR8-infected HLTEs from 5 donors at 8, 24 and 48 hpi. The Human IFN-α Platinum ELISA kit was used according to the procedure provided by the manufacturer (Affymetrix eBioscience, BMS216/BMS216TEN). Briefly, wash (20X) and assay (20X) buffers were diluted to a concentration of 1X each in distilled water. The HRP-conjugated antibody was diluted 1:100 with the assay buffer (1X). The lyophilized human IFN-alpha standard protein (1000 pg/ml) was reconstituted with distilled water for 10-30 min followed by gentle mixing. Standard dilutions were made in the microtiter plate. Each sample (standard, blank and control) was assayed in duplicate. 100 µl of assay buffer (1X) was added to the blank well. 100 µl of the sample supernatant was added in the sample well in duplicate. Then, 50 µl of HRP-conjugate was added to all wells. The microtiter plate was covered with adhesive film and incubated at room temperature for 2 hrs. After that, the film was removed and the wells were washed with 400 µl wash buffer. Next, 100-µl of TMP substrate solution was added to each well, and the plate was incubated for 10 minutes at room temperature. 100 µl of stop solution (phosphoric acid) was then added. The absorbance of each well was read with a spectro-photometer, using 450 nm as the primary wave length.

2.4. Histopathological examination and immunoistochemical (IHC) staining of virus NP

The histopathological and IHC staining was done as described in section 1.3.

2.5. Development of semi-quantitative scoring to evaluate viruses-induced histopathological lesions in HLTE

This part was done in collaboration with Dr. Peter Braubach, Institute of Pathology, Medical School of Hannover (MHH).

In this part, pieces of HLTE excised from 3 donors were either left non-infected or infected with 10^6 FFU of S-OIV wt and S-OIV NS PR8 viruses and the H&E-stained slides from these tissues were blindly scored for evaluating the differences between the histopathological lesions induced by both viruses using the criteria and grades mentioned in Table 4.
Results and Discussion

Understanding the virulence and pathogenicity of IAVs in humans is limited by either using single cell models that lack the cell-cell interaction or the use of non-human hosts. These factors hinder, to a large extent, the transferability of the results to humans. Therefore, using the HLTE model, which comprises healthy live human lung tissue, enables thorough analysis of host-virus interaction and further assist identifying the aspects of IAV-induced pathology, which, otherwise are not accessible to investigation.

1. Establishment of virus infection in the HLTE model

In order to investigate the infectivity of the HLTE with H1N1 virus, I infected tissues from 1 donor with 3 different viral doses (Fig. 6) and measured HA mRNA expression at 4, 8, 24 and 48 hpi using RT-qPCR. The viral transcription data indicated that a virus dose of $10^6$ FFU was sufficient to infect the HLTE since the virus transcription increased exponentially during the time course of the experiment, peaked at 48 hrs and declined thereafter. Lower doses ($4 \times 10^5$ plague forming unit (PFU)) of A/Bayern/63/2009 (A/Bay H1N1 pdm) were found by Weinheimer et al. to be able to infect the HLTE. The titer of A/Bay H1N1 pdm was comparable to that of the seasonal IAV A/New Caledonia/20/1999 (H1N1) virus [37]. Other studies reported using higher infection doses ($4 \times 10^6$ PFU) of H7N9 virus to infect the HLTE [133]. Collectively, my data suggest that the S-OIV wt could replicate efficiently in the HLTE when used at a high dose (e.g. $10^6$ FFU). The discrepancies in the virus doses among studies might be attributed to the virus strain used and to the tissue status. To determine if the infection of HLTE with S-OIV wt ($10^6$ FFU) induces a characteristic host response, relative mRNA levels of the anti-viral cytokines IFN-α and IFN-β and the chemokine CXCL-10 were quantified by RT-qPCR. Fig. 7 shows that S-OIV wt infection of HLTE induced expression of IFN-α, IFN-β and CXCL10 mRNAs. The level of IFN-α mRNA was higher than that of IFN-β and CXCL-10 mRNAs. It has been shown previously that HLTE infected with A/Bay H1N1 pdm induced a weak inflammatory response characterized by a low induction of IP-10, MIP-1β, IFN-β and IL-1β proteins. Whereas other IAVs A/duck/Alberta/60/1976
[H12N5] and A/Thailand/1(Kan-1)/2004 [H5N1] viruses caused a higher induction of these mediators [37]. The relatively low induction of IFN-β and CXCL-10 mRNAs was reported previously by Wu et al., who stated that infection of human lung tissue with A/Oklahoma/3052/09 (OK/09) for 24 h induced lower levels of these mediators than infection with the PR8 strain. They attributed this diminished anti-viral cytokine response to the suppressive ability imposed by the OK/09 strain on RIG-1 mediated sensing of the viral infection and, partially, to the virus-induced inhibition of NOD-like receptor expression [134]. Similar results were obtained using microarray analysis of A549 cells (a human lung epithelial cell line), where A/Jalna/NIV9436/2009 (H1N1 pdm) caused a suppression of host immune-related genes compared to seasonal H1N1. This inability to induce a strong immune response by H1N1 pdm could be the reason for its high transmissibility and pathogenicity [123].

To determine if the infection with the S-OIV wt leads to tissue lesions, pieces from HLTE infected with the 3 doses were processed for histopathological evaluation and IHC analysis. The specificity of the staining was confirmed by evaluating the - NP control slides (Fig. 8A and B). The non-infected tissues showed no staining for the NP protein (Fig. 8C). Infection of the tissue with the S-OIV wt at a dose of $10^6$ FFU showed inflammatory cell infiltrates at 8, 24 and 48 hrs post infection, which was more intense if compared with the tissue infected with lower viral doses (Fig. 9A, H&E staining panel). Furthermore, the IHC analysis indicated that the degree of NP antigen staining was much higher when the tissue was infected with a dose of $10^6$ FFU (Fig. 9A, IHC panel), whereas the tissue infected with lower virus doses showed only mild or no NP staining (Fig. 9B).

2. Relative quantification of HA mRNA of S-OIV wt and S-PIV NS PR8 viruses in the HLTE

Transcription of HA mRNAs of both viruses was observed as early as 8 hpi, reaching a FC of 1000 by 24 hpi. This indicates that the HLTE can support the replication of S-OIVs viruses. Weinheimer et al showed similar results for the A/Bayern/63/2009 strain [37]. The data also demonstrated that transcription of S-OIV NS PR8 was higher than that of the S-OIV wt at 8 and 48 hpi, but not at 24 hpi. At 72 hpi, there were no differences in the transcription between both viruses. In spite of the small
number of donors used in this study (n=3), S-OIV NS PR8 seems to have a slightly higher transcription efficiency compared to the S-OIV wt.

3. Differential expression of IFN-α mRNA in HLTE infected with S-OIV wt and S-OIV NS PR8 viruses

For some IAV viruses, the over expression of inflammatory cytokines is used as an indicator for increased virulence [135]. Therefore, I analyzed the level of IFN-α mRNA in HLTE infected with S-OIV wt and S-OIV NS PR8. The results indicated that both viruses triggered an IFN-α response, yet with different levels. IFN-α mRNA was more produced by the reassortant strain than by the wild type at the indicated time points (Fig. 11). Peterson H. et al. obtained similar results with regard to IFN-α and IFN-β mRNAs and other IFN stimulated genes in A549 cells. In this study, the authors indicated the lack of ability of the S-OIV NS PR8 to suppress IFN-β using a reported assay (Manuscript in preparation). These results suggest that the HLTE reacted more strongly to the infection with the S-OIV NS PR8.

4. ELISA-based relative quantification of IFN-α protein in HLTE infected with S-OIV wt and S-OIV NS PR8 viruses

In order to assess the impact of the NS reassortment on the virus-induced immune response in the HLTE, the levels of released IFN-α protein were measured in the supernatant of HLTE infected with both viruses. As shown in Fig. 12, I could not detect any IFN-α protein in the non-infected HLTE. Early in infection (8 hpi), S-OIV wt did not cause IFN-α protein production, whereas the S-OIV NS PR8 reassortant induced a slight IFN-α response. At 24 and 48 hpi, too, S-OIV NS PR8 caused a higher of induction of IFN-α protein. These data agree with the IFNα mRNA expression data shown in Fig. 11. These findings suggest that the presence of the NS segment from PR8 induced a brisker immune response in the HLTE and that this increased cytokine production might, at least in part, relate to the increased transcriptional activity of the S-OIV NS PR8 strain in this model.
5. Semi-quantitative scoring of histopathological lesions induced by S-OIV wt and S-OIV NS PR8 in HLTE

To characterize the differences in tissue damage caused by infection with S-OIV wt and S-OIV NS PR8 on HLTEs, infected and non-infected tissue sections from 3 donors were stained with H&E stain. In the infected tissue, there were variable degrees of epithelial tissue damage and delamination. Furthermore, there was endothelial cell damage associated with sloughing of the cells in the lumen of blood vessels (Fig. 13). In order to obtain a more quantitative description of the viruses-induced histopathological lesions, the slides were evaluated blindly by applying a semi-quantitative score. Table 4 summarizes the criteria and the associated damage scores and Fig. 14 shows the pair-wise comparison between HLTE infection with the two viral strains. Merging all the scoring criteria, the results revealed that the overall damage progressed over the time course of infection. The non-infected tissue, too, showed a slight damage at 8 hpi, which increased with increasing the incubation time. The observation of tissue damage in non-infected HLTEs is not uncommon. Similar observation was made by Jager et al, who demonstrated that RPMI-incubated non-infected HLTEs showed variable degree of changes in tissue architectures when incubated for 2, 24 and 48 hrs [136]. Nonetheless, it appeared that infection with S-OIV NS PR8 caused more endothelial damage and epithelial delamination than the S-OIV wt. The results of perivascular delamination and vascular destruction, on the other hand, varied. These preliminary results based on a small number of specimens suggest that histopathological changes may be more severe in S-OIV NS PR8 infection in the HLTE model, but that natural tissue disintegration in culture needs to be considered as a confounding factor. The observed destruction in alveolar epithelium is consistent with the known ability of IAVs to induce damage in tissue barriers and thus enhance viral spread to neighboring cells and organs [137-139]. Whether this invasiveness is a feature directly related to viral proteins or due to the secretion of tissue-destructive host molecules during inflammation, remains to be identified. particularly considering the observation that S-OIVs tend to induce diminished anti-viral cytokine response [123, 134].
Taken together, these findings emphasize the importance of the semi-quantitative scoring approach in determining differences in pathogenicity between IAV variants in this tissue-based model. Moreover, they suggest that relying on specific histopathological feature (e.g. endothelial damage and epithelial delamination) might be more advantageous over using a combined score in evaluating the S-OIVs-induced tissue lesions.
Table 4. Criteria and grades used to establish the semi-quantitative scoring system of the viruses-induced histopathological lesion in the HLTE model.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Grades of damage severity</th>
<th>Reference</th>
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<tr>
<td>Alveolar damage</td>
<td>(0) no damage, (1) sparse-some spreading of alveolar septa (AS), (2) pronounced spreading of alveolar septa (AS), (3) nearly complete disintegration of AS</td>
<td>[136]</td>
</tr>
<tr>
<td>Epithelial delamination</td>
<td>(0) no damage, (1) sparse, (2) pronounced, (3) complete. If all epithelial cells seemed missing, (3) was awarded</td>
<td>[136]</td>
</tr>
<tr>
<td>Endothelial damage¹</td>
<td>(0) no change, (1) some activated endothelia, (2) most endothelia activated, (3) endothelial delamination</td>
<td>[136]</td>
</tr>
<tr>
<td>Peri-vascular delamination¹</td>
<td>(0) no change in perivascular space, (1) some expansion/delamination in perivascular space, (2) pronounced changes (some vessels), (3) all vessels</td>
<td>[136]</td>
</tr>
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¹Perivascular delamination and endothelial damage were graded in small precapillary arterioles.

Figure 6. Relative quantification of S-OIV wt HA mRNA in HLTE infected with 3 viral doses (10⁵, 4x10⁵ and 10⁶ FFU). Human β-actin mRNA was used for normalization. FC in expression was calculated using the 2⁻(ΔΔCt) method and is plotted on a log₁₀ scale. Tissue pieces (n=3 technical replicate) from one donor were infected in this experiment.
Figure 7. Time-dependent induction of various inflammatory mediators upon infection of HLTE with S-OIV wt at dose of 10^6 FFU. Human β-actin mRNA was used for normalization. FC in expression were calculated using the 2^(-ΔΔCT) method. Virus infection of HLTE triggered the expression of IFN-α, IFN-β and CXCL10 mRNAs in a time-dependent manner indicating an ongoing inflammatory response. Tissue pieces (n=3 technical replicate) from one donor were infected in this experiment.

Figure 8. Control staining for IHC of S-OIV wt NP. A. Negative control: infected tissue stained only with the secondary antibody. B. Stain for the IAV NP antigen in various parts of H1N1-infected lung tissue. C. Stain for the IAV NP antigen in uninfected lung tissue.
Figure 9. **A.** IHC (NP) and H&E staining of HLTE infected with S-OIV wt using a virus dose of $10^6$ FFU. The figure shows tissue harvested at 8, 24 and 48 hpi. **B.** IHC staining for NP in HLTE infected with S-OIV wt used at doses of $4 \times 10^5$ and $10^5$ FFU) harvested at 48 and 24 hpi, respectively. The staining was done for tissues from one donor.
Figure 10. Relative quantification of HA mRNA in non-infected and infected (S-OIV wt and S-OIV NS PR8) HLTE in a 72-h time course. The data are from 3 donors (n=3 technical replicates each) and presented as mean±SEM of the FC on a log_{10} scale. Human β-actin mRNA was used for normalization. HA mRNA expression by S-OIV NS PR8 was higher than that of S-OIV wt at 8 and 48 hpi. Statistical significance was calculated by t-test (unpaired and two-tailed). The analysis was done with GraphPad Prism software, V.5. * (P ≤ 0.05), ** (P ≤ 0.01), *** (P ≤ 0.001).
Figure 11. Relative quantification of IFN-α mRNA in non-infected and HLTE infected with S-OIV and S-OIV NS PR8. The data are from 3 donors (n=3 technical replicate each) and presented as mean +/- SEM of the FC on a log_{10} scale. Human β-actin was used for normalization. The S-OIV NS PR8 induced higher IFN-α mRNA levels compared to the S-OIV wt. Statistical significance was calculated by t-test (unpaired and two-tailed). The analysis was done with GraphPad Prism software, V.5. * (P ≤ 0.05), ** (P ≤ 0.01), *** (P ≤ 0.001).
Figure 12. Quantification of IFN-α protein in the supernatants of control and infected (S-OIV wt and S-OIV NS PR8) HLTE in a 48-h time course. The data are from 5 donors (n=1 technical replicates each). Data are presented as means +/- SEM of IFN-α protein concentration (pg/ml). Statistical significance was calculated by t-test (unpaired and two-tailed). The analysis was done with GraphPad Prism software, V.5. * (P ≤ 0.05), ** (P ≤ 0.01), *** (P ≤ 0.001).
Figure 13. Histopathological lesions induced by S-OIV wt and S-OIV NS PR8 in HLTE (H&E stains). A. HLTE infected with S-OIV wt at 72 hpi showing epithelial damage and delamination (arrow). B. HLTE infected with S-OIV NS PR8 at 72 hpi showing alveolar damage and epithelial delamination (arrow). C. Infiltration of inflammatory cells in HLTE infected with S-OIV wt at 72 hpi. D. Endothelial damage (arrow) in HLTE infected with S-OIV NS PR8 at 8 hpi.
Figure 14. Overall and specific damage score of S-OIV wt and S-OIV NS PR8-infected HLTEs compared to non-infected tissue (n=3 donors). (A) For each time point and infection condition, histological damage was scored on a scale from 0 to 3 with the respect to the following parameters: alveolar damage, epithelial delamination, endothelial damage and perivascular delamination, yielding a maximum overall score of 9 (B-E). The mean +/- SEM of the values given to the 3 donors are plotted. The criteria for the damage include epithelial delamination (B), alveolar damage (C), perivascular delamination (D) and endothelial damage (E).
5. Discussion

RT-qPCR as a method to validate miRNAs relative quantification by deep sequencing (section 4.1 in Unpublished Data)

Previous reports indicated differences between mouse strains in their susceptibility to H1N1 infection [113]. More recently, NGS (RNA-seq) was employed to test whether DBA/2J and C57BL/6J mice exhibited differential miRNA responses upon infection with the mouse-adapted PR8 H1N1 strain [114]. These results revealed clear differences between the two mouse strains. In this part of my thesis, I used RNA extracted from the same experiment and RT-qPCR to quantify the relative expression patterns of selected 10 miRNAs identified by RNA-seq and, thereafter, compared the results of RT-qPCR with the RNA-seq results [114]. The results indicated that the calculation based on FC provided a better agreement between the two analytical approaches than if the agreement was calculated considering the direction of dysregulation pattern. The differences between the two approaches might be traced to differences in sensitivity and/or specificity. Nonetheless, these results do support the idea that RT-qPCR has some value for technical validation of miRNA expression results initially obtained with small RNA-seq. It is important to mention, though, that it is not at all clear whether RT-qPCR is a more accurate technique than RNA-seq or whether RNA-seq should be considered the gold standard [140] . The studied miRNAs were found to be important in multiple IAV-related signaling pathways (Fig. 4 in the Unpublished data). Among these pathways, the PI3K-Akt pathways has been shown to be activated by the NS1 protein of IAV and to promote viral replication [141]. The observation that PI3K and Akt, the two main genes within the PI3K-Akt pathway, are altered in response to PR8 infection (Fig. 5 in the Unpublished data) alongside with cellular miRNAs allows to hypothesize that miRNAs might shape the host response to H1N1 infection via modulating this pathway.
Cell- and virus-specific expression of cellular miRNAs (Manuscript III)

When studying the utility of miRNAs as potential biomarkers, the key questions arise: in which cell type and under which conditions are they expressed particular miRNA? These cell-specific signatures can potentially be detected in body fluids as indicators of the disease condition. This knowledge will assist in understanding the involvement of such miRNAs in signaling within the cell type in question. From the perspective that miRNAs can bind to specific target mRNAs, it has been shown previously that cell-specific mRNA expression can be inferred from cell-specific expression of the corresponding miRNAs [142]. Furthermore, defining the cellular origin of a particular miRNA will facilitate performing future functional studies on the cellular level.

The reassortment between IAVs is a frequent event and has constituted the origin of major influenza pandemics [143]. The non-structural (NS) segment of IAV is unique in that it helps establishing a favorable environment for IAV replication by different mechanisms [144, 145]. Therefore, it is expected that single reassortment of this segment will perturb viral replication and, possibly, virulence [146, 147].

In this part of my thesis, I studied the cell-type and virus strain-dependent miRNA expression of 6 miRNAs that have been previously identified to be associated with IAV susceptibility in a murine model [102] in A549 and dTHP-1 cells (see Manuscript III) using TLR agonists and both S-OIV wt and its reassortant S-OIV NS PR8 as a stimulant. Generally, TLR ligands (LPS and R848) tend to induce more miRNA expression in dTHP-1 than in A549 cells. Interestingly, the reverse was observed in the viral infections of these cell types (Fig. 2, 3 and 5 in Manuscript III). These differences suggest that miRNA regulation differs between the two cell lines, which is not surprising as viral infection usually is abortive in macrophages/monocytes, but productive in respiratory epithelial cells. The cell-specific expression of miRNA upon LPS stimulation is likely due to the fact that expression of the LPS sensing complex differs among cell types, including resident lung cells [148]. Generally, infecting both cell lines with the S-OIV wt or the S-OIV NS PR8 strain resulted in higher expression of miRNAs in A549 cells than in dTHP-1 cells (Fig. 5 in Manuscript III). Has-miR-223-3p exhibited the highest difference between the two cell types, especially upon infection with the S-OIV NS PR8 reassortant (Fig. 5a in Manuscript III). This response was more pronounced in A549 than in dTHP-1 cells (Fig. 6a in Manuscript
III). This indicates a relation of miR-223 with the virulence of S-OIV NS PR8, as previously suggested [149]. These authors also reported that miR-223 can indirectly repress the transcription factor CREB, which is responsible for maintaining cell survival and growth, and hypothesized that the lethal IAVs can induce cellular apoptosis via increasing miR-223 levels [99]. Of note, the reassortment of the NS segment lead to higher expression of has-miR-155-3p and has-miR-155-5p only in dTHP-1 cells (Fig. 6b and c in Manuscript III). The increased expression of has-miR-155, which can engage the suppressor of cytokine signaling (SOCS) [150], may lead to higher induction of cytokines which, in turn, may augment pathogenicity. Since has-miR-155 can target zinc finger proteins, it is tempting to speculate that S-OIV NS PR8 may down-regulate zinc finger proteins and thus hinder the anti-viral IFN response [151]. Collectively, the results indicated that the miRNA response to TLR agonists (LPS and R848) and pandemic H1N1 2009 variants is cell type-specific. Furthermore, reassortment of the NS segment influenced the expression of host miRNAs in a cell-specific manner, thus adding an additional layer of complexity.

The miRNA response to highly pathogenic IAV (H5N1) infection in its natural host, the duck (Manuscripts IV and V)

Given the importance of ducks (Anas platyrhynchos) in the ecology and transmission of the HPAI viruses of the subtype H5N1, coupled with the involvement of miRNAs in H5N1 virus-host interaction, there is a need to understand the regulatory function of miRNAs in the host response to H5N1 infection. Gaining this knowledge will assist in designing better strategies to combat H5N1 infection in poultry and subsequently reducing the potential of human transmission. Moreover, offering the chance to use these small molecules as infection indicators in case of outbreaks.

Dynamics of H5N1 virus in ducks (Anas platyrhynchos)
The initial characterization of A/chicken/Faquos/amn12/2011 (H5N1), an Egyptian strain belonging to HPAI clade 2.2.1.2, in duck (Anas platyrhynchos) revealed that the virus systemically disseminated throughout the host, reaching all internal organs examined, as well as the brain (Fig. 4 in Manuscript IV). Similar virulent HPAI (H5N1)
strains that were been isolated after 2002 showed the same lethality in ducks [152]. Although viral replication varied among organs, the brain of infected ducks represented an exception in that the overall viral transcription, viral growth rate and the associated tissue lesions were lower than in the other organs, notably the lung (Fig. 4 and 5 in Manuscript IV and Fig. 1 in Manuscript V). This was also coupled with an intermediate correlation (0.5) between viral transcription in lung and brain. (Fig. 6 in Manuscript IV). Apoptosis induction was much more pronounced and occurred earlier in lung than in brain (Fig. 2C in Manuscript V). Considering the importance of miRNAs as fine-tuners of gene expression during IAV pathogenesis [98, 107, 115, 153, 154] together with the differential transcription/replication efficiencies of this HPAI (H5N1) isolate in duck lung and brain, I hypothesized that miRNA expression during HPAI (H5N1) infection will differ significantly between these two organs.

Landscape of sncRNAs in duck lung and brain
Using the OASIS tool [155] (see Methods section in Manuscript V), I identified known and novel expressed miRNAs (Table 2 and S1A in manuscript V) that correspond to 52.7% of all identified sncRNAs (Fig. S1 in Manuscript V). This agreed with previous results involving chicken, ducks and mice [101, 156]. The abundance of the DE snoRNAs (44.4%) in the current study mirrors their significance in the host response to H5N1 virus infection (Fig. S1B in Manuscript V) [157]. Possible significances are that some snoRNAs might act as storage forms for miRNA precursors [158]. These nucleolar miRNAs mediate functions such as ribosomal protein modification or play roles in the late stages of ribosome biosynthesis [159]. The expressed and the DE snRNAs and rRNAs were present in low abundance in all libraries (Fig. S1A and B in Manuscript V).

Trends and patterns of DE miRNAs in H5N1-infected duck lung versus brain
Since we observed differential replication kinetics and growth rate of A/chicken/Faquos/amn12/2011 (H5N1) virus in lungs and brain, I next addressed the question of whether the cellular miRNA response will also differ between these organs. Although the specific pathogen-free (SPF) brain displayed a higher number
of expressed miRNAs than the lung (Fig. S1A in Manuscript V), miRNA reprogramming triggered by H5N1 virus infection was enormous to the degree that the number of DE miRNAs turned out to be higher in infected lung than in brain (Fig. 3A and S3B in Manuscript V). This also holds true for the overall number of DE non-miRNA sncRNAs (Fig. 4A and B in Manuscript V). miRNA reprogramming started earlier in lung than in brain and peaked during the late infection phase (72-120 hpi) in both organs (Fig. 5 in Manuscript V). Since data from two independent experiments showed that viral transcription peaked much earlier (between 40-48 hpi in both organs, Fig. 1 in Manuscript V), it is likely that miRNA reprogramming that is lagged behind viral transcription is a result, not a cause, of viral infection. This observation has been reported previously in PR8 H1N1-infected murine model [102].

Common and unique miRNA signatures in H5N1-infected duck: miRNAs as potential biomarkers

In this profiling study, I identified a set of miRNAs that are common DE in H5N1-infected lung and brain (n=13) (Fig. 3C in Manuscript V). Among the common DE miRNAs, miR-205 and miR-215 exhibited a consistent DE pattern. Nevertheless, their degree of the DE and the corresponding time phase varied between lung and brain (Fig. 3D and S3A in Manuscript V). miR-205 accounted for the highest up-regulated miRNAs in lung especially at 96 hpi. Both miRNAs formed single clusters in the PCA analysis (Fig. S3B in Manuscript V). Previous publication reported down regulation of miR-205 in H5N1-infected thymus, bursa and spleen of chicken and duck [101]. It was also found that miR-205 can target PB1, PB1-F2 of H5N1 genome [160]. miR-215 has been shown to be down regulated in the peripheral blood mononuclear cells of pandemic 2009 H1N1 patients [83]. This highlights their importance in H5N1 pathogenesis and raise possibilities of utilizing them as biomarkers. I also identified miRNAs that are unique DE in each organ (n=67 in lung, n=5 in brain) (Fig. 3C, 6A and 7A in Manuscript V). In lung, miR-125b and miR-130a constitute significantly expressed miRNAs in H5N1 infection irrespective of the time of infection as they were unique DE at all time points (Fig. S4A and 6A). This applied similarly for miR-183 in brain, which clustered alone in the PCA plot (Fig. 7A and B in Manuscript V). The observation that miR-183 was down regulated in our
experiment agrees with that reported by Zhao et al. who documented a similar pattern in brain of mice experimentally infected with rabies virus, indicating a general DE pattern in RNA viruses. The other unique miRNAs that are DE at certain time points in both organs are likely involved in fine tuning genes associated with the host response that exhibit temporal pattern such as immune-related genes [52]. Taken together, these data indicated that certain miRNAs might be good candidates as biomarkers for the organ-specific response and the associated clinical outcome in H5N1-infected ducks. The cluster analysis uncovered that the miRNA response in lung might be mediated at the individual level (more subclusters were identified) rather than as a cooperative interaction with other miRNAs. In contrast. Fewer subclusters were identified in brain, suggesting a higher degree of DE miRNAs co-regulation in this organ. The observation that higher clustering of the DE miRNAs occurred at 4 hpi and 5 hpi in lung and brain, respectively supports the notion that H5N1 virus induces earlier miRNA response in lung compared to brain.

Putative function of the DE miRNAs

As expected, the increased number of DE miRNAs in lung compared to brain was reflected on the numbers of mRNA targets of the DE miRNAs. In this regard, there were more putative mRNA targets identified in lung (n=481) than in brain (n=78) (Fig. 8 in Manuscript V). These mRNA targets were also more enriched in the late infection phase in both organs (Fig. S4B in Manuscript V). Obviously, the question of whether the infection-caused induction of organ-specific miRNAs is a part of the anti-viral host response or a virus-operated strategy to pave the way for established infection needs experimental validation. Nevertheless, correlating data about the organ-unique DE miRNA mRNA pairing might help unravel this puzzle. For instance, miR-145 and miR-125b, which were unique DE in lung (Fig. 6A in Manuscript V) were prediced to target the zinc finger protein genes; ZFYVE19 and ZBTB8OS, respectively. These targets were also uniquely found in lung (Table 3 in Manuscript V). Zinc finger proteins have previously been reported to mediate type-I IFN anti-viral responses in seasonal influenza H1N1 virus [151] and sindbis virus [161] infection. Therefore, it is hypothesized that the induction of these miRNAs in lung is driven by H5N1 virus to assist blocking host immunity. In contrast, miR-194, miR-
20b, miR-106 and miR-146, which were also unique DE in lung (Fig. 6A in Manuscript V) can target protein tyrosin phosphatase (PTPN2), a suppressor of interferon response [162, 163] (Table 3 in Manuscript V) thus supports the notion that this a host response geared toward augmenting the initiation of IFN-based antiviral activity. In summary, this part of the thesis illustrates the divergent roles played by miRNAs in lung and brain and advocates the notion that miRNAs might constitute biomarkers in the organ-specific response to H5N1 infection in ducks.

Putative regulatory circuits of DE miRNAs

As key participants in regulating gene expression, miRNAs can influence various biological networks [164]. The percentage of genes associated with each GO term or enriched pathway can indicate how significant is this Go term or pathway in the dedicated analysis. I identified the GO terms that are related to the biological and immunological processes in infected duck lung and brain. Combining all time points, I identified common GO terms (e.g. T-helper cell differentiation and positive regulation of JUN kinase activity) that were enriched in both organs. However, GO terms associated with neutrophil migration and myeloid cell differentiation were exclusively enriched in brain (Table S3 in Manuscript V), suggesting differential regulation of miRNA activities in the two organ. With regard to the enriched KEGG pathways, although a set of 32 KEGG pathways were commonly predicted to be enriched in lung and brain, for most of them, the percentage of associated genes were higher in lung than in brain with an overall percentages of 13.3% and 10.5% in lung and brain, respectively (Fig. 9 in Manuscript V). This argues that more miRNAs, and hence mRNAs, are implicated in regulating the biological network in H5N1-infected lung compared to brain. Many of these pathways are related to IAV infection. As one example of many, Notch signaling pathway constitutes the bridge between antigen presenting cells (APCs) and T cells. Mice deficient in notch signaling on macrophages exhibited high mortality, increased body weight loss and decreased IFN-γ production [165]. Of note, apoptosis signaling was predicted in lung at early phase, but only in the late infection phase in brain and also, as expected, with a lower percentage of associated genes (7.8% compared to 12.5% in lung). Coupling these findings with the semi-quantitative scoring of Casp 3 in both organs (Fig. 2 in
Manuscript V) suggests that H5N1 virus infection is associated with an early induction of apoptosis in lung, possibly as a defense mechanism to restrict viral spread [166], whereas apoptosis in brain likely occurs later, possibly as a virus-induced effect to support its replication [167]. I also identified unique enriched KEGG pathways in lung. Among these, IAV life cycle, Toll-like receptor signaling and NOD-like receptor pathways were identified (Fig. S5 in Manuscript V). These pathways were found previously to be related to IAV pathogenesis [168].

HLTE as a model to study the potential of miRNAs as biomarkers for IAV virulence (Section 4.2 in Unpublished data)

Despite the extensive use of lab animals and in vitro assays in human medicine, the overall success rate of transmitting the outcomes of these experiment directly to human benefit remains low. One prominent reason lies in the non-human background of the laboratory animals. Another reason is that some of the in vitro methods (e.g. cell lines and primary cells) lack the complexity of the human system. In this part of my thesis, I am establishing the HLTE model in order to later investigate the role of host-encoded miRNAs in discriminating between infection with pandemic 2009 H1N1 strains of variable virulence (see section 4.2 in Unpublished data).

HLTE support the infection with S-OIV wt

The HLTE system has proved to be infectable with S-OIV wt if used at a dose of $10^6$ FFU, but not lower (Fig. 6 in Unpublished data). The differences among establishment protocols, virus strains and the tissue condition might account for the variation among studies in the appropriate IAV dose that should be used to infect HLTE. The findings that S-OIV wt primed an immune response in the HLTE (Fig. 7 in the Unpublished data) agrees with other studies that involved human dendritic cells (DCs), macrophages [169] and pandemic 2009 H1N1 in A549 cells [123]. Autopsy samples from 50 patients with confirmed 2009 pandemic H1N1 infection indicated a remarkable elevation of IL-1RA, IL-6, IL-8, TNF-α and IP-10 mRNAs [170]. Thus, it can be reasonably assumed that induction of cytokines, which
correlates with virus gene expression, might be an extensive host response to S-OIV wt, in particular in fatal cases.

Replication competence of S-OIV wt and S-OIV NS PR8 in the HLTE model

Infection with IAVs of differential virulence has been reported to impact the miRNA profile [97, 100], suggesting potential roles of miRNAs as diagnostic and prognostic markers. It has been shown previously that S-OIV NS PR8 replicates at a higher rate than the S-OIV wt. This was coupled with a higher cytokine response in A549 and NPtTr (newborn pig trachea) cell lines (Peterson H. Et al., Manuscript in preparation). Along the same line, miR-223-3p was found to be more strongly induced upon infection of A549 cells with S-OIV NS PR8 if compared to the S-OIV wt. Whereas miR-155 showed the reverse pattern (Fig. 6a in Manuscript III).

Although these results provide hints about the impact of reassortment with the NS segment on the H1N1 pdm09, it remains important to investigate these differences in an established HLTE model.

At 8, 24 and 48 hpi, compared to the S-OIV wt, S-OIV NS PR8 had a higher transcription rate (Fig. 10 in the Unpublished results). This correlates with the level of IFN-α mRNA and the released IFN-α protein levels (Fig. 11 and 12 in the Unpublished results). In terms of tissue damage, the overall semi-quantitative scoring did not discriminate between both viruses or even between infected and uninfected tissue (Fig. 14 in Unpublished results). In contrast, endothelial damage and epithelial delamination were more prominent in S-OIV NS PR8 than in S-OIV wt infection, indicating that the a more aggressive infection might be caused by the reassortment of the NS segment. This might lead to a stronger induction of cytokines and IFN responses, as reported in macrophages infected with H5N1 virus [135].

Multi-organ involvement of pandemic 2009 H1N1 has been reported previously in fatal human cases [171] where the virus caused severe damage in lung, liver, heart and muscle tissues. Furthermore, Rodriguez et al. used an in vivo mouse model and cell lines to show that 2009 H1N1 strains isolated from fatal human cases replicated faster and induced higher cytokine levels than the strains isolated from mild cases. Moreover the strains isolated from fatal cases caused higher mortality and morbidity rate in mice than the ones isolated from the mild cases [172]. Taken together, my
own results support these previous observations that H1N1 strains can differ in virulence even when they differ in only one segment and that the HLTE model can be used to investigate both virus- and host-associated markers for progression of IAV infection, provided that artifacts due to aging of the tissue during culture are considered.

6. Conclusions and outlook

Summing up the results, it can be concluded that miRNAs are important mediators in the host response to IAV infection in mammals and in the natural host. My initial literature search clearly highlights played by miRNAs in both pathogenesis and clinical outcome of many infectious viral diseases that affect animal population. Furthermore, the ongoing laboratory trials suggests that the clinical use of small non-coding RNAs (miRNAs and siRNAs) in combatting animal viruses may be possible in the not too distant future.

While RNA-seq is highly sensitive in determining the level of miRNA expression, RT-qPCR technique has frequently been used to validate the results of RNA-seq. In my experiments, I found that results from both techniques agreed largely, particularly when considering the FC values of the miRNAs rather than the direction of their deregulation. In this case, matching results from both assays might be biased by the differences in the pre-defined FC cutoffs in both techniques. Since in the RNA-se analysis, one can obtain a large number of previously non-annotated reads that might correspond to novel miRNAs, it will be more interesting and valuable to use RT-qPCR to confirm the expression of these novel predicted miRNAs and to continue further by depositing them in public databases, such as miRBase.

This work proposed a cell-specific pattern of miRNA expression. Based on the results, I could assume that the miRNAs response to IAV in human lung is likely operates at the cellular level. Given the negative correlation between miRNAs and their target mRNAs, cell-specific miRNA signature can be used to predict the expression of cell-specific mRNAs. Studying these phenomena in a co-culture model comprising different cells will allow dissecting the role of miRNAs in cell-cell
communication. An open research question is why these miRNAs are expressed in one cell, but not the other. Whether this is corresponds to the miRNA transcription factors or other cell associated mechanisms, remained to be defined. Studying the phenomena.

In this study, I observed that reassortment of NS segment caused an alteration in miR-223 and miR-155 levels in A549 and dTHP-1 cells, respectively. This mirrors the influence of IAV reassortment on miRNA expression. It will be interesting to study the effect of reassortment by more than one IAV segment on miRNA reprogramming. Such information will be ameliorate our understanding of increased IAV virulence in humans.

There is a need to study the response to pathogens in their natural host, because this will assist understanding the mechanism behind the adaptation of these pathogens to humans. In this study, I analyzed the miRNAs response to the HPAI H5N1 in its natural host, the duck. The lung-specific preference of virus replication was associated with a more intense miRNA reprogramming and predicted function. I could identify a set of miRNAs that are unique to lung and brain. Those unique molecules might represent candidate biomarkers. As a future perspective, duck-derived lung and brain cell lines and/or primary cells could be used to validate the function of the identified unique and common miRNA in relation to virus transcription and other immune-related pathways. This can be done using miRNAs overexpression, knock down and reporter assays. Another area of research would be to compare the miRNAs response to HPAI H5N1 in ducks with that of human. This will provide invaluable insights into the mechanism of birds-to-human transmission of the virus and elucidate the host elements that are needed for the virus adaptation.

Despite the wide use of laboratory animals and in vitro assays to uncover different mechanisms behind IAV virulence, these approaches may not be conventional in all situations, particularly if it fails to reproduce the disease status that is seen in humans. The current work proposes the usefulness of the HLTE model in studying IAVs-host interaction. The system can support viral replication, which was associated with mounting an inflammatory response. Using this model to compare between miRNA DE in response to multiple IAV variants, which have differential
virulence, will help elucidating miRNAs role in IAVs-host cross talk and will pave the way for the future use of miRNAs as candidate biomarkers. Clearly, including more biological replicate in our experiments is required to minimize the variability among human donors. Further steps are needed to continue establishing this model before it can be used for miRNA studies.

In conclusion, the results of this study clearly suggest the involvement of miRNAs in the host response to IAV in mammals and in avian host. Furthermore, the identified cell- and organ-specific miRNAs highlight the possibilities of using miRNAs as candidate biomarkers. As a next step, experimental validation will be important to unravel different roles played by these miRNAs in IAV-host interaction.

7. References


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8. Affidavit

I herewith declare that I autonomously carried out the PhD-thesis entitled “MicroRNAs as biomarkers in the host response to influenza A virus infection in humans and animals”

No third party assistance has been used.

I did not receive any assistance in return for payment by consulting agencies or any other person. No one received any kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis.

I conducted the project at the following institution

*Institute of Experimental Infection Research. TWINCORE, Center for Experimental and Clinical Infection Research, GmbH.*

The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context.

I hereby affirm the above statements to be complete and true to the best of my knowledge.

15.02.2016, Mohamed Samir Ahmed
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27.03.2016, Hannover, Germany
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Publication

