RNAi AND miRNA PATHWAYS IN BIRDS

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PETR SVOBODA

Institute of Molecular Genetics, Academy of Sciences of the Czech Republic,
Videnska 1083, 142 20 Prague 4, Czech Republic

Correspondence to: Petr Svoboda, Institute of Molecular Genetics ASCR,
Videnska 1083, 142 20 Prague 4, Czech Republic, tel. # +420 241063147,
e-mail: svobodap@img.cas.cz.

ABSTRACT

RNA silencing denotes sequence-specific repression mediated by small RNAs. In vertebrates, there are two closely related pathways, which share several protein factors: RNA interference (RNAi) and microRNA (miRNA) pathway. The miRNA pathway regulates endogenous protein-coding gene expression and has been implicated in many biological processes. RNAi generally serves as a form of innate immunity targeting viruses and mobile elements. This text reviews miRNA and RNAi pathways in birds. Although the available literature on RNA silencing in birds is very limited, many features can be deduced from the genomic data in the public domain. miRNA, RNAi and other dsRNA-responding pathways in birds appear very much like those in mammals, important bird-specific features of RNA silencing pathways are yet to be identified. The miRNA pathway is likely the dominant small RNA pathway while the existence and functionality of endogenous RNAi remains unclear. Some variations may be present in the main bird antiviral interferon system.

Introduction

Birds (*Aves*) belong together with mammals and fishes to the group *Craniata* within chordates. Some of the birds are of high economic importance (food industry) or medical relevance (viral vectors causing zoonoses). Bird ancestors branched of mammalian ancestors over 300 MYA when the synapsid lineage leading to mammals branched of the sauropsid lineage leading to dinosaurs and birds. There are ~9000 extant bird species (Margulis and Schwartz, 1998). During their evolution, birds evolved numerous physiological adaptations in which they differ from mammals, including feathers, shelled eggs with external development, or different sex chromosome system, to name a few. At the same time, they are the closest mammal-related group covered in this series, in terms of synteny and sequence similarity. This is useful for assessing features of dsRNA and miRNA pathways because the available literature on RNA silencing in birds is very limited. However, many features can be deduced from the genomic data in the public domain. miRNA, RNAi and other

dsRNA-responding pathways in birds are very much like those in mammals and the literature does not report an important bird-specific feature in RNA silencing pathways. Since mechanistical principles of vertebrate miRNA and RNAi pathways were introduced in the first two reviews of this series (Svoboda, 2019a, b) and in further detail elsewhere (Bartel, 2018; Svoboda, 2014), I will focus here directly on features of these pathways described for birds.

Dicer

According to the complete genome sequences of chicken and Zebra Finch, birds have one Dicer protein. Chicken Dicer has been assigned to the chromosome 5 according to the radiation hybrid mapping (Tian et al., 2007) which is in agreement with the current chicken genome map. There is no detailed analysis of avian Dicer specificity and activity, which have to be inferred indirectly from other results. Chicken Dicer can process both, long dsRNA and miRNA precursors, as evidenced by induction of RNAi with long dsRNA (Mauti et al., 2008; Pekarik et al., 2003) and hundreds of avian miRNAs in the miRBase.

The common Dicer product size seems to be 21–23nt with a typical size of 22nt. This information can be inferred from available miRBase data (Fig. 1). Thus, the avian Dicer produces small RNAs with the same sizes as the mammalian Dicer (Fig. 1). Another possible substrate of Dicer in birds might be snoRNAs, although the biological significance of this observation remains unclear (Taft et al., 2009).

It is unclear if there are functionally different avian Dicer isoforms as is the case in murine oocytes and somatic cells (Flemr et al., 2013). There is one report of different Dicer splice variant in goose (*Anser cygnoides*) where one variant lacks a linker between DEAD box and helicase C domains at the N-terminus (gDicer-b) (Hu et al., 2014). The shorter isoform gDicer-b is present in multiple tissues, however its functional significance is unclear. The truncation is found in the N-terminus, which is associated with substrate selectivity and efficient processing. Therefore, one might speculate about some functional divergence in substrate processing between the two isoforms. However, there is no experimental evidence at the moment. The only available data, so far, concern cloning of the short isoform and expression analysis of several tissues and follicular stages by RT-PCR (Hu et al., 2014).

dsRBPs

dsRBP binding partners of Dicer have not been studied, so far. Interestingly, the chicken genome contains a dsRBP, which is related to TARBP2 and PACT, suggesting a more ancestral vertebrate state and a reduced crosstalk between RNAi and the interferon pathway.

Argonaute proteins

Argonaute family proteins are effectors of RNA silencing mechanisms. They are divided into two subfamilies: AGO proteins, which accommodate miRNAs and siRNAs, and PIWI

proteins, which accommodate piRNAs. Avian AGO proteins have not been characterized in a published report but public chicken genome data show that the setup is the same as in mammals: Studies in chicken revealed four AGO proteins, where AGO1, 3, and 4 are encoded within one locus on chromosome 23 and AGO2 is encoded separately on chromosome 2. This arrangement appears to be shared within mammals and birds (Zhou et al., 2010). Additional information about avian AGOs can be inferred indirectly from the existence of functional RNAi and miRNA pathways (discussed below), which implies that at least one AGO protein is a "slicer" (presumably AGO2, given its conserved role as a slicer from *Drosophila* to mammals). Avian AGO proteins can also mediate post-transcriptional silencing guided by imperfectly base paired miRNAs.

In addition, there were two publications found, which mention avian PIWI proteins, which primarily control genome integrity in the germline and are not within the scope of this report (Kim et al., 2012; Lim et al., 2013).

Other factors

Birds have additional proteins involved in other dsRNA responses, which are either associated with adenosine deamination (Herbert et al., 1995) or interferon response. Interferon response factors, which recognize some form of dsRNA and are also found in mammals, include MDA5 (Hayashi et al., 2014; Lee et al., 2012, 2014), RIG-I (Chen et al., 2015; Li et al., 2014a; Xu et al., 2015), and PKR (Gonzalez-Lopez et al., 2003; Lostale-Seijo et al., 2016; Zhang et al., 2014). Interestingly, chicken lack the RHA/DHX9 homolog (Sato et al., 2015). The antiviral response to dsRNA will be discussed further below.

miRNA pathway

According to miRBase (Kozomara and Griffiths-Jones, 2014), bird genomes encode hundreds of miRNAs (Table 1) During the systematic literature review, miRNA-related publications lacking a mechanistic molecular insight into the miRNA pathway were the most common class of annotated publications for birds (~50% of all selected publications). These publications fall into four basic categories:

a) annotations of novel miRNAs, including high-throughput expression analyses (for example (Godnic et al., 2013; Luo et al., 2012; Taft et al., 2009) and many others). This category also includes the original chicken and Zebra Finch genome annotation papers (International Chicken Genome Sequencing, 2004; Warren et al., 2010).
b) studies of miRNAs in different biological contexts, including reproduction (Lee et al., 2015; Lee et al., 2011), skeletomuscular apparatus (Chen et al., 2009a), bird song physiology (Gunaratne et al., 2011), growth/weight gain (Li et al., 2013), and many others; their comprehensive listing would be beyond the scope of this report.
c) studies of relationship between miRNAs and the immune system, especially antiviral – these will be discussed further below in the section 3.1.2.7. Other dsRNA response pathways

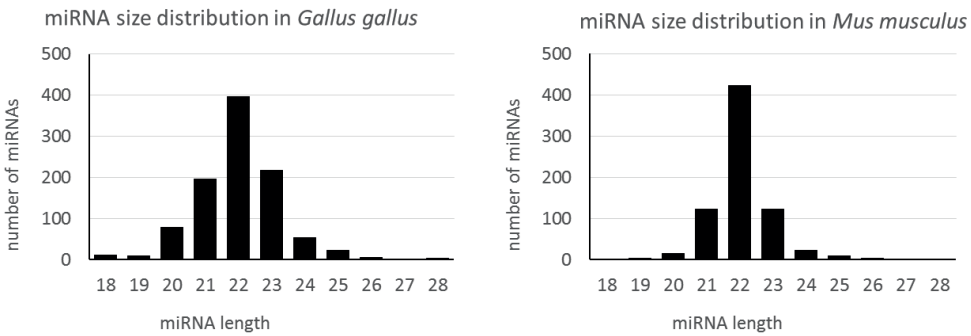

Figure 1 Avian miRNA lengths
The left graph depicts size distribution of all 994 chicken miRNAs deposited in the miRBase (release 21). For comparison, the right graph shows size distribution of 721 high-confidence murine miRNAs.

d) false positives of the search- reports describing mRNA knock-down through short hairpin RNAs adopting miRNA-like appearance. There is a series of nearly identical methodological papers, apparently published twice in 2006 and 2013, which fall in this category (Deng et al., 2015; Lin et al., 2006a; Lin et al., 2006b; Lin and Ying, 2006; Lin et al., 2013a; Lin et al., 2013b; Lin and Ying, 2013; Ying and Lin, 2009; Ying et al., 2010) and several other publications concerning development and adaptations of shRNA systems (e.g. (Andermatt et al., 2014; Chen et al., 2011; Das et al., 2006). These articles actually belong to the RNAi section below but due to the confusing use of nomenclature, they would also fall into the miRNA category.

Taken together, essentially all miRNA-related publications dealt with miRNA annotation, analysis of biological functions of miRNAs, and adoption of miRNAs for artificial knock-down systems allowing for suppressing any gene of interest. Avian miRNA-related publications did not reveal any avian-specific mechanistic insight into miRNA biogenesis, in which birds would differ from the general consensus for mammals, or other vertebrates in general. The complete list of all miRNA-related publications is available in a library accompanying this section.

Table 1 Bird miRNAs in the miRBase (release 22.1 (Kozomara and Griffiths-Jones, 2014)):

species [genome annotation]	miRNA precursors	mature miRNA
Gallus gallus [Gallus-gallus-4.0]	882	1232
Taeniopygia guttata [taeGlu3.2.4]	247	334

RNAi

Avian RNAi-related literature deals mainly with experimental knock-down of gene expression, which does not reveal much about the physiological role of RNAi pathway in birds. These studies cannot all be included in the report due to the high number, but they are

available in the reference library accompanying this section). What can be inferred from those studies is that birds have the complete molecular mechanisms for canonical RNAi and can efficiently execute it. This is evidenced by efficient knock-downs with long dsRNA (Mauti et al., 2008; Pekarik et al., 2003).

Published exogenous RNAi data provide insights into possible routes nucleic acids can become biologically active in birds and concern areas of EFSA main interests as various forms of RNAi technology (siRNAs or transgenic) were considered a way for preventing virulent strain circulation in poultry (O'Neill, 2007) although results of these efforts were relatively modest, being typically developed in cultured cells (Hutcheson et al., 2015; Sahare et al., 2015; Stewart et al., 2011; Yin et al., 2010). Exogenous RNAi in vivo required non-physiological manipulations such as 1) plasmid or siRNA electroporation (Andermatt et al., 2014; Baeriswyl et al., 2008; Mauti et al., 2008; Pekarik et al., 2003; Sato et al., 2004; Wilson and Stoeckli, 2011, 2012), 2) transfection (Dai et al., 2005; Lin et al., 2006a; Lin et al., 2013a; Wei et al., 2015), 3) recombinant virus (Lambeth et al., 2009b), or 4) recombinant lentivirus delivery (Chen et al., 2009b; Haesler et al., 2007). Altogether, these data suggest that exogenous RNAi would not be achieved by just exposing birds to small RNAs or their precursors in the environment or food.

Regarding the endogenous RNAi, it remains what its physiological role is. There are three possible roles for endogenous RNAi: antiviral defense, genome defense against retrotransposons and control of gene expression. These roles would be associated with production of viral siRNAs, retrotransposon siRNAs and mRNA-targeting siRNAs in vivo. However, an unequivocal evidence for existence of these classes and their function was not provided yet.

One report attempted to examine the role of Dicer in retrotransposon repression. It was shown that the loss of Dicer in chicken cells does not result in accumulation of chicken CR1 retrotransposon while introduction of a human L1 element into cells lacking Dicer results in accumulation of L1 transcripts and increased retrotransposition (Lee et al., 2009). However, these data are difficult to interpret as different scenarios could lead to the same observations, especially downstream effects of a perturbed miRNA pathway and chromatin-mediated silencing of CR1.

Other dsRNA response pathways

Chromatin regulation by small RNAs

Two studies involving bird models brought up a possible nuclear function of Dicer and its link to chromatin regulation, which is of the unsettled issues in vertebrate models. Despite a decade of research, there is still no proposed molecular mechanism explaining these phenomena while the literature contains a number of contradicting observations.

Fukagawa et al. produced a conditional loss-of-function Dicer mutant in a chicken-human hybrid DT40 cell line that contains human chromosome 21. The loss of Dicer resulted in cell death and accumulation of premature sister chromatid separation. Furthermore, aberrant accumulation of transcripts from human centromeric repeats was also found suggesting

loss of heterochromatin at centromeres. While localization of two heterochromatin proteins (Rad21 and BubR1) was abnormal, localization of core centromeric heterochromatin proteins CENP-A and -C was normal (Fukagawa et al., 2004). Although the article is highly cited (335 times up to date according to WOS core collection), the molecular mechanism of the effect remains elusive. It is possible that the phenomenon is an indirect consequence of perturbing the miRNA pathway. Furthermore, the model system is unique and human heterochromatin sequences might exhibit unusual behaviour in the chicken nuclear environment.

Giles et al. examined a 16 kilobase (kb) heterochromatin domain in the chicken erythroid progenitor cell line 6C2. RNAi-mediated downregulation of the enzyme Dicer resulted in increased histone acetylation and transcript levels from the heterochromatin locus while compact chromatin structure became more accessible to restriction endonucleases. It was also shown that chicken AGO2 homolog binds the 16 kb region in a Dicer-dependent manner and is necessary for a condensed chromatin structure (Giles et al., 2010). The article has been cited 26 times up to date according to WOS (core collection), yet there was no follow up providing any mechanistic explanation of the phenomenon. It is possible that the observed effects could be an indirect effect of suppression of the miRNA pathway or even an experimental artefact. Additional controls and experiments would be needed to address these concerns and clarify inconsistencies with other reports. Therefore, this report should be considered an interesting observation without a clear mechanistic explanation.

Taken together, small RNA-mediated chromatin changes in birds remain an open question. Without knowing the molecular mechanism, especially that of biogenesis of small RNAs regulating chromatin and their mode of action, there is simply not enough information for qualified conclusions.

Antiviral defense – interferon response and crosstalk with RNA silencing

Many studies deal with various aspects of viral infections in birds or avian cells. The most studied model for viral infections in birds is Marek's disease, which is a consequence of a Herpesvirus infection in poultry. Publications linked to Marek's disease addressed virus encoded miRNAs (Coupeau et al., 2012; Luo et al., 2011; Morgan et al., 2008; Muylkens et al., 2010; Strassheim et al., 2012; Xu et al., 2011; Yao et al., 2008; Zhao et al., 2011; Zhao et al., 2009), changes in host miRNA expression during infection (Dinh et al., 2014; Han et al., 2016; Lambeth et al., 2009a; Li et al., 2014b; Li et al., 2014c; Lian et al., 2015a; Lian et al., 2015b; Stik et al., 2013; Tian et al., 2012; Xu et al., 2010; Yao et al., 2008), or attempts to block the virus with RNAi (Chen et al., 2009b; Chen et al., 2008; Lambeth et al., 2009b). A similar set of articles has been found for other studied viruses infecting birds – e.g. avian influenza virus H5N1 and H9N2, bursal disease virus, subgroup J avian leucosis virus. The complete list is available in the library accompanying this section.

Reports concerning host and virus-encoded miRNAs generally represent adaptations manipulating the miRNA pathway for the benefit of the pathogen. At the same time, these articles did not reveal some unique adaptation of the chicken miRNA pathway, which would differ from molecular mechanisms and principles described in the previous section.

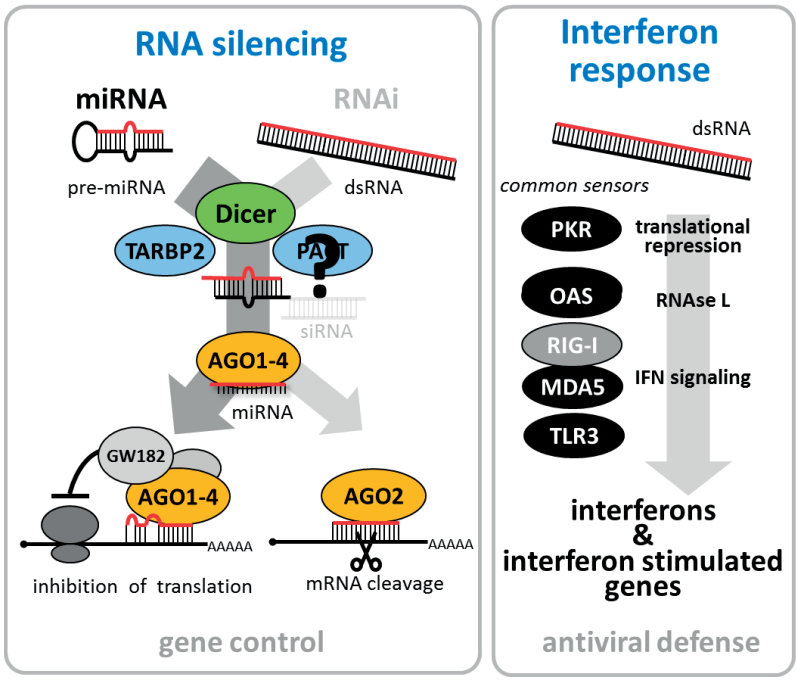

Figure 2 Overview of avian pathways
dsRNA and miRNA pathways in birds are very much similar to the mammalian ones with some minor exceptions. Birds have only a single dsRBP homologous to TARBP2, and lack PACT ortholog.

The last group of articles reviewed here represent publications covering the interferon system, the common antiviral system induced by dsRNA and other RNA species (Karpala et al., 2008; Kint et al., 2015; Lostale-Seijo et al., 2016). Birds generally utilize the same antiviral interferon system including its key dsRNA sensing proteins: PKR (Gonzalez-Lopez et al., 2003; Lostale-Seijo et al., 2016; Zhang et al., 2014), RIG-I (Chen et al., 2015; Li et al., 2014a; Xu et al., 2015), and MDA5 (Hayashi et al., 2014; Lee et al., 2012, 2014), 2',5'-OAS (Lee et al., 2014; Villanueva et al., 2011). However, there seem to be some species-specific variations. For example, RIG-I is found in some birds, such as ducks or pigeons (Chen et al., 2015; Xu et al., 2015) but not in chicken, which lack RIG-I and the RNA sensing RHA/DHX9 helicase homolog (Sato et al., 2015). Although the lack of RIG-I is partially compensated by chicken MDA5 activity (Hayashi et al., 2014; Karpala et al., 2011) the absence of RIG-I-like function may contribute to the chicken's susceptibility to highly pathogenic influenza (Karpala et al., 2011; Li et al., 2014a).

Adenosine deamination

Birds have also adenosine deaminases that act on RNA (Herbert et al., 1995) but their physiological significance in birds is unknown at the moment.

Summary

In terms of the mode-of-action of dsRNA and miRNA pathways, birds are closely resembling mammals despite over 300 millions of years of separate evolution. The molecular mechanism of RNAi and miRNA pathways seems to be essentially identical to that of mammals except of a single dsRBD instead of two different ones. The significance of this difference is unclear. The miRNA pathway seems to be the dominant small RNA pathway while the existence and functionality of endogenous RNAi remains unclear. Some variations were found in the interferon system (lack of RIG-I in chicken), which appears to be the main antiviral system in birds.

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