RNAi AND miRNA PATHWAYS IN MAMMALS II – BIOLOGICAL ROLES

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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 mammals, 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. It has been implicated in many biological processes and majority of mammalian genes appear to be directly or indirectly exposed to miRNA-mediated regulations. RNAi generally serves as a form of innate immunity targeting viruses and mobile elements, although it occasionally also acquired function in protein-coding gene regulation. The function of RNAi in mammals is still poorly understood but it is clear that proteins supporting RNAi are also involved in miRNA biogenesis and function. Because of the large volume of the existing literature, the review of mammalian miRNA and RNAi pathways was divided into two parts, where first one reviewed components of the pathways and the second one, presented here, reviews roles and significance of the pathways.

Introduction

In the first part of the review off mammalian RNAi and miRNA pathways, I focused on mechanistic description of the pathways. Here, I will provide an overview of biological roles and biological phenomena associated with mammalian RNAi and miRNA pathways (Fig. 1).

miRNA-mediated control of gene expression – important functional aspects

The current miRBase (Kozomara and Griffiths-Jones, 2014) edition 22.1 annotates 1917 human miRNA loci that give rise to 2654 annotated miRNAs. There are 1234 precursors and

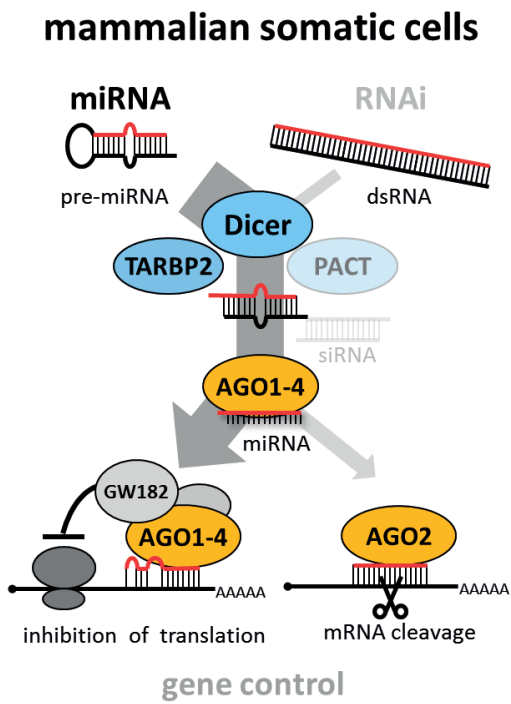

Figure 1 Mechanistical merging of miRNA and RNAi pathways in mammals

1978 mature miRNAs annotated in mouse. A simple connection of these counts with the fact that only nucleotides 2–8 of a miRNA are sufficient for target recognition and suppression implies that miRNA-mediated repression is a widespread and extremely evolvable regulatory system for gene expression. At the same time, one should not forget the above-mentioned stoichiometry between miRNAs and their target sites that is needed for efficient silencing.

Evolution of miRNAs is fast – there are only a few miRNAs conserved between *Drosophila* and mammals. Given the diversity of canonical and non-canonical miRNAs, it is conceivable that miRNAs to emerge from random formation of Drosha/Dicer substrates. Newly evolving miRNAs likely form a considerable portion of annotated miRNAs, especially in species where miRNAs are deeply sequenced and low-abundant miRNAs are identified.

According to the evolutionary theory, new miRNAs would either acquire function and become fixed during evolution or they would be lost. In addition, the target repertoire of existing miRNAs can also rapidly evolve since a single point mutation can weaken an existing regulation or create a new one. This idea is consistent with the data showing that mammalian mRNAs are under selective pressure to maintain and/or avoid specific 7-nucleotide seeding regions (Farh et al., 2005). It can be nicely exemplified on the Texel sheep phenotype where a single mutation creating a novel miRNA target site in myostatin causes the exceptional meatiness of this breed (Clop et al., 2006).

The set of miRNAs in each cell type forms a combinatorial post-transcriptional regulation system stabilizing gene expression pattern. miRNAs have widespread impact on expression and evolution of protein-coding genes (Farh et al., 2005). The number of mRNAs that have functionally important interaction with miRNAs (i.e. suppression of this interaction yields a phenotype) in a studied model system is presumably small and certainly difficult to discern among the possible interactions. Thus, every search for functionally important interactions between miRNAs and their targets has to face the fact that miRNAs represent a dynamically evolving system with countless random interactions, which are not biologically relevant.

Extracellular microRNAs

An interesting research field developed around the release of miRNAs from cells, detection of extracellular miRNAs, and transfer between cells. Importantly, the vast majority of the references provided descriptive and correlative data documenting presence of circulating miRNAs under different conditions (e.g. (Arroyo et al., 2011; Bellingham et al., 2012; Huang et al., 2013b; Luo et al., 2009; Novellino et al., 2012; Turchinovich et al., 2011). I will not review the bulk of the circulating RNA literature, which provides data concerning biomarker potential of circulating miRNAs, undoubtedly of extreme clinical relevance but of minimal relevance for this review. Below, I summarize results, which admittedly raise more questions than provide satisfactory answers.

Small RNAs can be transmitted from one cell to another under physiological conditions, as evidenced, for example, by systemic RNAi in arthropods or plants. Small RNAs can utilize dedicated transporters, common communication channels, or secretory vehicles. It was also reported that Gap junctions can serve for miRNA transfer from microvascular endothelial cells to colon cancer cells (Thuringer et al., 2016). Circulating mammalian miRNAs were reported 2008 when they were found in serum of lymphoma patients; they were immediately recognized as potential non-invasive biomarkers for cancer diagnostics and treatment (Lawrie et al., 2008). The same year, placental miRNAs were found circulating in maternal plasma (Chim et al., 2008), which was one of the discoveries leading to the notion that miRNAs could be a mobile regulating molecule (Iguchi et al., 2010) and that could even mediate transgenerational epigenetic heritance (Sharma, 2015) or be transmitted across species (Buck et al., 2014; Zhang et al., 2012). Since then, extracellular miRNAs were identified in a broad range of biological fluids, including plasma, aqueous humour, cerebrospinal fluid, nasal mucus, or milk (Baglio et al., 2015; Dismuke et al., 2015; Huang et al., 2013b; Izumi et al., 2015; Kropp et al., 2014; Pegtel et al., 2011; Wu et al., 2015a). miRNAs were identified in the cargo of exosomes, membranous vesicles 40 to 100 nm in diameter, which are constitutively released by almost all cell type and are found essentially in every biological fluid (reviewed, for example, in Rak, 2013; Yoon et al., 2014)

However, extracellular miRNAs do not need to be necessarily encapsulated in extracellular vesicles, as two studies showed that 95–99% of extracellular miRNA are not in extracellular vesicles but associated with AGO proteins in serum and cell culture media (Arroyo et al., 2011; Turchinovich et al., 2011). Furthermore, most individual exosomes in standard

preparations do not seem to contain biologically significant numbers of miRNAs (Chevillet et al., 2014). The molecular mechanism of miRNA release, either as a cargo in a vesicle or free, is poorly understood and the current knowledge does not allow for building a coherent model as the literature is scarce. Non-templated nucleotide additions were found to distinguish between cellular miRNAs, which were 3' end adenylated in cells whereas 3' end uridylated isoforms appeared overrepresented in exosomes suggesting a possible role of 3' terminal modifications in sorting miRNAs into extracellular vesicles (Koppers-Lalic et al., 2014). Recently, ALIX, an accessory protein of the endosomal sorting complex, it has been implicated in sorting miRNAs into extracellular vesicles based on its interaction with AGO2 and reduced miRNAs levels in extracellular vesicles upon *Alix* knock-down (Iavello et al., 2016).

Importantly, any model where miRNAs would be carried over to regulate gene expression by the canonical miRNA activity must face the kinetic data mentioned above (Wee et al., 2012). While one cannot exclude a non-canonical signalling function of circulating miRNAs (which has not been conclusively demonstrated yet), the literature on circulating RNAs may include misleading statements, which are unsupported by experimental evidence.

Taken together, while existence of circulating miRNAs has been demonstrated beyond a doubt, experimental evidence for their function (if any) is not conclusive. Exosomal vesicles can carry miRNAs and siRNAs – in the latter case, exosomes were adapted for a delivery tool for siRNAs, which has a good potential for further development of siRNA therapy (El-Andaloussi et al., 2012; Kumar et al., 2015; Lasser, 2012; Lee et al., 2012; Nguyen and Szoka, 2012; Shtam et al., 2013; Wahlgren et al., 2012; Wahlgren et al., 2016).

RNAi pathway in mammals– important functional aspects

It should reiterated that the, "so-called" RNAi knock-down with siRNAs in mammalian cells is essentially using the miRNA pathway with retained the ability to cleave perfectly complementary targets by AGO2. The mammalian canonical RNAi (i.e. long dsRNA-driven) is a dormant pathway, at best. By that is meant that the protein factors present in every mammalian cells (Dicer, TARBP2, and AGO2) are competent to support RNAi but long dsRNA does not efficiently induce RNAi in most mammalian cells (Nejepinska et al., 2012). This notion is supported by the reconstitution of human RNA interference in budding yeast demonstrates that Dicer, TARBP2, and AGO2 are sufficient to functionally reconstitute RNAi (Suk et al., 2011). This demonstrates that these three proteins constitute the essential core of RNAi mechanism although RNAi is not properly reconstituted when bona fide RNAi precursors were co-expressed (Wang et al., 2013). The problem is apparently at the level of Dicer processing as the human slicer AGO2 RNAi role is so conserved that it could function in RNAi in the early divergent protozoan *Trypanosoma brucei*, demonstrating conservation of basic features of the RNAi mechanism (Shi et al., 2006). In an analogous experiment, human AGO2 could not replace *Arabidopsis thaliana* AGO1 in the miRNA pathway (Deveson et al., 2013). In a sense, these different results are not that surprising considering the minimal requirements for RNAi and the complexity of the miRNA pathway, which provides a larger space for evolution of incompatible adaptations.

However, there are some cases indicating that RNAi is still active in mammals and, under unique circumstances, may be even an essential pathway.

The main bottleneck for canonical RNAi in mammals is efficient production of siRNAs from long dsRNA, which is poor in most mammalian cells (Flemr et al., 2013; Nejepinska et al., 2012). However, several reports showed that induction of RNAi with intracellular expression of long dsRNA can be achieved in transformed and primary somatic cells (Diallo et al., 2003; Elbashir et al., 2001; Gan et al., 2002; Shinagawa and Ishii, 2003; Tran et al., 2004; Yi et al., 2003). These data imply that RNAi can occur if there is a sufficient amount of long dsRNA, which is directed preferentially to RNAi but not into other dsRNA pathways. Under these circumstances, the limiting factor is just Dicer's ability to produce siRNA (Flemr et al., 2013).

Endogenous RNAi in the germline

Retrotransposon repression in mouse oocytes

RNAi-mediated mobile element silencing has also been documented in the mouse germline (Tam et al., 2008; Watanabe et al., 2006; Watanabe et al., 2008). Mutations in the piRNA pathway components are detrimental to sperm development, suggesting that piRNAs are the dominant class of small RNAs controlling mobile element activity in the male germline (reviewed in Toth et al., 2016). In contrast, female mice lacking functional piRNA pathway are fertile with no obvious defects in oocytes (Carmell et al., 2007). Endo-siRNAs suppress TEs silencing in mammalian oocytes as documented by derepression of some retrotransposons in oocytes depleted of Dicer or AGO2 (Murchison et al., 2007; Watanabe et al., 2008). As already proposed for invertebrates, the piRNA and endo-siRNA pathways likely cooperate in creating a complex silencing network against mobile elements in the mammalian germline. Long terminal repeat MT elements and SINE elements are strongly upregulated in *Dicer*$^{-/-}$ oocytes, while the levels of IAP transposon are elevated in the absence of MILI protein but not in *Dicer*$^{-/-}$ oocytes (Murchison et al., 2007; Watanabe et al., 2008). Still many loci composed of other types of TEs, e.g. LINE retrotransposons, give rise to both piRNAs and endo-siRNAs, again suggesting that the biogenesis of these small RNAs is interdependent. The role of endogenous RNAi in TE silencing extends from germ cells to preimplantation embryo stages. Apart from maternally derived piRNAs and endo-siRNAs, which persist in the embryos for a large part of preimplantation development, zygotic endo-siRNAs are generated *de novo* mainly to control the activity of zygotically activated MuERV-L retrotransposon (Ohnishi et al., 2010; Svoboda et al., 2004). SINE-derived endo-siRNAs also increase in abundance in early embryo stages, which is consistent with the observation that B1/Alu SINE endo-siRNAs account for a vast majority of endo-siRNAs sequenced from mouse ES cells (mESCs) (Babiarz et al., 2008). Whether these SINE endo-siRNAs play an active role in TE silencing in mESCs similarly to other TE-derived endo-siRNAs in oocytes remains to be determined. RNAi-dependent silencing of LINE transposons has also been described in cultured HeLa cells, where endo-siRNAs derived from bidirectional transcripts of sense and antisense L1 promoter were proposed to control L1 activity (Yang and Kazazian, 2006). Although some evidence for retrotransposon-derived endo-siRNAs

from mammalian somatic cells was obtained from deep sequencing data (Kawaji et al., 2008), a convincing support for the function of endo-siRNAs in TE silencing in mammalian somatic tissues, has yet to be provided.

Control of endogenous genes in mouse oocytes

In mice, perturbation of the endo-siRNA pathway in oocytes is responsible for severe meiotic defects and resulting female infertility. Targeted oocyte-specific knockout of both *Dicer* and *Ago2* lead to similar phenotypes including chromosome misalignment and defective spindle (Kaneda et al., 2009; Murchison et al., 2007; Tang et al., 2007). These effects were originally attributed to the loss of maternal miRNAs. However, miRNA pathway is suppressed in mouse oocytes and oocytes lacking *Dgcr8*, which is required for canonical miRNA biogenesis, can be fertilized and do not show any significant disturbance of the transcriptome (Ma et al., 2010; Suh et al., 2010). This means that the canonical miRNA pathway is non-essential and largely inactive in mouse oocytes despite intact biogenesis of miRNAs (Fig. 2). In fact, the spindle phenotype is caused by the loss of a highly active RNAi pathway in mouse oocytes. High-throughput analysis of small RNAs in mouse oocytes revealed a unique class of endo-siRNAs derived from processed pseudogenes (Tam et al., 2008; Watanabe et al., 2008). Transcriptomes of oocytes lacking *Dicer* and *Ago2* (including oocytes expressing catalytically-dead AGO2) are similarly affected (Kaneda et al., 2009; Stein et al., 2015). At the same time, genes matching pseudogene-derived endo-siRNAs are enriched in the group of upregulated genes in both knockouts (Kaneda et al., 2009; Stein et al., 2015; Tam et al., 2008; Watanabe et al., 2008).

In addition, putative endo-siRNA targets are enriched in cell cycle regulators and genes involved in microtubule organization and dynamics (Tam et al., 2008). These findings suggest that regulation of protein-coding genes by endo-siRNAs controls the equilibrium of protein factors required for proper spindle formation, chromosome segregation and meiosis progression in mouse oocytes. As pseudogenes are rapidly evolving source of dsRNA for endo-siRNA production, it will be interesting to investigate whether the role of RNAi in spindle formation during meiotic maturation of oocytes is conserved in mammals.

The reason for high levels of endo-siRNAs and the high RNAi activity in mouse oocytes is the aforementioned truncated Dicer isoform that lacks the N-terminal helicase domain (Flemr et al., 2013) (Fig. 2). It efficiently generates siRNAs from long dsRNAs, and is sufficient for enhancing RNAi in cultured cells while its loss in mouse oocytes yields the same phenotype as conditional knock-outs of *Dicer* or *Ago2* (Flemr et al., 2013).

Endo-siRNAs have also been proposed to contribute to the self-renewal and proliferation of mouse embryonic stem cells (mESCs), since the proliferation and differentiation defects observed in *Dicer*$^{-/-}$ mESCs are more dramatic than in *Dgcr8*$^{-/-}$ mESCs (Kanellopoulou et al., 2005; Murchison et al., 2005; Wang et al., 2007). A population of endo-siRNAs derived mostly from hairpin forming B1/Alu subclass of SINE elements was identified in mESCs (Babiarz et al., 2008). Fragments of SINE elements are commonly present in untranslated regions of protein-coding transcripts and it is therefore possible that SINE-derived endo-siRNAs participate in posttranscriptional gene silencing in mESCs. However, this hypothesis has not been tested experimentally.

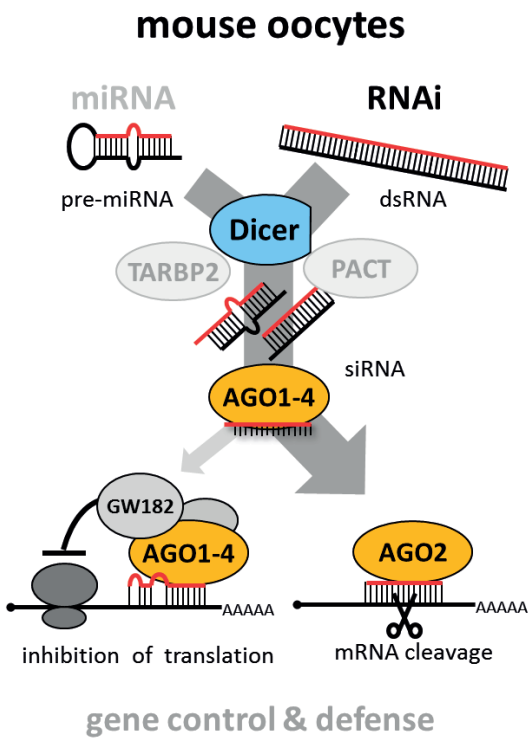

Figure 2 miRNA & RNA arrangement in mouse oocytes

Endogenous RNAi in the soma

Little evidence is available for potential role of endo-siRNAs in the regulation of protein-coding mRNAs in mammalian somatic tissues. The natural antisense transcription in somatic cells, which has a potential to generate dsRNA, yields low levels of endo-siRNAs, whose biological relevance is questionable. At the same time, endo-siRNAs derived from natural antisense transcripts of *Slc34a* gene were identified in mouse kidney, where Na/phosphate cotransporter exerts its physiological function (Carlile et al., 2009). However, changes in expression levels of *Slc34a* upon suppression of the endo-siRNA pathway have not been addressed. In mouse hippocampus, deep sequencing revealed a set of potential endo-siRNAs generated from overlapping sense/antisense transcripts and from hairpin structures within introns of protein-coding genes (Smalheiser et al., 2011) . The most abundant endo-siRNAs from *SynGAP1* gene locus were also found in complexes with AGO proteins and FMRP *in vivo*. Interestingly, a large part of potential hippocampal endo-siRNA targets encode for proteins involved in the control of synaptic plasticity and the number of endo-siRNAs derived from these gene loci increased significantly during olfactory discrimination training (Smalheiser et al., 2011). Given the fact that vast majority of identified endo-siRNA sequences mapped to intronic regions, the endo-siRNAs could

act co-transcriptionally on nuclear pre-mRNAs, perhaps similarly to the mechanism of RNAi-mediated inhibition of RNA Pol II elongation described in *C. elegans* (Guang et al., 2010). Alternatively, endo-siRNAs could control correct distribution of target mRNAs as unspliced pre-mRNA can be exported from the neuronal nucleus and transported to dendrites for processing (Glanzer et al., 2005). In any case, these findings open an attractive hypothesis that endo-siRNAs participate in synaptic plasticity during learning process and the neuronal endo-siRNA pathway might be also linked to various neurodegenerative disorders (Smalheiser et al., 2011).

Antiviral RNAi

In contrast to nematodes and insects, data supporting involvement of mammalian RNAi in antiviral defense is weak (reviewed in detail in Cullen, 2006; Cullen et al., 2013). It is unlikely that RNAi substantially acts as an antiviral mechanism in mammals where long dsRNA induces a complex sequence-independent antiviral response, commonly known as the interferon response (reviewed in Gantier and Williams, 2007). Consistent with this, no siRNAs of viral origin have been found in human cells infected with a wide range of viruses (Pfeffer et al., 2005). Occasional observations, such as detection of a single siRNA in HIV-1 infected cells (Bennasser et al., 2005) does not provide any conclusive evidence that RNAi is processing viral dsRNA and suppresses viruses under physiological conditions *in vivo*.

It must be stressed that circumstantial evidence suggesting the role of RNAi in viral suppression must be critically examined and interpreted. One has to keep in mind, for example, that data, which appear as evidence for viral suppression by RNAi, could reflect miRNA-mediated effects. Since viruses co-evolve with different hosts and explore all possible strategies to maintain and increase their fitness, it is not surprising that viral reproductive strategies come into contact with mammalian RNA silencing pathways, particularly the miRNA pathway, which shares components with the RNAi pathway. For example, Epstein-Barr virus (EBV) and several other viruses encode their own miRNAs (Parameswaran et al., 2010; Pfeffer et al., 2005; Pfeffer et al., 2004; Sullivan et al., 2005) or take advantage of host cell miRNAs to enhance their replication (Jopling et al., 2005).

Another evidence for an interaction between viruses and RNA silencing is the presence of putative suppressors of RNA silencing (SRS) in various viruses. As viral genomes rapidly evolve, SRS should be functionally relevant. For example, B2 protein in Nodaviruses (e.g. FHV) is essential for replication, inhibits Dicer function, and B2-deficient FHV can be rescued by artificial inhibition of RNAi response (Li et al., 2002). B2 protein also enhances the accumulation of Nodaviral RNA in infected mammalian cells (Fenner et al., 2006; Johnson et al., 2004). Other potential SRS molecules have been identified in viruses infecting vertebrates, such as Adenovirus VA1 noncoding RNA (Lu and Cullen, 2004), Influenza NS1 protein (Li et al., 2004), Vaccinia virus E3L protein (Li et al., 2004), Ebola virus VP35 protein (Haasnoot et al., 2007), TAS protein in primate foamy virus (Lecellier et al., 2005), or HIV-1 TAT protein (Bennasser et al., 2005).

The existence of SRS in viruses infecting mammals does not prove that these viruses are targeted by mammalian RNAi. First, viruses may have a broader range of hosts (or vectors),

including, e.g. blood sucking insects. Thus, a virus can be targeted by RNAi in one host and by another defense mechanism in another one. For example, the Dengue virus, whose life cycle takes place in humans and mosquitoes, is targeted by RNAi in mosquitoes and it likely evolved an adaptation to circumvent RNAi (Sanchez-Vargas et al., 2009). Second, viral SRS in mammalian cells may have other purpose than counteracting viral suppression by RNAi. Since biogenesis and mechanism of action of mammalian miRNAs overlaps with RNAi, it is possible that the role of such SRS is to modify cellular gene expression by suppressing the activity of miRNAs. Third, the main effect of SRS may be aimed at other defense mechanisms recognizing and responding to dsRNA and, as a consequence, SRS effects on RNAi are observed.

Systemic RNAi in mammals

Non-cell autonomous RNA with an extent similar to that of *C. elegans* or in some insects is highly unlikely to function in vertebrates. However, a limited environmental or systemic RNAi may exist there as the homologs of sid-1 have been found in all sequenced vertebrate genomes (Jose and Hunter, 2007). Two sid-1 homologs (SidT1 and SidT2) are present in mice and humans with a documented role for SidT1 in dsRNA uptake in humans (Duxbury et al., 2005; Wolfrum et al., 2007). Furthermore, experimental overexpression of human SidT1 significantly facilitated cellular uptake of siRNAs and resulted in increased RNAi efficacy (Duxbury et al., 2005). As it will be discussed later, the mammalian immune system employs a number of proteins responding to dsRNA independently of RNAi (Gantier and Williams, 2007), while RNAi does not seem to participate in the innate immunity (Cullen, 2006; Cullen et al., 2013). Thus, the primary role of a dsRNA uptake mechanism in mammals is likely not involving RNAi even though it could have served such a role in an ancestral organism.

Nuclear function of small RNAs

The literature search yielded a large heterogeneous group of publications concerning nuclear localization of Dicer and AGO proteins as well as nuclear effects, including transcriptional gene silencing. Some of these observations might come from physiologically relevant nuclear silencing mechanisms. However, when critically evaluating published studies, not enough evidence was found, to establish a model for transcriptional silencing in mammals; except of the PIWI-induced transcriptional silencing in the germline (REF). Here, I will provide an overview of nuclear aspects of RNA silencing and highlight those observations which might be related to the miRNA pathway or long dsRNA response.

Homology-dependent phenomena and observations that may reflect nuclear mechanisms involving small RNAs can be sorted into several areas, which will be discussed further below:

Indirect effects of miRNAs on chromatin
Nuclear RNAi (nuclear post-transcriptional silencing)
Transcriptional regulations (stimulation/repression) by exogenous small RNAs

Transcriptional regulations (stimulation/repression) by miRNAs
27-nt RNA – mediated regulation of endothelial nitric oxide synthase gene
Splicing regulation by small RNAs
DNA repair-associated small RNAs

First, it is necessary to discuss nuclear localization of miRNA and RNAi factors, since their nuclear localization is a pre-requisite for nuclear silencing. Pioneering RNAi work indicated that silencing occurs in the cytoplasm because dsRNA against intronic sequences had no silencing effect (Fire et al., 1998) and the RISC complex co-purified with ribosomes (Hammond et al., 2000). Early studies in mammalian cells also suggested that RNAi is cytoplasmic (Billy et al., 2001; Zeng et al., 2002). However this notion was subsequently challenged by a series of studies reporting nuclear RNAi and small RNA-induced transcriptional silencing (Morris et al., 2004; Robb et al., 2005; Ting et al., 2005).

Nuclear localization of Dicer

A number of works directly or indirectly implies nuclear localization of Dicer (Doyle et al., 2013; Drake et al., 2014; Gagnon et al., 2014a; Gullerova and Proudfoot, 2012; Haussecker and Proudfoot, 2005; Neve et al., 2016; Ohrt et al., 2012; Sinkkonen et al., 2010; White et al., 2014), which contrasts with a recent *in vivo* study on mouse Dicer where tagging of an endogenous Dicer gene with an antibody epitope yielded exclusively cytoplasmic localization in all analyzed tissues with an extremely sensitive detection limit for nuclear Dicer (Much et al., 2016). Thus, despite the collection of the reports below, one should still approach the nuclear aspect cautiously. One of the earlier implications for nuclear localization of Dicer was the reported Dicer-dependent turnover of intergenic transcripts from the human beta-globin gene cluster (Haussecker and Proudfoot, 2005). However, this study showed mostly correlation of abundance of nuclear transcripts. Nuclear Dicer processing was also implicated by several other studies (Flemr et al., 2013; Neve et al., 2016; Valen et al., 2011; White et al., 2014). In terms of function, nuclear Dicer was thought to be involved in nuclear dsRNA processing (?) (White et al., 2014), selection of alternative polyadenylation sites (Neve et al., 2016) or rRNA processing (Liang and Crooke, 2011). Several studies documented Dicer nuclear localization by microscopy. Dicer was detected in cultured mammalian cells with several different antibodies in the chromatin where it resided in rDNA clusters on acrocentric human chromosomes (Sinkkonen et al., 2010). Finer mapping using chromatin immunoprecipitation suggested that Dicer localizes in the proximity of the rRNA transcribed region (Sinkkonen et al., 2010). However, this study failed to reveal any functional significance of Dicer localization and it is not clear if the localization is related to the later reported role of Dicer in pre-rRNA processing (Liang and Crooke, 2011) or to rDNA-derived small RNAs (Wei et al., 2013).

Furthermore, localization of Dicer to rDNA is distinct from the nuclear localization of Dicer detected by fluorescence correlation/cross-correlation spectroscopy (FCS/ FCCS) (Ohrt et al., 2012), since FCS/ FCCS can detect diffusing and not rDNA chromatin-bound Dicer. FCS/FCCS identified Dicer in the nucleus in HeLa cells and suggested that nuclear Dicer is alone while the cytoplasmic Dicer exists in a large complex with AGO2 (presumably RLC) (Ohrt et al., 2012). This would imply that while Dicer could process nuclear

substrates, they would not be loaded on AGO proteins in the nucleus. However, a later study suggested that Dicer, TARBP2, AGO2 and GW182 associate into a large complex in the nucleus although, consistently with FCS/FCCS data, loading of nuclear small RNA duplexes was not detected (Gagnon et al., 2014a). Regarding the nuclear localization mechanism of Dicer, it does not employ a canonical nuclear localization signal (NLS). A pyruvate kinase fusion system suggested that dsRBP of Dicer could function as an NLS similarly to ADAR dsRBD (Doyle et al., 2013). However, this study did not prove nuclear localization of full-length Dicer under physiological conditions. Interestingly, an additional report implied phosphorylation of Dicer in nuclear localization in nematodes, humans and mice (Drake et al., 2014). Remarkably, one of the two reported phosphorylation sites for ERK was in the dsRBD, providing a hypothetical link to analysis of the study implying dsRBD in nuclear localization of Dicer. Yet, as mentioned above, EGFP tagging of Dicer in mice does not support nuclear localization of Dicer (Much et al., 2016). Taken together, nuclear localization/function of Dicer is still poorly understood and further research is needed to build a more coherent picture from the contradictory observations.

Nuclear AGO1–4

A similarly unclear situation exists for nuclear AGO1–4 proteins. AGO1–4 proteins were observed in the nucleus under different circumstances including immunofluorescent staining, such as, for example (Ahlenstiel et al., 2012; Allo et al., 2014; Aporntewan et al., 2011; Bai et al., 2014; Berezhna et al., 2006; Gagnon et al., 2014a; Huang et al., 2013a; Jang et al., 2012; Janowski et al., 2006; Kim et al., 2012; Kim et al., 2006; Liang and Crooke, 2011; Nishi et al., 2013; Ohrt et al., 2008; Ohrt et al., 2012; Robb et al., 2005; Rudel et al., 2008; Sharma et al., 2016; Tan et al., 2009a). Notably, one should be cautious about nuclear localization detected by antibodies and cellular fractionation in the absence of appropriate controls. While antibody cross-reactivity often cannot be excluded, biochemical fractionations suffer from impurities and endoplasmic reticulum contamination is frequently not examined. In any case, if there would be any consensus about nuclear AGO localization, it seems that a small fraction of AGO proteins is indeed in the nucleus and can engage complementary RNAs (Berezhna et al., 2006; Gagnon et al., 2014b; Ohrt et al., 2008; Robb et al., 2005). The mechanism of nuclear import of AGO proteins is unclear. AGO proteins do not carry a canonical NLS. It has been proposed that AGO proteins could be imported into the nucleus by GW182 (Nishi et al., 2013) or via Importin 8 (Weinmann et al., 2009).

The next section reviews effects of small RNA mechanisms in the nucleus, starting with indirect ones.

Indirect effects of miRNAs on chromatin

Studies of miRNA targets suggest that 10 to 30% of human genes are potential miRNA targets (John et al., 2004; Lewis et al., 2003). Also, experiments with delivering miRNAs into different cell types suggest that individual miRNAs can down-regulate a large number of genes (Lim et al., 2005). Thus, it is not surprising that many genes regulating chromatin structure are directly or indirectly regulated by miRNAs. One of the recent examples of

such a connection is the regulation of DNA methylation in murine embryonic stem cells (ESCs). Phenotype analysis of Dicer$^{-/-}$ cells revealed that the loss of Dicer in ES cells leads to defects in differentiation and it may (Kanellopoulou et al., 2005) or may not (Murchison et al., 2005) lead to aberrant changes in centromeric chromatin. It has been speculated that Dicer functions in a pathway similar to that of *Schizosaccharomyces pombe* (Kanellopoulou et al., 2005). This interpretation was consistent with previous analysis of chicken-human hybrid DT40 cell line lacking Dicer, where defects in heterochromatin were also observed (Fukagawa et al., 2004). However, cloning of small RNAs from WT and Dicer$^{-/-}$ ES cells suggests that ES cells do not naturally produce endogenous siRNAs and that Dicer exclusively produces miRNAs (Calabrese et al., 2007).

Detailed analysis of the transcriptome of murine Dicer$^{-/-}$ ES cells (Sinkkonen et al., 2008) correlated changes of gene expression with the presence of binding sites for AAGUGC-seeded miRNAs (miR-290 cluster and other miRNAs), which were previously found in human and murine ES cells (Houbaviy et al., 2005; Suh et al., 2004). The loss of Dicer and miRNAs resulted in down-regulation of de novo DNA methyltransferases and defects in de novo DNA methylation during differentiation. This defect could be rescued by over-expressing de novo DNA methyltransferases or by transfection of the miR-290 cluster miRNAs (Sinkkonen et al., 2008). These data were complemented by the study of Benetti et al., who showed that the loss of Dicer leads to decreased DNA methylation, concomitant with increased telomere recombination and telomere elongation (Benetti et al., 2008).

Regulation of de novo DNA methyltransferases by miRNAs is likely much more complex than described above because other miRNAs were also implicated in their direct regulation in other cell types (Duursma et al., 2008; Fabbri et al., 2007). Importantly, the genetic background or culturing conditions can also influence epigenetic changes in studied cells, which might explain why a third study of Dicer-deficient ESCs observed normal DNA methylation dynamics (Ip et al., 2012).

In any case, it is very likely that miRNAs play a similar role in other aspects of chromatin formation. Considering that up to 60% of the genes are possibly regulated by miRNAs, data from experiments which directly or indirectly affect the miRNA pathway (including siRNA off-targeting), should be handled with open mind and great caution.

Post-transcriptional regulations by small RNAs in the nucleus – nuclear RNAi

In mammalian cells, Robb et al. showed that nuclear RNAs can be targeted by RNAi (Robb et al., 2005). In addition they also provided biochemical data showing that AGO1 and AGO2 localize into the nuclear RISC (Robb et al., 2005). While these data did not make a conclusive evidence as contamination of the nuclear fraction with AGO proteins associated with outer nuclear envelope could not be excluded, nuclear localization of AGOs was later backed up by other data (Berezhna et al., 2006; Gagnon et al., 2014b; Ohrt et al., 2008). Berezhna et al. showed that siRNAs accumulate in the nucleus in a cognate mRNA dependent manner (Berezhna et al., 2006). Ohrt et al. reported that siRNAs against firefly luciferase microinjected into HeLa cells enter nucleus but are actively excluded from non-nucleolar space in Exportin-5 dependent manner (Ohrt et al., 2006).

Taken together, loaded AGO2 seems to be able to engage nuclear RNAs and, in case of perfect complementarity, it can cleave its targets. How nuclear RNAi incorporates nuclear and cytoplasmic small RNA precursors and how it is working under physiological conditions on perfectly complementary and partially complementary targets remains largely unknown.

Transcriptional regulations (stimulation/repression) by exo-siRNAs

In plants and fungi, RNA silencing mechanisms also mediate transcriptional silencing. Similar transcriptional silencing phenomena were intensely searched also in mammals. The first two reports of transcriptional silencing in mammals were published in 2004 when two groups reported siRNA-mediated transcriptional silencing coupled with DNA methylation (Kawasaki and Taira, 2004; Morris et al., 2004) and laid problematic foundations of transcriptional silencing research in mammals. The reason is that reproducibility of both reports was quickly questioned and one of them was later forced retracted, formally because "a proper data notebook is not available as evidence to support our findings, which constitutes non-adherence to ethical standards in scientific research. In accordance with the recommendations from the National Institute of Advanced Industrial Science and Technology, K.T. therefore wishes to retract this paper." (Taira, 2006). While the second report has not been retracted, DNA methylation has been doubted as a key silencing effect (Ting et al., 2005). Furthermore, the report did not analyse induction of DNA methylation by informative bisulfite sequencing but relied on suboptimal methylation-sensitive restriction digest. Another troubling aspect of the second report is a technically impossible transcriptional silencing experiment shown in Fig. 1B, which raises a question how careful was the peer reviewing process. In any case, transcriptional misregulation by exogenous oligonucleotides complementary to promoter sequences has been reported by different laboratories for different promoters in different cells arguing that some complementarity-based transcriptional regulation by small RNAs functions in mammalian cells. Importantly, the underlying mechanism was not conclusively revealed after a decade and is still a matter of debates.

The siRNA-induced transcriptional silencing involves changes in the chromatin structure such as loss of "active" histone modifications (H3K4 methylation, histone acetylation) (Janowski et al., 2006; Morris et al., 2004), appearance of "inactive" histone modifications (H3K9 and H3K27 methylation) (Castanotto et al., 2005; Janowski et al., 2006; Jiang et al., 2012; Kim et al., 2006; Kim et al., 2007; Weinberg et al., 2006), and occasionally DNA methylation (Morris et al., 2004). These are all common features of transcriptional repression and could be either directly induced by siRNA-containing complexes or they be a consequence of transcriptional silencing. DNA methylation is apparently a secondary effect (Ting et al., 2005) although a systematic analysis revealed a group of gene promoters whose methylation was dependent on Dicer (Ting et al., 2008).

Small RNAs used for silencing were either "classical" synthesized siRNAs (agRNAs) (Ahlenstiel et al., 2012; Castanotto et al., 2005; Hawkins et al., 2009; Janowski et al., 2005a; Jiang et al., 2012; Kim et al., 2007; Morris et al., 2004; Napoli et al., 2009; Ting et al., 2005) or shRNAs expressed from a plasmid (Castanotto et al., 2005; Kim et al., 2007). Importantly, transcriptional silencing could be induced by a variety of antisense oligomers

targeting promoter sequences such as single-stranded antigene (ag) peptide nucleic acid (PNA) (Janowski et al., 2005b), PNA-peptide conjugates (Hu and Corey, 2007), locked nucleic acid (LNA) (Beane et al., 2007) or duplex RNA (siRNA) oligos (Janowski et al., 2005a). These results represent a remarkably comprehensive set of data concerning inhibition of human progesterone receptor A (hPR-A) and B (hPR-B) isoforms. Despite all approaches achieved inhibition of gene expression, these silencing oligonucleotides have radically different properties and it is questionable whether they would all operate loaded on an AGO protein in a RISC-like complex.

Furthermore, studies of oligonucleotides targeting promoter sequences revealed that some oligonucleotides have a positive effect on transcription (Janowski et al., 2007; Li et al., 2006). The activating effect of small RNAs also appeared later in other reports (Hu et al., 2012; Wang et al., 2015a; Zhang et al., 2014). "Scanning" a promoter with oligonucleotides revealed the existence of sites whose targeting results in transcriptional repression as well as sites whose targeting promotes expression (Janowski et al., 2005a; Janowski et al., 2007). According to these data, a shift by several nucleotides could have an opposite effect on gene expression that would correlate with changes in histone modifications. These data would imply existence of a still-unknown RISC-like nuclear complex acting as a switch or siRNA strand-selection onto a single RISC-like complex, where the opposing effects would stem from targeting sense and antisense RNAs in the locus. Alternatively, the observed silencing stems from the oligonucleotide binding and is not mechanistically involving AGO proteins.

To date, the active agent of the silencing has not been conclusively identified and critically assessed. It still remains a question whether there is a dedicated transcriptional silencing machinery in mammalian cells involving AGO proteins and some binding partners as proposed by some reports (Cho et al., 2014; Hawkins et al., 2009; Hu et al., 2012; Janowski et al., 2006; Kim et al., 2006; Suzuki et al., 2008), which are partially contradicting themselves and are not supported by proteomic analysis of AGO complexes (Hauptmann et al., 2015; Hock et al., 2007; Meister et al., 2005), or various artificial disturbances of lncRNAs generate the observed effects.

Transcriptional regulations (stimulation/repression) by miRNAs

A peculiar phenomenon of miRNA-associated transcriptional activation was reported from cultured mammalian cells for the E-cadherin and cold-shock domain-containing protein C2 (CSDC2) promoters and the miR-373 (Place et al., 2008). The initial observation leading to discovery of this phenomenon was that transfection of siRNAs homologous to E-cadherin, p21WAF1/CIP1, and VEGF promoters lead to unexpected transcriptional activation (Li et al., 2006). Subsequent sequence analysis of the E-cadherin promoter revealed high complementarity between the miR-373 and the sequence at position -645 relative to the transcription start site (Place et al., 2008). Delivery of miR-373, pre-miR373 and a synthetic siRNA sequence targeting -640 position could stimulate E-cadherin expression by ~5–7-fold in PC-3 cells. Interestingly, intact miRNA pathway was required for the E-cadherin stimulation as partial down-regulation of Dicer protein by morpholino oligonucleotides abolished the stimulatory effect of pre-miR-373. Since then, several other reports of

miRNA-mediated transcriptional silencing appeared, including transcriptional inhibition of *HOXD4* expression by miRNA-10a (Tan et al., 2009b), miR-320-mediated transcriptional silencing of *POLR3D* (Kim et al., 2008), and others (Adilakshmi et al., 2012; Benhamed et al., 2012; Kim et al., 2011)

It remains an open question whether effects of exogenous siRNAs represent the same molecular mechanism as those attributed to nuclear activities of miRNAs. It would be supported by non-cleaving AGO1 implicated in transcriptional regulation (Huang et al., 2013a; Janowski et al., 2006; Kim et al., 2006) and transcriptional silencing by miRNA mimics targeting gene promoters (Younger and Corey, 2011). In terms of the possible miRNA-mediated nuclear silencing, it was reported that POLR3D silencing involves miRNA-mediated promoter association with a complex including AGO1 and EZH2 (H3K27 histone methyltransferase) (Kim et al., 2008). It was also suggested that AGO1 interacts with polymerase II (Huang et al., 2013a; Kim et al., 2006). At the same time, others implicated GW182 as the factor important for nuclear localization and function of nuclear AGO proteins (Nishi et al., 2013; Nishi et al., 2015). However, the mechanistic link between AGO-GW182 and histone modifications still remains elusive.

27nt RNAs – mediated regulation of endothelial nitric oxide synthase gene

This phenomenon has been observed in endothelial nitric oxide synthase (eNOS) where a 27nt repeat polymorphism in intron 4 was a source of predominantly nuclear 27nt small RNAs, which could be either a novel class of small RNAs, or atypical miRNAs (Zhang et al., 2008b). In any case, these 27nt RNAs were implicated in eNOS suppression by altering histone acetylation and DNA methylation in regions adjacent to the 27nt repeat element and core promoter (Zhang et al., 2008a). Whether this phenomenon is related to other ones described here remains unclear.

Regulation of splicing

In addition to transcriptional and post-transcriptional silencing, one of the reported effects of small RNAs transfected into mammalian cells was also an impact on alternative splicing (Allo et al., 2009). A mechanism was proposed, which involves AGO1 recruitment to transcriptional enhancers (Allo et al., 2014), while other reports implicated AGO2 (Liu et al., 2012; Liu et al., 2015) or both (Ameyar-Zazoua et al., 2012).

Small RNAs associated with DNA repair

The last nuclear phenomenon associated with small RNAs is their emergence upon DNA damage. It was reported that DICER and DROSHA-dependent small RNAs emerge as DNA-damage response and are functionally associated with it through the MRE11 complex (Francia et al., 2012). In addition, knock-down of Dicer or AGO2 in human cells reduced double-stranded break repair (Wei et al., 2012). It was proposed that small RNAs emerging from DNA –damage loci may function as guide molecules directing chromatin modifications or the recruitment of protein complexes to facilitate repair (Francia et al.,

2012; Wei et al., 2012). What is somewhat confusing in DNA-repair associated small RNAs is the role of miRNA-specific factors Drosha (Francia et al., 2012) or DGCR8 (Swahari et al., 2016).

Despite the heterogeneity of the nuclear effects and many unknowns, some common themes emerged, allowing for formulating testable hypotheses that could be critically evaluated. First, nuclear effects can be mediated by small RNAs provided in trans. Second, small RNAs recruit AGO proteins in a sequence-specific manner, most likely recognizing a local transcript (perhaps an ncRNA). Third, the effect involves a change in the chromatin structure. Thus, by carefully examining essential exogenous siRNA properties in previously reported nuclear effects, one should be able to demonstrate that the silencing phenomenon truly involves an AGO-loaded small RNA engaging another nuclear RNA and whether the effect requires the "slicer" activity. Detailed examination of the seed sequence would also discern between specific nuclear effects and off-targeting. Furthermore, should the effect involve small RNA loaded AGO protein, the kinetics of the phenomenon should be in agreement with known RISC kinetics discussed above. Finally, if the aforementioned phenomena rely on localized recruitment of AGO-loaded small RNAs, one should be able to mimic those effects by tethering AGO proteins through sequence-specific DNA binding modules such as those employed by TALEN or CRISPR nucleases. These research directions should be combined with validated antibodies for chromatin immunoprecipitation and immunofluorescence (or epitope knock-in into candidate genes), more extensive use of mutants defective in RNA silencing, detailed quantitative analysis of cellular fractionation and identifications of interacting partner, studies of putative nuclear import and export signals of Dicer and AGO proteins, and advanced imaging techniques.

Other dsRNA–associated mechanisms I – dsRNA sensing in the interferon pathway

Long dsRNA is not a usual RNA molecule in eukaryotic cells while RNA viruses produce dsRNA during replication. A common mechanism repressing viruses in non-vertebrate species is RNA silencing (Wang et al., 2006; Wilkins et al., 2005). However, response to foreign long dsRNA in mammals is much more complex and involves a set of sequence-independent sensors triggering expression of a defined set of genes known as interferon-stimulated genes (ISGs). The interferon pathway is the most ubiquitous sequence-independent pathway induced by dsRNA in mammalian cells (reviewed in detail in de Veer et al., 2005). Among the relevant sensors recognizing cytoplasmic dsRNA are protein kinase R (PKR), the helicase RIG-I, MDA5, 2',5'-oligoadenylate synthetase (2',5'-OAS), or Toll-like receptors (TLR3, 7, 8) (reviewed in Gantier and Williams, 2007; Sadler and Williams, 2007). Notably, there are also dsRNA-independent mechanisms that can activate interferons in mammalian cells. Altogether, different stimuli are being sensed and converge on activation of overlapping but distinct sets of ISGs (Geiss et al., 2001). The situation is even more convoluted by cellular diversity as some cell types, particularly immune cells, can elicit the interferon response by additional, cell-type-specific pathways (reviewed in Schlee and Hartmann, 2010).

PKR

PKR is the oldest known mammalian dsRNA sensing protein. A pioneering work by Hunter et al. showed that different types of dsRNA can block translation in reticulocyte lysates (Hunter et al., 1975). Analysis of the phenomenon identified PKR that is activated upon binding to dsRNA and blocks translation by phosphorylating the alpha subunit of eukaryotic initiation factor 2 (eIF-2α) (Meurs et al., 1990). Activation of PKR also includes activation of the NFκB transcription factor and a large number of interferon-stimulated genes (ISGs) (Geiss et al., 2001). PKR response to viral dsRNA can be coordinated with other dsRNA sensors, such as RIG-I and MDA5 (Sen et al., 2011). PKR can also respond to endogenous RNAs in unique physiological regulations (Bevilacqua et al., 1998; Bommer et al., 2002). However, endogenously expressed long dsRNA does not necessarily induce canonical PKR response with interferon activation, although PKR binding to dsRNA and restricted translational repression can be observed (Nejepinska et al., 2012; Nejepinska et al., 2014). It was believed that dsRNA <30-bp in length does not induce PKR. However, Marques et al. reported that, siRNAs can bind and activate PKR *in vitro* regardless of siRNA termini (Marques et al., 2006) arguing against the long-established 30-bp length as the minimal size-limit for PKR activation. There are also other data indicating sensitivity of PKR to dsRNA motifs shorter than 30-bp (Puthenveetil et al., 2006; Reynolds et al., 2006; Zheng and Bevilacqua, 2004).

RIG-I-like receptors (RIG-I, MDA5, LGP2)

Mammalian somatic cells can respond to dsRNA in a sequence-independent manner.. In addition to PKR, several other proteins recognizing dsRNA are integrated to the interferon response, including helicases RIG-I (retinoic-acid-inducible gene-I, also known as DDX58), MDA5 (IFIH1), and LGP2 (DHX58), which sense cytoplasmic dsRNA and activate interferon expression.

RIG-I is a cytoplasmic sensor differentiating between endogenous and foreign RNAs structures. In particular, RIG-I is activated by blunt-ended dsRNAs with or without a 5'-triphosphate, by single-stranded RNA marked by a 5'- triphosphate, and by polyuridine sequences. RIG-I domains organize into a ring around dsRNA, capping one end, while contacting both strands; the structure is consistent with dsRNA translocation without unwinding and cooperative binding to RNA (Jiang et al., 2011a; Jiang et al., 2011b). Like RIG-I and LGP2, MDA5 preferentially binds dsRNA with blunt ends (Li et al., 2009a). RIG-I, MDA5, and LGP2 exhibit differences in recognizing specific RNA structures and different types of viruses providing a broader range of coordinated sensitivity do different potential threats (Kato et al., 2006; Li et al., 2009b; McCartney et al., 2008; Sen et al., 2011; Slater et al., 2010; Wu et al., 2015b). Interestingly, RIG-I can become activated also with siRNAs lacking 2-nt 3' overhangs (Marques et al., 2006). These data imply that 2-nt 3' overhangs generated by Dicer are the structural basis for discriminating between Dicer products and other short dsRNA. Roles of MDA5 and LGP2 in siRNA-mediated interferon response remains to be addressed. Furthermore, recognition 5' triphosphate RNA ends RIG-I (Hornung et al., 2006; Pichlmair et al., 2006) highlights importance of appropriate

processing of 5' termini of RNAs produced by phage polymerases when such RNAs are used in mammalian cells.

It is not clear how PKR and RIG-I pathways are integrated. RIG-I binds siRNAs (with or without 2-nt 3' overhangs) *in vitro* and it shows greater unwinding of blunt-ended siRNAs. Unwinding is then translated into the interferon activation mediated via IRF-3.

Toll-like Receptor 3 (TLR3)

TLR3 is a member of the Toll-like receptor (TLR) family and functions as a sensor of extracellular, intracellular and viral dsRNAs (Amarante et al., 2011; Seo et al., 2013; Wang et al., 2015b; Wu et al., 2015b; Yang et al., 2006b). TLR3 has distinct or complementary roles to RIG-I and related helicases in sensing foreign molecules and activating downstream responses (Livengood et al., 2007; McCartney et al., 2009; Slater et al., 2010; Wu et al., 2015b).

Oligoadenylate Synthetase (OAS)

Interferon and dsRNA also activate 2',5'-oligoadenylate synthetase (2',5'-OAS) that produces 2',5' oligoadenylates with 5'-terminal triphosphate residues that subsequently induce activation of RNAse L; a protein responsible for general RNA degradation (de Veer et al., 2005).

TARBP2 and PACT

Interactions between RNAi, miRNA, and interferon response are poorly understood. There are two clear mechanistic connections between these two pathways. First, TARBP2 and PACT, two dsRNA binding proteins, which were mentioned earlier as Dicer-interacting proteins, interact also with PKR. Notably, while TARBP2 inhibits PKR (Cosentino et al., 1995; Park et al., 1994), PACT has the opposite role (Patel and Sen, 1998). While cytoplasmic long dsRNA in somatic cells apparently triggers the interferon response, it is not clear if the same dsRNA is also routed into the RNAi pathways. Experiments in oocytes and undifferentiated embryonic stem cells (Stein et al., 2005; Yang et al., 2001) suggest that RNAi dominates response to cytoplasmic long dsRNA in the absence of a strong interferon response and that the interferon pathway dominates when its relevant components are present. On the other hand, this view may be too simplistic as it does not explain the lack of both, RNAi and interferon response, in somatic cells expressing long dsRNA (Nejepinska et al., 2012; Nejepinska et al., 2014). In any case, understanding the role of TARBP2 and PACT isoforms in routing long dsRNA into RNAi and interferon pathways requires further studies.

There is a clear evolutionary connection between RNAi and interferon response. The above-mentioned mammalian RNA helicases RIG-I, LGP2 and MDA5 are the closest homologs of helicases involved in processing of long dsRNA during RNAi in *C. elegans*. Notably, RIG-I is an established component of the interferon response to long dsRNA (Yoneyama et al., 2004). This suggests that the interferon response, which has a common

trigger and evolved after the RNAi pathway, adopted several components from the latter pathway. It remains to be determined whether these and other components of RNAi lost their function in RNAi entirely or mediate some form of a cross-talk between RNAi and interferon response.

Finally, there is also a complex relationship between miRNA and interferon pathways (Ingle et al., 2015; Ostermann et al., 2012; Shapiro et al., 2014; Xu et al., 2011). One connection is exemplified by viral miRNAs, which viruses use to regulate the host response, in particular factors of the interferon pathway (Ostermann et al., 2012) or other cellular signalling (Xu et al., 2011). However endogenous cellular miRNAs may also act to suppress the interferon response factors, such as the case mir-485, which has a dual role in targeting RIG-I as well as the influenza virus H5N1 (Ingle et al., 2015).

Other dsRNA-associated mechanisms II – Adenosine deamination

A-to-I editing is a covalent RNA modification system of broad significance (reviewed in Nishikura, 2016). It is mediated by adenosine deaminases acting on RNA (ADARs), enzymes that carry two or three dsRBD and recognize both inter- and intramolecular dsRNAs longer than 20–30 bp (Nishikura et al., 1991). ADARs convert adenosines to inosines, which base pair with cytosines, which are interpreted as guanosines during translation. Thus, RNA editing affects coding potential, fidelity of RNA replication reverse transcription, or formation/stability of RNA secondary structures where a change of a single base in a sequence may result either in dsRNA destabilization (inosine-uridine pair) or stabilization (inosine-cytidine pair) (Nishikura, 2010). Such transition in the local and global stability of dsRNA structure can influence further processing of dsRNA, such as the selection of the effective miRNA strand (Bartel, 2004; Meister and Tuschl, 2004).

Mammals (and vertebrates in general) have three ADAR genes (reviewed in Nishikura, 2016) (Fig. 3). Two encode proteins carrying deaminase activity: ADAR1, which is interferon-inducible, and ADAR2, which is constitutively expressed. ADAR3 is mostly expressed in the brain but its editing activity has not been shown yet. The specificity of the ADAR1 and ADAR2 deaminases ranges from highly site-selective to non-selective, dependent on the duplex structure of the substrate RNA.

The complete ADAR structure has not been solved yet but structure of several domains is known – the Z alpha domain of the human editing enzyme ADAR1 (Schwartz et al., 1999) and dsRBDs of ADAR2 (Stefl et al., 2010). The analysis of dsRBDs provided an insight into editing of a specific substrate and revealed that dsRBDs of ADAR not only recognize the shape but also the sequence of the dsRNA (Stefl et al., 2010). The unexpected direct readout of the RNA primary sequence by dsRBDs is achieved via the minor groove of the dsRNA and this recognition is critical for both editing and binding affinity of edited RNA (Stefl et al., 2010). It was also shown that ADAR2 forms dimers *in vivo* and that dsRBDs are necessary and sufficient for dimerization of the enzyme (Poulsen et al., 2006).

ADARs exhibit complex regulation of localization. For example, it was shown that mouse ADAR1 isoforms are differentially localized in cellular compartments and that their

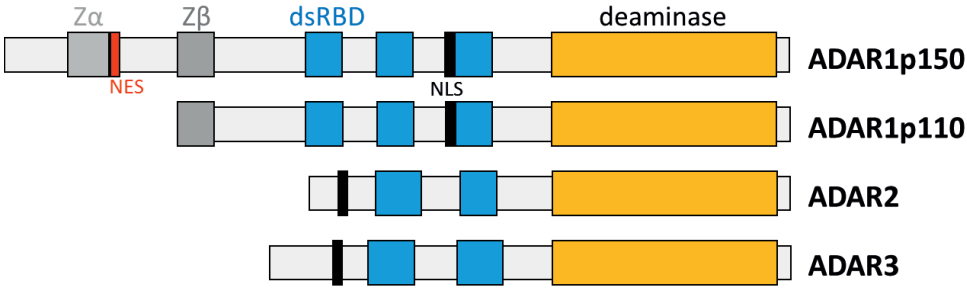

Figure 3 Domain composition of mammalian ADAR proteins
NES, nuclear export signal, NLS, nuclear localization signal; dsRBD, dsRNA binding domain.

localization is controlled by several independent signals, which include a nuclear localization signal (NLS), the nucleolar localization signal (NoLS), the nuclear exporter signal (NES) near the N terminus (Nie et al., 2004). ADAR1 interacts with TUDOR-SN nuclease (Nishikura, 2010; Scadden, 2005; Weissbach and Scadden, 2012; Yang et al., 2006a) and localizes to stress granules upon stress induction (Weissbach and Scadden, 2012) while tudor-SN degrades hyperedited dsRNA (Scadden, 2005).

RNA editing concerns a broad range of RNAs including viral and cellular RNAs. Many long perfect dsRNAs (>100 bp) undergo extensive editing with a conversion of approximately 50 % of adenosines to inosines (Nishikura et al., 1991; Polson and Bass, 1994). Extensive editing (hyperediting) is linked with nuclear retention (reviewed in DeCerbo and Carmichael, 2005). On the other hand, short RNAs (~20–30 bp) or imperfect long dsRNAs are edited selectively; usually only a few adenines at specific sites are deaminated (Lehmann and Bass, 1999). High throughput analyses revealed the extent of RNA editing of mammalian RNAs in terms of substrate diversity and frequency of editing in the transcriptome (Carmi et al., 2011; Peng et al., 2012). Edited endogenous RNAs (Dawson et al., 2004; Hundley et al., 2008; Morse et al., 2002; Salameh et al., 2015) include mRNAs, repetitive sequences (mainly Alu (Athanasiadis et al., 2004)), and miRNAs. It was predicted that more than 85% of pre-mRNAs may be edited, predominantly in the non-coding regions (Athanasiadis et al., 2004).

Several pri-miRNAs (e.g. miR-142) are known to undergo editing, which inhibits Drosha cleavage or even causes degradation of pri-miRNA by Tudor SN (Nishikura, 2010; Scadden, 2005; Yang et al., 2006a). In other cases, pri-miRNA editing does not influence Drosha activity but inhibits processing of pre-miRNA by Dicer (e.g. miR-151) (Kawahara et al., 2007a). Last but not least, RNA editing might also inhibit export of miRNAs from the nucleus (Nishikura, 2010). A systematic analysis of edited miRNAs in the human brain showed that editing of miRNAs affects several miRNAs but it is not widespread (Alon et al., 2012). A similar picture was obtained from analysis of embryonic miRNAs (Garcia-Lopez et al., 2013; Vesely et al., 2012)

One of the roles of ADARs in immunity is to prevent innate immune sensing of self-RNA (Heraud-Farlow and Walkley, 2016). ADARs also affect viral RNAs in various ways – ADARs are both antiviral and proviral; the effect on virus growth and persistence depends

upon the specific virus. (Samuel, 2011). Viruses targeted by ADARs in mammals include HIV (Clerzius et al., 2009), herpesvirus (Gandy et al., 2007), HRSV (Martinez and Melero, 2002), HCMV (Nachmani et al., 2014), VSV (Nie et al., 2007), and HDV (Wong and Lazinski, 2002).

Crosstalk between RNA editing and other dsRNA pathways.

ADARs affect other dsRNA pathways in several ways. In RNA silencing, ADARs can compete with RNAi for dsRNA substrates (including siRNAs). The ADAR1 isoform (ADAR1p150) strongly binds siRNA and reduces thus the availability of dsRNA for RNAi, resulting in less efficient RNAi in normal cells compared to Adar1$^{-/-}$ cells (Yang et al., 2005). Interestingly, injection of high doses of siRNAs enhances ADAR1 expression, suggesting a role of ADAR1 in a cellular feedback mechanism in response to siRNA (Hong et al., 2005).

Editing affects base pairing quality of dsRNA substrates as well as target recognition since a single nucleotide mismatch between siRNA and target mRNA can reduce RNAi efficacy (Scadden and Smith, 2001) or modify target specificity, especially when occurring in the seed sequence (Kawahara et al., 2007b). MiRNAs would be affected in a similar way. A moderate deamination (one I-U pair per siRNA) does not prevent Dicer processing to siRNAs (Zamore et al., 2000) but, hyperediting (~50 % of deaminated adenosines) can make dsRNA resistant to Dicer processing (Scadden and Smith, 2001).

Thus, ADARs are factors conferring to formation of RNAi resistance (Hong et al., 2005), which may be one of the viral strategies to avoid being targeted through a dsRNA-responding pathway (Zheng et al., 2005). ADARs influence the innate immunity either indirectly by preventing sensing of self-RNA (Heraud-Farlow and Walkley, 2016) or by interacting with innate immunity factors, such as PKR (Clerzius et al., 2009). The immunosuppressive role of ADAR1 could explain the phenotype of the Aicardi-Goutieres syndrome (AGS, OMIM# 225750), an autoimmune disorder caused by ADAR1 mutations (Rice et al., 2012). It has been proposed that in the absence of ADAR1, accumulation of cytoplasmic dsRNA may provoke interferon signalling and cause upregulation of interferon-stimulated genes, which is observed in AGS (Rice et al., 2012).

Summary

In mammals (Fig. 4), the miRNA pathway seems to be the dominant small RNA pathway in the soma while the existence and functionality of endogenous RNAi remains unclear. The only cell type with well documented robust and mechanistically explained endo-RNAi is the mouse oocyte. Somatic cells typically respond to long dsRNA with a sequence-independent interferon response, which is employing multiple dsRNA sensors, which trigger a complex interferon response.

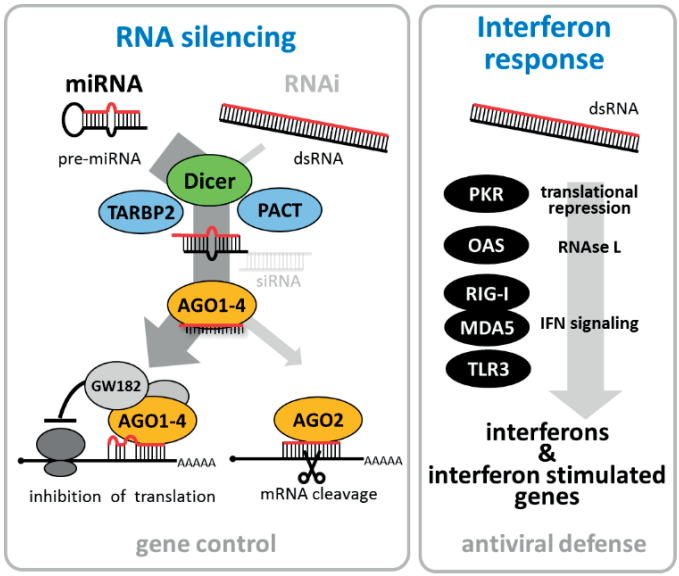

Figure 4 Summary of mammalian RNA silencing and dsRNA response pathways. On the right are schematically depicted dsRNA sensors in the sequence-independent interferon response.

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