RNAi and RNA-Based Regulation of Immune System Function

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Abstract
Gene regulation by short RNAs is a ubiquitous and important mode of control. MicroRNAs are short, single-strand RNAs that bind with partial complementarity to the 3’ untranslated region of several genes to silence their expression. This expanding class of endogenous short RNAs are evolutionarily conserved and participate in control of development and cell-specific gene function. Several of these microRNAs have been cloned uniquely from mammalian lymphocytes suggesting specialized roles in lymphocyte development and function. In addition, several genes linked to RNAi in lower eukaryotes have mammalian homologs with specialized roles in adaptive immunity. For example, in worms, the nonsense-mediated decay (NMD) and RNAi pathways appear to be intrinsically linked. NMD plays a key role in regulating antigen-receptor expression in lymphocytes and there are mammalian homologs for factors identified in worms that appear to be common in both RNAi and NMD pathways. On the other hand, RNA editing and RNAi have an inverse relationship and RNA editing has an important role in viral immunity. These observations indicate unique roles for dsRNAs in the mammalian immune system.

1. Introduction
In 1998, researchers described a new process of gene silencing triggered by double-strand (ds) RNAs called RNA interference (RNAi) (Fire et al., 1998).
Simply, introduction of dsRNAs into the nematode worm, *Caenorhabditis elegans* caused potent silencing of a gene containing sequences complementary to the dsRNAs. The discovery of RNAi resonated around the world and sparked a revolution in how we think about regulation of gene expression. RNAi is an evolutionarily ancient and highly conserved pathway of gene silencing mediated by short RNAs (reviewed in Novina and Sharp, 2004). An impacting result of the RNAi revolution was the surfacing of several biological processes across phyla, which had been incompletely understood. Viral gene silencing in plants, quelling in fungi, and cosuppression in multiple organisms subsequently emerged as processes mediated by short RNAs derived from dsRNA (reviewed in Tomari and Zamore, 2005).

RNAi has been genetically linked to other pathways of short RNA-directed gene silencing (reviewed in He and Hannon, 2004). In the process of RNAi, long dsRNAs are cleaved by an endoribonuclease called Dicer into short dsRNAs called short interfering RNAs (siRNAs). An endogenous class of genes expressing hairpin RNAs are also cleaved by Dicer into short, single-strand RNAs called microRNAs (miRNAs). siRNAs and miRNAs are incorporated into a microribonucleoprotein complex (miRNP) that contains members expressed from an evolutionarily ancient family of Argonaute genes (reviewed in Carmell et al., 2002; Schwarz and Zamore, 2002).

The short RNAs recruit the miRNP to mRNAs containing the complementary sequence. The fate of the miRNP-loaded mRNA depends partly upon the degree of complementarity between the targeted mRNA and the short RNA. The short RNA may block protein production in two ways. Perfectly complementary base pairing between the short RNA and the targeted mRNA will lead to mRNA cleavage and degradation. Alternatively, partially complementary base pairing between the short RNA and the targeted mRNA will lead to repression of translation of the targeted mRNA (Fig. 1). Thus, gene expression may be repressed or derepressed at a distal point in the gene expression pathway with rapid kinetics that would not require de novo transcription, mRNA splicing, processing, or export. Though short noncoding RNAs that control the precise timing of developmental transitions were first described in worms (Lee et al., 1993; Reinhart et al., 2000), miRNAs have since been implicated in developmental transitions in virtually every multicellular organism.

There are more than 250 miRNAs expressed in humans (Lim et al., 2003); however, very few have been assigned a biological function. The importance of miRNA function is underscored by recent reports that predict that miRNAs target one fifth (Xie et al., 2005) to more than one third (Lewis et al., 2005) of genes in the human genome. A more complete understanding of the processes regulated by miRNAs has been confounded by imperfect base pairing between the miRNA and its cognate mRNA. Typically, miRNAs control cell-fate
decisions and are involved in cell-specific gene expression (reviewed in Bartel, 2004). For example, the pancreatic islet-specific miRNA, miR375, regulates insulin secretion (Poy et al., 2004) and miR143 regulates adipocyte differentiation (Esau et al., 2004). Several miRNAs have been cloned selectively from different populations of immune cells (Chen et al., 2004). The importance of some of these miRNA functions in lymphocytes is highlighted by dysregulated miRNA activities that are correlated with leukemias (Calin et al., 2002; 2004a,b; Gauwerky et al., 1989).

It has become clear that RNAi is a mechanism of genome defense in several organisms, in which short RNAs perform adaptive immune-effector functions. In worms, mutations in RNAi pathway genes increase susceptibility to mutations by transposons (Ketting et al., 1999; Tabara et al., 1999). In plants, the RNAi pathway serves as an immune system protecting against virus attack.
siRNAs play very important roles as the effector arm of the antivirus response (Hamilton and Baulcombe, 1999). Short RNAs associated with specific gene-silencing phenomena were first reported in virus-infected or transgenic plants (Hamilton and Baulcombe, 1999). Conversely, plant viruses have evolved counter-defense mechanisms against the plant RNAi response, thereby promoting infection (reviewed in Lecellier and Voinnet, 2004). Recently, miRNAs were cloned from B cells infected with Epstein-Barr Virus (EBV) (Pfeffer et al., 2004), indicating that there may be very specialized roles for short RNAs in immune system functions. It is not surprising that the war between host cell and parasitic genetic elements is being waged on the RNA battlefields.

Short RNA-directed gene silencing also occurs in the nucleus at the transcriptional level. In plants, short RNAs direct transcriptional gene silencing (TGS) through chromatin modifications (reviewed in Matzke and Matzke, 2004; Matzke et al., 2004). The most efficient TGS was observed in plants when promoter sequences were inserted into a replicating virus which resulted in methylation of the endogenous promoter sequences (Lindroth et al., 2001; Wassenegger et al., 1994). In mammals, introduction of siRNAs complementary to promoter sequences led to promoter methylation and TGS (Kawasaki and Taira, 2004; Morris et al., 2004). Short RNAs have been proposed to play a role in nuclear events in the mammalian immune system such as regulating the chromatin structure at antigen receptor and cytokine loci (Chowdhury and Novina, 2005). Consistent with the notion that RNAi constitutes an intracellular immune system that functions in genome defense, yeast with mutations in RNAi genes demonstrate desilencing of centromeric repeat DNA (Griffiths-Jones, 2004; Grishok et al., 2001; Hutvagner et al., 2001; Lee et al., 2003; Schramke and Allshire, 2003; Verdel et al., 2004). Similarly, mammals with homologous disruption in the gene coding for Dicer demonstrate defects in heterochromatin formation (Lewis et al., 2003) and desilencing of centromeric repeat DNA (Fukagawa et al., 2004; Kanellopoulou et al., 2005). In flies, short RNAs promote TGS of high copy number transgenes through chromatin modifications (Pal-Bhadra et al., 2004) and in Tetrahymena thermophila, short RNAs specify sites of genome fragmentation and DNA deletion (Mochizuki et al., 2002). Paradigms of short RNA-directed gene silencing established in other eukaryotes may have homologous functions in mammalian lymphocytes.

A defined role for RNAi in mammals has yet to be described. Much of our understanding about RNAi and short-RNA function is derived from worms. RNAi is linked to RNA editing and nonsense-mediated decay in worms. Several of the PAZ-PIWI-Domain (PPD) proteins implicated in RNAi-related pathways in worms and other eukaryotes have homologs with specialized functions in lymphocytes which require dsRNA for proper function and which are
distinct from their functions in other mammalian cell types. This review describes recent data on short RNAs in mammalian lymphocyte development and function and explores the unique relationships between RNAi genes and dsRNA intermediates in processes required for antigen-receptor expression, indicating that lymphocyte-specific gene expression may require RNAi.

2. Short RNAs in the Mammalian Immune System

Short RNAs have been cloned from cells of the hematopoietic lineages. Identification of these short RNAs unique to these cell lineages and from a variety of cellular contexts suggests their importance in the generation and maintenance of immune system functions. In addition to the evolving roles for short RNAs in the nucleus, short RNAs in the cytoplasm may play pivotal roles in lymphocyte-restricted gene expression.

miRNAs are conserved and have been implicated in developmental transitions in numerous organisms (reviewed in Bartel, 2004). miRNAs are expressed as long polII transcripts that are processed by the RNaseIII enzyme Drosha (Lee et al., 2003) to produce shorter hairpin RNAs that are exported to the cytoplasm where they are further processed by Dicer (Grishok et al., 2001; Hutvagner et al., 2001) into mature miRNAs. Therefore, miRNAs are related to siRNAs in the Dicer processing step (Fig. 1). Unlike siRNAs that are derived from long dsRNAs typically from exogenous sources such as transgenes, miRNAs are expressed from endogenous genes to silence target genes expressed in trans (reviewed in Bartel, 2004). A comprehensive list of known miRNAs and their predicted targets can be found in the miRNA registry (Griffiths-Jones, 2004).

To better understand the roles for miRNAs in mammalian development, researchers cloned ~100 unique miRNAs from mouse bone marrow (Chen et al., 2004). Three of these miRNAs (miR181, miR223, and miR142) were preferentially or uniquely expressed in hematopoietic tissues. To characterize the role of these miRNAs in hematopoiesis and to investigate their pattern of expression during differentiation, these miRNAs were ectopically expressed in hematopoietic progenitor cells cultured in a cocktail of cytokines. Expression of miR181 doubled the number of B cells with no effect on the number of T cells. Conversely, ectopic expression of miR142 and miR233 had a modest but opposite effect that increased T-cell but not B-cell number. The effect of miR181 in cultured cells was reproduced in vivo by adoptive transfer of miR181-expressing bone marrow cells in lethally irradiated mice. Though the target gene(s) of miR-181 have not been determined, these observations implicate miRNAs in regulation of mammalian immune system gene expression.
To gain insight into the roles of miRNAs in mammalian immune system function, several laboratories are attempting to identify the target genes through genetic and biochemical approaches while other laboratories are using a bioinformatics approach to predict the mRNA target genes for the miRNAs. More than one-third of human genes appear to the conserved miRNA targets (Lewis et al., 2005). miRNA-directed silencing is specified by perfect Watson-Crick complementarity among nucleotides 2–7 relative to the 5′ end of the miRNA (Doench and Sharp, 2004; Lewis et al., 2003, 2005). Using this and other parameters, mRNAs with conserved pairing to the 5′ region of the miRNA can be identified as candidate genes regulated by cloned miRNAs. Translational inhibition requires imperfect nucleotide base pairing between the mRNA and the miRNA and the degree of translational inhibition is thought to depend on the number of miRNAs bound to the mRNA. Approximately 3% of these targets are genes involved in the immune system suggesting cell-type-specific roles for these short RNAs. The identities of some of these targets are listed in Table 1.

miRNAs have been implicated in cell-cycle control, and dysregulation of miRNA pathways can lead to initiation of cancer. For example, in flies, bantam encodes a miRNA required for cell-cycle progression and activation of the proapoptotic pathway (Brennecke et al., 2003). In lymphocytes, there are examples in which loss of miRNA genes and oncogene dysregulation correlates directly with cancer formation. Chronic lymphocyte leukemia (CLL) is the most common form of adult leukemia in the western world. Croce and colleagues demonstrated that miR15 and miR16 are located at chromosome 13q14 in a region that is deleted in most patients with CLL (Calin et al., 2002). Genome-wide expression profiling of miRNAs in human CD5+B cells from CLL patients reveal distinct expression patterns compared to normal controls (Calin et al., 2004a). One way of interpreting these observations is that these miRNAs have tumor suppressor roles. As many as 98 of 186 miRNA genes are located in genomic regions or in fragile sites, suggesting that miRNA loss-of-function may be involved in cancer progression (Calin et al., 2004b).

Alternatively, miRNAs could promote cancer if an oncogene is translocated 3′ relative to a miRNA promoter. An example of this mechanism is the translocation of the myc gene to a site 4 nucleotides 3′ relative to miR142, causing an aggressive B-cell leukemia due to upregulated MYC expression (Gauwerky et al., 1989). One consequence of myc translocation is loss of a conserved 20 nucleotide element 3′ relative to miR142 (Gauwerky et al., 1989). It is possible that loss of this element causes inefficient processing of the miR142/MYC fusion transcript resulting in a reduction of miR142, which leads to increased translation and higher levels of MYC protein.
3. miRNAs in Mammalian Virus Infection

Mammals produce short RNAs during viral infections. Researchers cloned short RNAs from a Burkitt’s lymphoma cell line latently infected with EBV (Pfeffer et al., 2004). The EBV miRNAs were produced from two regions of the EBV genome. The first cluster is located within the mRNA of the BHRF1 gene, encoding a distant Bcl-2 homolog. The other EBV miRNAs cluster in the intronic region of the BART gene whose function is still unknown.

Researchers examined the expression of the miRNAs in cell lines infected with the EBV virus in different stages of latency. Interestingly, BART miRNAs were detected in every stage but were significantly higher in cells in the lytic phase. BHRF miRNAs appeared to be downregulated at the early stages of latency but were present during the later stages of latency and the lytic phase. Although BHRF1 is a lytic-stage protein, earlier studies have shown that

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Families</th>
<th>Target gene</th>
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<tr>
<td>miR17/20/106</td>
<td></td>
<td>IL17E Interleukin 17E</td>
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<tr>
<td>miR17/20/106</td>
<td></td>
<td>PTEN Phosphatase and tensin homolog</td>
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<tr>
<td>miR17/20/106</td>
<td></td>
<td>RUNX1 Runt-related transcription factor 1</td>
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<tr>
<td>miR25/32/92/367</td>
<td></td>
<td>CD69 CD69 antigen (p60, early T-cell activation)</td>
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<tr>
<td>miR15/16/195, miR-181, miR182</td>
<td></td>
<td>BCL2 B-cell lymphoma 2 protein</td>
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<tr>
<td>miR181</td>
<td></td>
<td>BCLAF1 BCL2-associated transcription factor 1</td>
</tr>
<tr>
<td>miR181</td>
<td></td>
<td>CD4 CD4 antigen (p55)</td>
</tr>
<tr>
<td>miR30</td>
<td></td>
<td>PRDM1 PR domain containing 1, with ZNF domain isoform (BLIMP1)</td>
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<tr>
<td>miR30</td>
<td>STIM1</td>
<td>Stromal interaction molecule 2</td>
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<tr>
<td>miR30</td>
<td>NFAT5</td>
<td>Nuclear factor of activated T cells 5</td>
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<tr>
<td>miR30</td>
<td>CBFβ</td>
<td>Core-binding factor, beta subunit</td>
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<td>miR30</td>
<td>BCL9</td>
<td>B-cell CLL/lymphoma 9</td>
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<tr>
<td>miR200b</td>
<td>BCL11B</td>
<td>B-cell CLL/lymphoma 11B</td>
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<tr>
<td>miR200b</td>
<td>TCF8</td>
<td>Transcription factor 8 (represses interleukin 2)</td>
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<tr>
<td>miR130/301</td>
<td>BTG1</td>
<td>B-cell translocation protein 1</td>
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<tr>
<td>miR130/301</td>
<td>IRF1</td>
<td>Interferon regulatory factor 1</td>
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Several of the known miRNAs (column 1) have predicted target sites in the 3’ UTR of genes involved in immune-specific functions. Based partly upon exact nucleotide complementarity between the 5’ end of the miRNA and the target mRNA, miRNAs have predicted targets in the immune system (column 2). Targets include transcription factors required for B-cell-specific transcriptional activation and differentiation markers for B- and T-cell lineages. As observed in Table 1, one particular miRNA may target more than one gene, and one gene may have binding sites for more than one particular miRNA. Note that some microRNAs, such as miR16, may be expressed in nonhematopoietic tissues but may have regulatory targets that are immune-restricted. A more comprehensive listing of miRNA target predictions can be found at http://genes.mit.edu/targetscan/. (Adapted from Lewis et al., 2003.)
latent-stage EBV seems to transcribe this gene (Murray et al., 1996; Oudejans et al., 1995). It is possible that these transcripts are processed to miRNAs during this stage and there is no BHRF protein production. However, it remains unclear how the virus overcomes this block during the lytic phase and produces the BHRF protein. The authors speculate that during the lytic phase, the expression level of the BHRF genes increases and high transcript levels exceed cellular miRNA processing capacity and the unprocessed transcripts then get translated. The BART miRNAs target the mRNA of virally encoded DNA polymerase BALF5 for degradation. This could be a key step in initiating the lytic phase of the virus. Therefore, the working model for EBV-derived miRNA function is regulation of the transition between latent and lytic phases of the replication cycle. One surprising observation was that the noncoding hairpins EBERs1 and 2 (Glickman et al., 1988), which are the most abundant transcripts in the latent phase and are absent in the lytic phases, did not demonstrate detectable miRNA production.

Though miRNAs appear to be a mechanism used by the virus to subvert the molecular immune system, there could be another intended function for miRNAs used by mammalian cells. Similar to the paradigm established in plants, short RNAs produced by mammalian cells in response to virus infection could be used to prevent virus infection and establish intracellular immunity against infecting viruses. These apparently paradoxical “proviral” versus “antiviral” effects of short RNA may be explained by the idea that large DNA viruses such as EBV have evolved mechanisms to co-opt the virally derived short RNA, produced by the host and intended to cleave viral transcripts, and instead use these short RNAs to silence genes that effect the transition between latent and lytic stages of the virus replication cycle.

4. RNAi, NMD, and TCR

In addition to miRNA-directed translational repression, RNAi may play important roles in dsRNA-directed surveillance events in lymphocytes. Antigen-receptor expression requires successful recombination of several repetitive and highly related genetic elements including variable (V) diversity (D) and joining (J) region gene segments. Recombination leading to premature termination codons creates a cellular stress that could lead to lymphocyte death. During V(D)J recombination, there are two intermediate steps that add to antigen-receptor diversity. Recombinase Activating Genes (\textit{rag})1 and 2 initiate V(D)J recombination by introducing a DNA double-strand break at the border of the recombination signal sequence, leaving hairpin-sealed coding ends and blunt signal ends that are usually excised from the chromosome (reviewed in Gellert, 2002). The hairpins generated at the end of the
coding segment are resolved by endonucleolytic cleavage by the newly identified factor Artemis, and there is transfer of nucleotides (P-addition) from the complementary strand to the coding strand, thereby adding to the diversity (Ma et al., 2002; Pannicke et al., 2004). Secondly, during repair of intermediates in V(D)J recombination, nongermline-encoded sequence (N-addition) is added to broken ends before joining through the action of terminal deoxynucleotidyl transferase (TdT). TdT is a template-independent DNA polymerase expressed only in cells actively undergoing V(D)J recombination (Gilfillan et al., 1995).

Although these mechanisms for the addition of nontemplated nucleotide addition to antigen-receptor transcripts lead to variability in nucleotides at the junction of V, D, and J segments and increase the receptor repertoire, they also change the reading frame and therefore two of three rearrangement events can generate premature termination codons (PTCs). If the truncated proteins encoded by PTC-bearing antigen-receptor genes were translated, they could be deleterious as they act as dominant-negative mutants that inhibit the function of the wild-type immunoglobulin (Ig) and T-cell receptor (TCR) proteins (Herskowitz, 1987). Earlier work demonstrated that truncated Ig proteins also induce a stress response in the endoplasmic reticulum (ER) if they are overexpressed or misfolded (Pahl and Baueerle, 1997). Recent work shows that the unfolded protein response (UPR), a multifaceted signaling pathway emanating from the ER membrane, plays a key role in the differentiation of mature B cells to plasma cells. UPR upregulates the expression of ER chaperones, folding enzymes and factors like X-box binding protein 1 (XBP1) which are known to be crucial for the terminal differentiation of B cells (reviewed in Gass et al., 2004). One can envisage a scenario in which in the absence of nonsense-mediated decay (NMD) there would be an overload of truncated Ig molecules in the ER leading to UPR signaling which could adversely affect B-cell development.

How does the adaptive immune system get around this problem? It was observed over a decade ago that the steady-state level of PTC containing Ig and TCR mRNAs (Aoufouchi et al., 1996; Baumann et al., 1985; Carter et al., 1995; 1996; Connor et al., 1994; Jack et al., 1989; Li et al., 1997; Lozano et al., 1994) is dramatically lower than that of productively rearranged counterparts, although the transcriptional rates of these genes are comparable. This posttranscriptional quality-control mechanism is termed nonsense-mediated mRNA decay. NMD distinguishes the product of a successful rearrangement and selectively allows its translation. It is important to note that this selection is being made at the level of RNA, thus allowing the system to be promiscuous at the level of DNA and generate greater diversity, but then imposes a conservative checkpoint at the level of protein.
In worms, regulation of premature termination codons by nonsense-mediated decay is genetically linked to RNAi. Worms that are mutated in certain genes required for nonsense-mediated decay are also defective in RNAi. The following sections explore the unique relationships between RNAi genes and processes required for antigen receptor expression.

5. NMD and TCR Expression

In mammalian cells, NMD is triggered by pre-mRNA processing (Maquat, 2004). Pre-mRNA is bound by cap-binding proteins CBP80 and CBP20 at the 5’ cap and by poly(A) binding protein PABP2 at the 3’ poly(A) tail (Fig. 2). Splicing deposits an exon junction complex (EJC) 20–24 nucleotides 5’ of the exon–exon junction. Minimally, the EJC consists of RNPS1, SRm160, UAP56, Y14, REF, and TAP-P15, (Lejeune et al., 2002; Reichert et al., 2002) proteins involved in splicing and mRNA transport. Then this complex acquires proteins involved in NMD starting with Upf3/3X, a nuclear protein that is exported to the cytoplasm with the mRNA (Gehring et al., 2003; Kim et al., 2001). A perinuclear protein Upf2 is then recruited to the complex forming the pioneer translation initiation complex (Lykke-Andersen et al., 2000; Serin et al., 2001). NMD is initiated during this preliminary round of translation when a PTC resides more than 50–55 nucleotides away from the 5’ end of an exon–exon junction (Ishigaki et al., 2001). The EJC-Upf3/3X-Upf2 complex together with Upf1 triggers NMD. Upf1 is a RNA-dependent ATPase and 5’ to 3’ helicase, which unlike other Upfs, does not appear to form a stable complex with EJC (Mendell et al., 2002; Sun et al., 1998). Upf1 phosphorylation by SMG1 (phosphatidylinositol kinase-related protein kinase) is vital for NMD (Denning et al., 2001; Yamashita et al., 2001). The RNA degradation involves decapping followed by 5’ to 3’ decay, as well as deadenylation followed by 3’ to 5’ decay (Lejeune et al., 2003). In order for normal translation to proceed, the initial translation initiation complex must be remodeled. The EJC and the Upf proteins are removed, CBP80 and CBP20 are replaced by eukaryotic initiation factor 4E, and at the 3’ end, PAPB2 is replaced by PABP1 (Lejeune et al., 2002).

Intron position within a pre-mRNA is an important determinant of NMD. According to the established rule, PTCs followed by an intron located 50–55 nucleotides downstream usually elicit NMD (Nagy and Maquat, 1998). The exception is provided by both the TCRβ and IgH transcripts that are susceptible to NMD even when the distance is less than 50–55 nucleotides (Carter et al., 1996; Wang et al., 2002). The reason for this exception to the rule is unknown. One possibility is the presence of cis-elements within these transcripts that catalyze NMD, other than an exon–exon junction. This possibility
Figure 2  NMD and RNAi are directly related. Pre-mRNA consists of the exons (colored boxes) and the introns (line between boxes). Splicing results in the removal of introns and deposition of a complex of proteins called the exon junction complex (EJC) 20–25 nucleotides (nt) 5' relative to the exon–exon junction. The processed mRNA is bound by cap-binding proteins (CBP20 and CBP80) at the 5' cap and by the polyA binding protein (PABP2) at the 3' poly A tail. The EJC recruits Upf3 (ortholog of SMG4) or Upf3X, the first factors involved in NMD that associate with mature mRNA. Upf3 or Upf3X is thought to recruit Upf2 (ortholog of SMG3) leading to the first (pioneer) round of translation. It is not clear whether multiple ribosomes are involved in this process, although the substrate for this event is a messenger ribonucleoprotein (mRNP) complex that is very different from the mRNPs during subsequent rounds of translation. Events during the pioneer round of translation determine whether the mRNA is going to be translated or destroyed. In the absence of a premature termination codon (PTC), the pioneer translation initiation complex is modified to the steady-state translation initiation complex. The events numbered 1–3 lead to translation of mRNA. (1) The EJC complex and Upf proteins are removed. (2) CBP20 and CBP80 are replaced by the eukaryotic initiation factor eIF4E. (3) The poly-A binding protein PABP2 is replaced by PABP1. When a PTC is located 50–55 nucleotides (nt) 5' relative to the exon–exon junction, a series of events are triggered (numbered 4–6) that lead to decay of mRNA. (4) Upf1 (ortholog of SMG2) is recruited to the EJC and likely functions in translation termination. (5) Decapping of the mRNA occurs, followed by 5'-to-3' exonucleolytic decay. (6) Deadenylation of the mRNA occurs, followed by 3'-to-5' exonucleolytic decay. Several of the SMG proteins (orthologs of Upfs) have been implicated in the siRNA pathway.
is supported by the identification of regulatory sequences from two different TCRβ transcripts that were localized to a region composed of the rearranged V(D)J exon and the immediately flanking intron sequences (Gudikote and Wilkinson, 2002).

Typically, NMD is 10- to 100-fold more efficient in TCR and Ig transcripts relative to other mammalian transcripts such as β-globin, v-src, or triosephosphateisomerase (TPI) (Baumann et al., 1985; Carter et al., 1996; Cheng and Maquat, 1993; Jack et al., 1989; Naeger et al., 1992; Simpson and Stoltzfus, 1994). Introduction of the VDJ exon, together with immediately flanking introns of TCRβ, was sufficient to trigger robust downregulation in response to nonsense codons in other mammalian genes like TPI (Gudikote and Wilkinson, 2002). Importantly, PTCs located within 3′, but not 5′ of the VDJ exon triggered strong downregulation of gene expression by NMD (Gudikote and Wilkinson, 2002). The same holds true for IgH transcripts, where a 177-long sequence comprising the 5′ half within the VDJ exon is necessary for efficient NMD (Buhler et al., 2004).

Another distinctive feature of NMD in lymphoid cells is the cell specificity. Transcripts from a TCRβ construct with a PTC in the penultimate exon is only downregulated in T cells and not in a nonlymphoid cell line (Carter et al., 1996). Biochemical assays using B-cell nuclear extracts showed a decreased level of PTC-bearing processed Igκ transcripts, a result not observed in nuclear extracts from other cell types (Aoufouchi et al., 1996). In the same system, the B-cell nuclear extracts preferentially downregulated PTC-bearing Igκ transcripts compared to β-globin transcripts. This lymphoid specificity might be a consequence of quantitative differences in trans-acting factors present rather than a fundamental difference in the mechanism of NMD.

6. RNAi and NMD Are Genetically Linked

The NMD and RNAi mRNA degradation pathways are linked in worms. SMG (suppressor with morphogenic effect on genitalia) factors were shown to be involved in NMD in worms. A study in C. elegans revealed that three smg genes, smg-2, smg-5, and smg-6, were required for persistence of RNAi as long as the target mRNA was continuously transcribed (Domeier et al., 2000). Animals with a mutation in either of these genes initially demonstrated silencing at levels comparable to wild-type worms but rapidly recovered from RNAi. These results suggest that these proteins do not have a role in initiating RNAi-mediated silencing but function in maintenance of the silencing signal. The SMG proteins are required for NMD and mutations that block the phosphorylation of SMG2 by SMG1, and those that impair the subsequent dephosphorylation by SMG5, SMG6, and SMG7, eliminate NMD. Interest-
ingly, smg-2, smg-3, and smg-4 in C. elegans have orthologs Upf1, Upf2, and Upf3, respectively, in mammals. Based upon sequence conservation, all the other SMG proteins also have mammalian orthologs.

Posttranscriptional gene silencing by RNAi and mRNA surveillance by NMD was also connected in the green algae, Chlamydomonas reinhardtii. Researchers showed that the DEAH-box RNA helicase, Mut6 is involved in both processes (Wu-Scharf et al., 2000). Mut6 appears to be part of a nuclear complex comprised of a single-stranded RNA-binding protein and a ribonuclease containing a staphylococcal nuclease-like domain. There is speculation that Mut6 might be part of a nuclear RISC-like complex. Studies in C. elegans suggest that RNAi does not target pre-mRNA. But they do not rule out the possibility that a nuclear RISC-like complex might selectively target nonpolyadenylated transcripts from transposons.

In mammals, RNAi has been shown to be restricted to the cytoplasm (Hutvagner and Zamore, 2002; Martinez et al., 2002) although there are alternate possibilities. A fraction of the RISC-complex may be located at the nuclear pore, potentially accessing the transcripts prior to complete export from the nucleus. This mechanism would allow the RISC complex to scan the RNA that is being exported and function as a quality-control checkpoint.

The subcellular location of NMD of the TCR and Ig transcripts is controversial (Wilkinson and Shyu, 2002). Two independent approaches were used to demonstrate that inhibition of mRNA export did not affect the downregulation of PTC-containing TCRβ transcripts in the nuclear fraction of mammalian cells (Buhler et al., 2002; Li et al., 1997). These observations provide strong evidence for intranuclear NMD. Translation is a necessary event for NMD and whether translation can occur in the nucleus is also a matter of debate (Kapp and Lorsch, 2004; Strudwick and Borden, 2002). By one estimate, nuclear translation may constitute as much as 10–15% of reporter gene-translation activity in mammals (Iborra et al., 2001).

RNAi and NMD are distinct but genetically related RNA degradation pathways that serve the common function of removing potentially deleterious RNA from the cell. Though there are obvious mechanistic differences between these two pathways, there are likely some overlapping mechanisms. In vitro studies revealed that RISC is bound to ribosomes in cell-free extracts (Hammond et al., 2000) potentially connecting RNAi to translation and as discussed earlier, initiation of translation is an integral part of NMD.

Identification of common factors in lower organisms that are required for both RNAi and NMD has raised the tantalizing possibility that RNAi and NMD may be linked in the mammalian adaptive immune system. Because antigen receptor genes acquire PTCs at very high rates and the NMD pathway functions at its highest efficiency in lymphocytes relative to other cell types
(Baumann et al., 1985; Carter et al., 1996; Cheng and Maquat, 1993; Jack et al., 1989; Naeger et al., 1992; Simpson and Stoltzfus, 1994), it is logical to investigate potential links between RNAi and NMD in the adaptive immune system.

7. RNAi, ADARs, and Viruses

Although RNAi and NMD are two pathways acting synergistically to affect RNA degradation using some common factors; in worms, RNAi has an inverse relationship with other RNA-directed processes such as the RNA-editing pathway. Changes in mRNA sequence may result in diversifying or correcting information encoded by the genome. RNA editing is another posttranscriptional mechanism to alter RNA sequence important for cellular and viral genes. Two types of RNA editing have been described in nuclear-encoded mRNA. Inosine formed by adenosine deamination (A-I editing) is the most frequent form of editing (Bass, 2002; Seeburg, 2002) while uridine formed by cytidine deamination (C-U editing) (Balnc and Davidson, 2003) is relatively rare. An A-I editing enzyme was first discovered in Xenopus through its ability to unwind long dsRNA by deaminating multiple As to Is, which result in unstable I:U base pairs (Bass and Weintraub, 1987; 1988) (Fig. 3A). A-I editing is catalyzed by adenosine deaminases that act on RNA (ADARs).

7.1. RNAi Versus ADAR Activities

The substrate for ADAR activity is dsRNA and in worms, RNA editing is competitively related to RNAi. Worms with high-ADAR activity are poor at RNAi. Conversely, worms with low-ADAR activity are efficient at RNAi. Wild-type and adr-1;adr-2 mutant worms trigger RNAi in response to injected long dsRNA directed against a reporter. Only in somatic tissues of adr-1;adr-2 mutant worms but not in somatic tissues of wild-type worms, long dsRNAs produced from a transgene expressing repetitive arrays triggered RNAi. In these experiments, ADAR's ability to antagonize RNAi triggered by a transgene required a dsRNA intermediate (Knight and Bass, 2002) and worms were capable of editing injected, long dsRNA (Domeier et al., 2000). Furthermore, adr-1;adr-2 double-mutant worms demonstrated a reduced ability to silence transgene-triggered RNAi when the complexity of the repetitive transgene was reduced (when random DNA was inserted in the repetitive array) (Knight and Bass, 2002). These observations suggest host cells are capable of discerning the origin of the trigger dsRNA.

In another study, it had been observed that deletions in each or both of the C. elegans adr genes (adr-1 and adr-2) produced animals with defects in
Figure 3 Competition between ADAR activity and RNAi. (A) ADARs convert adenosines to inosines by hydrolytic cleavage. Inosine is translated as guanosine, which base pairs with cytidine. (B) dsRNA is the substrate for both ADARs and the RNAi machinery. RNAi and ADAR activities appear to function in a mutually exclusive fashion suggesting a model of noncompetitive inhibition. Editing could change the secondary structure of long dsRNA making it a poor substrate for Dicer processing of long dsRNA into siRNAs (left panel). Conversion of long dsRNA into siRNA could antagonize the activity of ADARs because siRNA duplexes are expected to be too short for adenosine deamination (right panel). Viral dsRNA and cellular single strand RNAs that form intramolecular dsRNA duplexes may be targeted by different ADARs in the nucleus prior to export to the cytoplasm to prevent Dicer activity. Conversely, ADAR1(p150) and Dicer may directly compete for long dsRNA binding in the cytoplasm. These mechanisms are not well understood.
chemotaxis (Tonkin et al., 2002). When *C. elegans* strains lacking ADARs were crossed with RNAi-defective mutants *rde1* and *rde4*, the *rde-1* and *rde-4* alleles rescued the chemotaxis defects of the *adr-1;adr-2* animals (Tonkin and Bass, 2003). In flies, ADAR activity competes with RNAi in vitro (Scadden and Smith, 2001). Also, subcellular localization or alternate contexts may be important determinants in the relationship between ADAR activity and RNAi.

ADARs have the ability to create sequence and structural changes in dsRNA, which could affect the RNAi pathway in several ways. First, editing could prevent dsRNA from being recognized by Dicer and make it a poor substrate for siRNA production. Second, the mRNA targeting step of RNAi could be inhibited by the substitution of I:U for A:U base pairs between the siRNAs and the target mRNA. Finally, I:U base pairs within the siRNA duplexes could interfere with the ability of RNA-dependent RNA polymerase (RdRP) to recognize the siRNA as a primer. Conversely, RNAi could also impede ADAR activity. Conversion of dsRNA to siRNA would antagonize the activity of ADARs, because siRNA duplexes are expected to be too short for adenosine deamination. Thus, in the absence of ADAR activity, the RNAi pathway should be more effective, whereas any deficiency in the RNAi pathway should enhance ADAR activity.

Three ADAR family members are expressed in mammals. ADARs from all organisms have multiple dsRNA binding (DSRB) domains at varying distances from each other and a highly conserved C-terminal catalytic domain (reviewed in Bass, 2002). ADAR1 has two forms: a long form (150 kDa) produced in response to interferons, which is localized in the cytoplasm and a short form (110 kDa) which is constitutively nuclear (George and Samuel, 1999b). Because RNAi is restricted to the cytoplasm and RNA editing is both nuclear and cytoplasmic depending upon the ADAR activity, RNA editing may have a noncompetitive relationship with RNAi for dsRNA binding in the nucleus (Fig. 3B). ADAR activity may be prevented from antagonizing RNAi and the relationship between ADAR activity and RNAi may differ from tissue to tissue depending upon the triggering source of the dsRNA and other factors present in a particular cell type.

ADARs target mRNAs in coding regions, as well as in noncoding sequences such as 5’ and 3’ UTRs. Most of the known cellular substrates of the ADARs are in the nervous system (Bass, 2002; Sanders-Bush et al., 2003). ADARs target mRNAs of five glutamate receptor subunits and all the editing occurs within the dsRNA that forms between an exon sequence and an intron with an exon-complementary sequence (Seeburg et al., 1998). ADARs also target the mRNA encoding the 2C subtype of the serotonin receptor (Niswender et al., 2001). Other examples of neuronal substrates (calcium and sodium channel
forming proteins) of ADARs are found in flies (Hanrahan et al., 2000; Palladino et al., 2000; Reenan et al., 2000).

The ADAR1 knockout mouse revealed a heterozygous embryonic lethal phenotype and though most of the heterozygous embryos died before embryonic day 14 (E14), each had defects in hematopoiesis (Wang et al., 2000). The expression level of ADAR1 was reported to increase in the liver from day E13 to E14. It is feasible that ADAR1 targets in the liver need to be edited at this point for further embryonic development. Because early development of the adaptive immune system occurs in the fetal liver, one may speculate that ADAR substrates are involved in this developmental process.

7.2. ADARs and Viruses

Although there are no known targets of the ADARs in the immune system, ADARs play a significant role in the cellular response to viral pathogens. ADAR activity on transcripts of measles virus, polyoma virus, and hepatitis delta virus (HDV) has been well characterized. Soon after the discovery of ADARs, a viral RNA isolated from the brain of a patient who died of a persistent measles virus infection corresponded to changes resembling an ADAR substrate (Cattaneo et al., 1988). The functions of these mutations are still unclear, but there is speculation that these mutations somehow lead to viral persistence. ADAR1 edits HDV in vitro, an event reportedly required for the virus life cycle. HDV encodes two forms of a single protein (HD-Ag). RNA editing converts a stop codon to a tryptophan resulting in the long form (HD-Ag-L) as opposed to the short form (HD-Ag-S) of the viral protein (Casey and Gerin, 1995; Luo et al., 1990; Polson et al., 1996). Both proteins are essential for the viral cycle: the short form is necessary for replication (Kuo et al., 1989) and the long form for assembly (Chang et al., 1991). In the case of polyoma virus, conversion of adenosines to inosines serves a novel purpose. Transcripts with a large number of inosines are retained in the nucleus by a complex containing an inosine-specific RNA-binding protein (Zhang and Carmichael, 2001). These transcripts are not translated, leading to downregulation of the protein products. The early transcripts of the polyoma virus are regulated via ADARs in this fashion allowing the late transcripts to be selectively exported (Kumar and Carmichael, 1997).

Several other observations provide indirect evidence that A-I editing plays a role in inhibiting viral replication and helps modulate cellular responses to virus infection (Jayan, 2004; Jayan and Casey, 2002; Macnaughton et al., 2003). Genes induced by interferons are known to function in viral defense. Although there is no direct evidence to support this idea, unlike other ADARs,
the cytoplasmic localization of the long form of ADAR1, and its production from an interferon-inducible promoter (George and Samuel, 1999a) gives some credence to the theory that the long form of ADAR1 functions in viral defense.

The other type of RNA editing which is less frequent in the mammalian system is C to U. The best-characterized example of this form of RNA editing involves the nuclear transcript encoding intestinal apolipoprotein B (apo B) (Lau et al., 1991). The enzyme responsible for this editing activity is APOBEC-1 (apo-B editing catalytic subunit 1). Recently, two members of the APOBEC family, APOBEC3G and AID (activation induced cytidine-deaminase) have been the focus of intensive investigation by immunologists (Wedekind et al., 2003). APOBEC3G induces hypermutations on newly synthesized viral DNA, acting as an antiviral agent. A virus like HIV-1 has developed a mechanism to counter APOBEC3G activity. The viral protein called viral infectivity factor (Vif) binds to APOBEC3G triggering its polyubiquitination and rapid degradation preventing its entry into progeny virions. Without Vif, the encapsidated APOBEC3G would damage the viral reverse transcripts causing their degradation (Bhagwat, 2004; Schrofelbauer et al., 2004). AID is necessary for both class switching and somatic hypermutation in B cells. These proteins have RNA- and DNA-editing activity in vitro though their principle substrate may be DNA in vivo. There are two distinct schools of thought regarding how AID functions. The more popular idea is that AID edits DNA, causing C-to-U conversions which leads to AP-endonuclease-mediated breaks necessary for class switching (Chaudhuri and Alt, 2004; Conticello et al., 2004). The alternative view suggested, is that AID edits an unidentified mRNA and the edited form of mRNA codes for a DNA endonuclease (Honjo et al., 2004). However, there has been no reported link between C-to-U RNA editing and RNAi.

8. Conclusions

Recent observations in the studies of RNAi and short-RNA function provide evidence that RNAi and related pathways could be critical for lymphocyte-specific gene expression and development. microRNAs are expressed from endogenous genes that are conserved (reviewed in Bartel, 2004). The common theme for miRNA function across phyla is their participation in developmental transitions and cell-specific gene expression. When small RNAs are derived from transgenes or mobile genetic elements, the common theme that emerges is their participation in genome defense. In plants, short RNAs act as an antivirus “immune system” (reviewed in Lecellier and Voinnet, 2004). Recent evidence indicates that the RNA arms race may have reached
mammals. Mammalian B cells may possess similar short RNA-directed antiviral defense mechanisms observed in plants (Pfeffer et al., 2004). Conversely, influenza and vaccinia viruses express factors that inhibit mammalian RNAi (Li et al., 2004).

RNAi has not yet been implicated in antigen-receptor expression in lymphocytes, directly. However, there are several posttranscriptional quality-control mechanisms in place that may perform a surveillance function in antigen-receptor expression in addition to RNA editing and NMD. Curiously, extensive antisense transcripts have been identified in immunoglobulin heavy chain variable (IgHv) region genes. It may be possible that long dsRNA complementary to promoter regions in IgHv is processed by Dicer and leads to short RNAs that affect accessibility at the IgHv promoters. Indeed, antisense transcription has been implicated in methylation, TGS, and human disease (Tufarelli et al., 2003). Observations such as these could provide the link between RNAi and control of antigen receptor expression.

An intracellular immune system helps an organism defend against genetic challenges. Ruthless evolutionary pressure selects for genetic outliers, that is, rare organisms that possess advantageous genetic changes. However, the majority of challenges resulting in genetic changes are disadvantageous and lead to the death of the organism. In one model, an RNA-directed silencing pathway that potentially helps maintain genomic integrity may constitute an intracellular “immune system” with parallel functions to the cellular adaptive immune system (Chowdhury and Novina, 2005). In another model, an RNA-directed silencing pathway has been compared to the activation kinetics of the adaptive immune response (McManus, 2004).

Because it plays a central role in preservation of the integrity of an organism, the intended (immune) functions of RNAi may have been conserved through evolution, even if specific RNAi gene functions have been adapted to other processes. Unique relationships exist between dsRNAs and the genes mediating short-RNA function in lower eukaryotes. It is tempting to speculate that the homologs of genes implicated in RNAi in lower eukaryotes may be conserved in mammals. A better understanding of their roles in mammals may illuminate the biological roles of RNAi in lymphocytes.

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References


Further Readings


