

Review

Shhh! Silencing by microRNA-155

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Small RNAs mediate a diverse pot-pourri of post-transcriptional silencing mechanisms, ranging from ‘classical’ RNA interference (RNAi), to gene repression by microRNAs (miRNAs), to maintenance of genomic stability by repeat-associated small RNAs. Here, we review recent findings on the function of miR-155, particularly its roles in mammalian innate and adaptive immunity, viral infection and oncogenesis.

Keywords: RNAi; miRNA; miR-155; innate immunity; adaptive immunity

1. INTRODUCTION

Since the first proposition of RNA as an information-bearing molecule (Woese 1967; Crick 1968; Orgel 1968), an astounding breadth of RNA function has been revealed over the last several decades. Far from serving as mere intermediaries between DNA and protein, RNA molecules have proven to be dynamic entities bearing beautifully complex secondary structures capable of diverse molecular behaviours that alter gene expression. Messenger RNAs (mRNAs) throughout the living world undergo *cis* (and sometimes *trans*)-splicing reactions, at times with the option of alternative exons; editing by cytidine and adenosine deaminases (Smith 2008); and can even function as direct metabolite-sensing mediators of gene expression (Tucker & Breaker 2005). Non-coding RNAs hardly rank as the inferior cast-offs of their information-rich mRNA relatives. To cite but a few examples: ribosomal RNA (rRNA) and transfer RNA are universal components of the translation machinery; catalytic RNAs intimately participate in biochemical reactions (Strobel & Cochrane 2007); small nucleolar RNAs guide chemical modifications to rRNA (Kiss 2001); and small guide RNAs target mRNA editing events in kinetoplastid mitochondria (Simpson *et al.* 2000).

One can hardly discuss non-coding RNAs without mentioning those of the miniature persuasion, which have been implicated in post-transcriptional gene regulation. Early observations of an unexplained silencing phenomenon in floral pigmentation were termed ‘co-suppression’ (later known as RNA interference or RNAi). This was the unexpected outcome of the experiments performed by Jorgenson and colleagues, where transgenic overexpression of a pigment biosynthesis gene, *chalcone synthase*, in petunia plants often resulted in the production of flowers with variegated pigmentation or even complete lack of

colour, instead of more vividly coloured flowers (Napoli *et al.* 1990). What could have been dismissed as trivia for horticulture aficionados instead proved to be the first phenotypic evidence of a gene silencing mechanism that was also observed by others in fungi (Romano & Macino 1992) and nematodes (Guo & Kemphues 1995).

The mystery of this phenomenon was later unravelled in the landmark studies of Fire and Mello who uncovered a double-stranded RNA (dsRNA)-triggered gene silencing mechanism in *Caenorhabditis elegans* (Fire *et al.* 1998). The molecular mechanism of RNAi was further elucidated by Hamilton and Baulcombe who identified small, approximately 25 nt long RNAs complementary to silenced genes in plants undergoing transgene-dependent co-suppression (Hamilton & Baulcombe 1999). Biochemists and geneticists proceeded to describe the means of biogenesis and function for these small interfering RNAs (siRNAs). Through pathways conserved in fungi, plants and animals, dsRNAs are progressively chopped into small RNA duplexes by the RNaseIII-type enzyme Dicer (Hammond *et al.* 2000; Zamore *et al.* 2000; Bernstein *et al.* 2001; Hutvagner *et al.* 2001). Single-stranded 21–23 nt siRNAs derived from these duplexes then integrate into and guide the ribonuclease activity of the RNA-induced silencing complex (RISC) to an mRNA target in a sequence-specific manner (Hammond *et al.* 2001), leading to cleavage and silencing. This nucleolytic activity lies in the Argonaute (Ago) protein component of RISC (Hammond *et al.* 2001; Liu *et al.* 2004). Armed with an understanding of the mechanisms driving RNAi, molecular biologists have been able to adapt what began as a puzzling observation in plants into a powerful technique in the modern laboratory toolkit.

Parallel to the discovery of siRNA-mediated silencing, a related class of endogenously encoded small RNAs was described in *C. elegans* (Lee *et al.* 1993; Wightman *et al.* 1993). These microRNAs (miRNAs) are largely indistinguishable from siRNAs

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One contribution of 17 to a Discussion Meeting Issue ‘DNA deamination in immunity, virology and cancer’.

in terms of their biochemical make-up, and also engage many of the same molecular agents as siRNAs. They arise from the multi-step processing of a long primary miRNA (pri-miRNA) transcript that is 5'-capped and polyadenylated (Lee *et al.* 2002; Cai *et al.* 2004), and contains one or more hairpin structures each encompassing a mature miRNA sequence (Bartel 2004). Distinct ribonuclease-containing protein complexes in the nucleus and cytoplasm whittle the hairpin structures into small RNA duplexes (Lee *et al.* 2003), and as with siRNAs, one strand of each duplex is selected for incorporation into an effector protein complex (Hutvagner & Zamore 2002), which we will refer to here as RISC (which also goes by other similar names, depending on one's preferred terminology). Plant miRNAs function prevalently as siRNAs, binding with full complementarity to their cognate mRNAs and targeting them for endonucleolytic cleavage (Llave *et al.* 2002; Rhoades *et al.* 2002). Animal miRNAs, by contrast, are believed to latch onto mRNA target sequences with partial complementarity, and mediate silencing primarily through translational repression (Bartel 2004), although examples of mRNA destabilization have also been observed (Mansfield *et al.* 2004; Yekta *et al.* 2004). Although the current miRNA registry is by no means comprehensive, miRNAs have been identified in most eukaryotic model organisms, with the striking exception of *Saccharomyces cerevisiae* (Griffiths-Jones *et al.* 2008). In humans, the known miRNAs number in the several hundreds, some with evolutionary conservation reaching back to nematodes and arthropods (Griffiths-Jones *et al.* 2008). The miRNA gene pool is much like any other generic gene family: some miRNAs are phylogenetically ubiquitous, while others are restricted to single species; some are present in multiple cell types, while others are constrained in time and space; and some exist in single forms, while others comprise families of related 'isoforms' that differ by only a few nucleotides. An estimated 30 per cent of eukaryotic genes are subject to miRNA regulation (Lewis *et al.* 2003; Yu *et al.* 2007), implicating this mechanism as a substantial means by which organisms modulate their gene expression profiles. Unsurprisingly, this seeming prevalence of miRNA-mediated regulation throughout evolution and the living world has inspired many (including the authors of this review) to embark upon scientific quests to identify specific targets of miRNA regulation.

Kin of siRNAs and miRNAs have also been implicated in the silencing of repetitive elements in the genome. The centromeric repeats of fission yeast give rise to 22 nt heterochromatic small RNAs (Reinhart & Bartel 2002) that recruit an Ago-containing silencing complex distinct from RISC, called RITS (RNA-induced initiation of transcriptional gene silencing), to maintain the silenced heterochromatic character of the centromere (Volpe *et al.* 2002, 2003; Verdel *et al.* 2004). The most recent additions to the small RNA clan are the Piwi-interacting small RNAs (piRNAs), 25–31 nt long species enriched in metazoan germ cells (Hartig *et al.* 2007; O'Donnell & Boeke 2007). Unlike their more diminutive small RNA cousins, piRNAs arise in a Dicer-independent fashion, probably from a single-stranded

RNA precursor (Vagin *et al.* 2006; Houwing *et al.* 2007). They partner with the Piwi subfamily of Argonaute proteins to silence transposons in the germ line, and may play additional unknown roles in mice, whose piRNA repertoire includes only a handful matching to repetitive transposon sequences.

The burgeoning literature on small RNA function reflects on the diversity of essential tasks they perform in nearly all clades of life. Most of these small RNAs function in what one could broadly classify as self-protection—against exogenous sources of dsRNA or against endogenous selfish genetic elements. Plants generate siRNAs from invading viral genomes as one component of their antiviral immune defences (Ding & Voinnet 2007), although the necessity of mechanism appears to have dwindled in evolution with the advent of more complex immune systems, as similar virus-derived immune siRNAs have not been described in higher eukaryotes. siRNAs and piRNAs also shield the genome from damage by transposable elements, maintaining them in silenced and non-mobile states (Slotkin & Martienssen 2007). The heterochromatic small RNAs of fission yeast also play a role in the maintenance of genomic integrity, as they preserve the silencing of important chromosome structure elements (Volpe *et al.* 2002, 2003; Verdel *et al.* 2004).

With regard to function, animal miRNAs stand slightly apart—not necessarily final arbiters of silencing, but rather fine-tuners of gene expression with the capacity for coordinate regulation of groups of genes. In this review, we will discuss one microRNA, miR-155, as a representative example of the influence that a single non-coding small RNA can wield on multiple physiological processes.

2. miR-155

A perusal of miRBASE, the online miRNA registry, shows that miR-155 is quite well conserved in the animal lineage, having been identified in sea squirts, fishes, frogs and mammals (Griffiths-Jones *et al.* 2008). With the development of techniques to assay for global patterns of miRNA expression (by small RNA cloning and sequencing, or by array methods), it is possible to survey tissue-specific patterns of miRNA expression. One such dataset for human and mouse tissues shows that miR-155 is prominently expressed in many haemopoietic cell types (Landgraf *et al.* 2007). This is a fortuitous convergence, as modern immunologists have at their disposal detailed knowledge of immune cell lineages, cell surface markers that differentiate cell subsets and numerous assays both *in vitro* and *in vivo* for immune function—in other words, laboratory immunology is an excellent milieu in which to study the impact of a specific miRNA on cell development, maturation or effector function. Indeed, over the last few years, several complementary stories have implicated miR-155 as a key regulator of diverse immune processes.

3. miR-155 AS ONCOMIR

The story of miR-155 (although it was not thus named at the time) originates with studies in chickens inflicted with avian leukosis virus-induced lymphomas, which were known to harbour retroviral insertions at

proto-oncogenes such as *myc* and *myb* (Clurman & Hayward 1989). An additional preferred site of proviral insertion was identified in these lymphomas, named *bic*, for B-cell integration cluster (Clurman & Hayward 1989). Retroviral activation of *bic* was correlated with *myc* activation and tumour metastasis, suggesting collaboration between oncogenes to promote cancer progression. Homologues of *bic* were later identified in mouse and human (Tam 2001), but the functional significance of *bic* remained unknown for some time, as the gene lacked conserved open reading frames. The most conspicuously conserved feature in the Bic RNA was a predicted double-stranded fold-back motif, which would later be recognized as the precursor hairpin encoding miR-155 (Tam 2001; Eis *et al.* 2005).

Similar associations between Bic/miR-155 expression and human B-cell cancers began to emerge: Bic/miR-155 is highly overexpressed in lymphomas of activated-B-cell origin, including Hodgkin's lymphoma (van den Berg *et al.* 2003; Kluiver *et al.* 2005) and diffuse large cell B-cell lymphoma (Eis *et al.* 2005; Kluiver *et al.* 2005). Upregulation of miR-155 does not appear to be a universal feature of B lymphomas, however, as Burkitt's lymphomas express very little Bic (Kluiver *et al.* 2006), and furthermore demonstrate an ill-described defect in the processing of mature miR-155 from the Bic precursor (Kluiver *et al.* 2007). These correlational observations were complemented by the work of Croce and colleagues who created transgenic mice overexpressing miR-155 in B cells (Costinean *et al.* 2006). These mice developed pre-B-cell lymphoproliferative disorders, which later progressed to full B-cell lymphomas. The authors then assayed for changes in the transcriptome of these transgenic animals by microarray analysis, and found that 200 proliferation genes were upregulated—an unsurprising result, given that the general model of miRNA function holds that most miRNAs are capable of regulating multiple targets (Krek *et al.* 2005).

Not an exclusive bane of lymphoid cells, miR-155 was also detected at elevated levels in the bone marrow of some patients suffering acute myeloid leukaemia (O'Connell *et al.* 2008). Overexpression of miR-155 in haemopoietic stem cells in the mouse resulted in gross expansion of myeloid lineages in the bone marrow and peripheral blood at the expense of erythroid and lymphoid populations. These mice exhibited downregulation of approximately 1000 transcripts; of those containing putative miR-155 target sites, several genes involved in myeloid proliferation or genesis were highlighted as candidate miR-155 targets responsible for the myeloproliferative disorder.

The patently obvious clinical relevance of miRNAs to cancer has been demonstrated not only for miR-155, but for numerous others as well, thus designating a class of oncogenic miRNAs dubbed 'oncomiRs'. Given that miR-155 overexpression has additionally been observed in solid tumours of diverse origin (breast, lung and colon), assays for miR-155 expression could potentially serve as clinical diagnostic tools (Volinia *et al.* 2006). Furthermore, knowledge of specific miRNA expression can serve as a springboard for identification of tandemly regulated sets of genes whose downregulation may contribute to oncogenesis.

4. miR-155 IN INNATE AND ADAPTIVE IMMUNITY

The hazards of deranged miR-155 expression are clearly demonstrated by the diversion of lymphoid and myeloid cells to an oncogenic fate, but what is the normal role of miR-155 in the immune system? The earliest Bic enthusiasts observed low expression of Bic in haemopoietic and lymphoid organs of healthy chickens (Tam *et al.* 1997), suggesting some kind of inherent function outside of oncogenesis. As the miRNA field came to prominence, several groups noted that mature miR-155 was induced upon activation of myeloid and lymphoid cell types in the mouse (O'Connell *et al.* 2007; Rodriguez *et al.* 2007; Thai *et al.* 2007; Teng *et al.* 2008).

Baltimore and colleagues noted miR-155 upregulation as a consistent feature of the mammalian inflammatory response (O'Connell *et al.* 2007). Inflammation is a hallmark of innate immunity, which performs the first wave of anti-pathogenic defence. Specialized cells such as macrophages and dendritic cells recognize conserved pathogenic molecular motifs via Toll-like receptors (TLRs), triggering cytokine and chemokine production, recruitment of additional effector cells and the initiation of the later-acting adaptive immune response. Various TLR ligands that can simulate viral or bacterial infection *in vitro* induced miR-155 expression in monocyte and macrophage cell lines (O'Connell *et al.* 2007; Tili *et al.* 2007). This induction was dependent on the signalling pathways initiated by TLR activation, implicating miR-155 as a downstream player in innate immune function (O'Connell *et al.* 2007). However, the direct targets downregulated by miR-155 during inflammation have not been unequivocally confirmed.

Both B and T lymphocytes, key to the adaptive immune response, also display similar induction of Bic/miR-155 in response to activating stimuli (Haasch *et al.* 2002; Thai *et al.* 2007; Teng *et al.* 2008). Here, we will focus mainly on the findings in B lymphocytes. During an *in vivo* infection, the immediate innate immune response is later supplanted by the adaptive immune response, which can provide a greater degree of antigen specificity, as well as the generation of immunological memory. One component of this response is provided by the B lymphocytes, which manufacture antigen-recognizing immunoglobulins (Ig). These molecules arise on the B-lymphocyte cell surface during early development, and undergo additional functional maturation upon contact with their cognate antigens. These secondary maturation processes include: affinity maturation, which refers to the generation of Ig variants with increased affinity for their cognate antigens (achieved through somatic hypermutation, or SHM, of the Ig gene); and class switch recombination (CSR), which changes the Ig isotype (and hence, effector function). Mice deficient in miR-155 show clear defects in both of these processes, exhibiting reduced overall titres of serum Ig, and specifically, decreased titres of high-affinity and class-switched hapten-specific Ig (Rodriguez *et al.* 2007; Thai *et al.* 2007; Vigorito *et al.* 2007). These B-lymphocyte defects, along with faulty antigen presentation by dendritic cells and disturbed T lymphocyte maturation, fed into the gross phenotype

of the immunocompromised miR-155-deficient animal, which was unable to generate immunological memory, and thus could not protect itself from repeated infections with the same pathogen (Rodriguez *et al.* 2007). Transcriptome profiling revealed that approximately 60 putative miR-155 target genes were upregulated in these mice compared with wild-type counterparts (Vigorito *et al.* 2007). One of these potential targets was Pu.1, a transcription factor known to function in B-lymphocyte development (Scott *et al.* 1994; McKercher *et al.* 1996). Indeed, overexpression of Pu.1 in wild-type B lymphocytes recapitulated the CSR defect observed in miR-155-deficient cells, suggesting the existence of Pu.1-mediated regulation of Ig maturation (Vigorito *et al.* 2007).

Concomitantly, we and the Nussenzweig group independently identified activation-induced cytidine deaminase (AID) as a miR-155 target (Dorsett *et al.* 2008; Teng *et al.* 2008). AID provides the enzymatic impetus for both SHM and CSR in B lymphocytes (Muramatsu *et al.* 1999, 2000), and the AID mRNA harbours a very well-conserved miR-155 target site in its 3' UTR. Instead of evaluating the effects of global miR-155 deficiency, we examined the effects of specifically disrupting the interaction between miR-155 and its target site in the AID mRNA *in vivo*. B lymphocytes from mice bearing a mutated AID-miR-155 target site showed increased expression of AID mRNA and protein in activated B lymphocytes, as well as promiscuous expression in B-lymphocyte populations where AID activity should no longer be present. These expression defects were furthermore associated with increased CSR frequency, defective affinity maturation reminiscent of that reported by our colleagues and increased frequency of AID-mediated chromosomal translocations (Dorsett *et al.* 2008; Teng *et al.* 2008). Thus, miR-155-mediated regulation of AID serves a dual purpose—controlling abundance and timing of AID expression during the natural immune response, and prohibition of potentially oncogenic chromosomal aberrations.

The immune deficiencies of the miR-155-deficient mouse clearly reflect the unbalanced expression of a suite of genes far more complex than simply Pu.1 and AID. The challenge in the coming years will be to validate the panel of predicted target genes, and somehow integrate this knowledge to understand how a single miRNA can exert such diverse influence over multiple cell types to contribute to the coordination of a concerted cellular immune response.

5. miR-155 AND VIRUSES

Since the first computational and biological identification of virally encoded small RNAs (Pfeffer *et al.* 2004, 2005), a number of miRNA-mediated functions have been proposed on both sides of the virus–host equation (for review, see Gottwein & Cullen 2008). To date, only the dsDNA subset of viruses has been found to encode its own miRNAs, which largely regulate the expression of viral gene products (Gottwein & Cullen 2008). Viruses have also been known to exploit host miRNAs as survival mechanisms (Gottwein & Cullen 2008), and fascinatingly can even encode viral

doppelgangers of host miRNAs. One such miR-155 mimic has been described in Kaposi's-sarcoma-associated herpesvirus (KSHV; Gottwein *et al.* 2007; Skalsky *et al.* 2007). The KSHV miR-K12-11 seed region (the 5'-most eight nucleotides of an miRNA responsible for its targeting specificity) shares complete homology to that of miR-155, and both miRNAs can regulate a communal set of targets (Gottwein *et al.* 2007; Skalsky *et al.* 2007). Thus, in addition to all the insidious viral mechanisms of subverting host immunity, viral homologues of cellular miRNAs may further manipulate host gene expression to create an environment more palatable for viral survival and propagation. Exactly what functional parallels exist between viral infection and normal B-lymphocyte activation, both of which depend on the suppression of miR-155-responsive targets, remains to be seen. Gottwein and Cullen have also speculated on a role for viral miR-155 homologues in lymphomagenesis, noting that the MDV-1 herpesvirus, oncogenic in chickens, also expresses an miR-155-like miRNA, while its non-oncogenic cousin MDV-2 does not (Gottwein & Cullen 2008). This hypothesis is consistent with our knowledge of viral-transformation-induced Bic expression in avian lymphomas. Incidentally, the same is true in humans—Epstein–Barr virus (EBV), an oncogenic virus that latently infects human B lymphocytes, also induces host miR-155 expression (Yin *et al.* 2008a).

In this miR-155-mediated interplay between virus and host, we glimpse a fascinating cellular mutiny—part of the natural B-lymphocyte maturation programme is unfortunately diverted onto an alternative path leading to persistent viral infection, transformation and cancer.

6. miR-155 AS MULTITASKER

Although, miR-155 has been largely characterized as an immune-specific miRNA, its expression profile indicates that this is not necessarily the case. Outside of haemopoietic lineages, miR-155 is also expressed in mammalian reproductive tissues, fibroblasts and epithelial tissues, and the central nervous system (Landgraf *et al.* 2007). In fact, one of the earliest described miR-155 targets was the endothelial angiotensin II type 1 receptor (AT1R), whose ligand, angiotensin II, contributes to the development of cardiovascular disease (Martin *et al.* 2006, 2007). A single nucleotide polymorphism (SNP) in the 3' UTR of the human AT1R gene had long been associated with cardiovascular pathologies (Martin *et al.* 2007). It was shown that this SNP disrupted an miR-155 target seed region, impeding miR-155-mediated downmodulation of AT1R expression, thus allowing for increased pathological bioactivity of angiotensin II (Martin *et al.* 2007; Sethupathy *et al.* 2007). Contrasting to the previously described deleterious consequences of miR-155 expression, in this case miR-155 plays a protective role as a molecular safeguard against cardiovascular disease.

However, in keeping with the known immunoncogenic character of this miRNA, pancreatic cancer researchers have also noted the overexpression

of miR-155 in pancreatic ductal adenocarcinoma cells. The pro-apoptotic TP53INP1 (tumour protein 53-induced nuclear protein 1) was found to be suppressed by miR-155 in these pancreatic tumours. As loss of TP53INP1 has been observed in a number of other epithelial cancers, it is possible that miR-155 may contribute to a standard mechanism of oncogenesis in these types of tissues (Gironella *et al.* 2007).

Although miR-155 is by no means a ubiquitously expressed miRNA, it is neither snobbishly restricted to immune cells. What non-oncogenic purposes, if any, may it serve in these other tissues? Only time (and newly developing miRNA target validation strategies) will tell...

7. PERSPECTIVE AND FUTURE DIRECTIONS

RNAs perform some of the most astonishing acrobatics in biology, and the explosive discovery of small-RNA-mediated activities in the last few years has only spurred the rapt captivation of RNA devotees (the authors included). We discuss here the diverse contributions of one small RNA, miR-155, to many physiological processes, sometimes teetering on the edge of normal function and disease. Our understanding of miR-155 function is by no means complete, and we imagine that many of the remaining questions will be addressed by our colleagues in the coming years.

What controls the expression of miR-155 itself? Conflicting reports have implicated AP-1 (O'Connell *et al.* 2007; Yin *et al.* 2008b) and NF- κ B (Yin *et al.* 2008a) sites as control elements for Bic transcription, but the definitive set of transcriptional regulatory factors for the gene is not known. Two alternatively polyadenylated forms of Bic have also been detected (Tam 2001): are these isoforms equally competent at producing mature miR-155? Is one Bic isoform preferentially expressed in certain B lymphomas, and not others?

One cannot consider miRNA-mediated gene expression without querying the set of targets that it regulates. miR-155, being a relatively well-characterized representative of the miRNA family, could be an excellent candidate around which one could design an integrative scheme to look at global miRNA target regulation. That is, can we better understand miR-155 function by somehow cross-referencing miRNA target prediction algorithms, miRNA expression data, tissue-specific transcriptome data and proteomic profiles (using SILAC-based methods, for example)—an endeavour no doubt currently underway by industrious bioinformaticians. Subsequent target-by-target validation of resulting computational findings would be relatively easy to address in established cell- and animal-based models that have been described above.

From an evolutionary standpoint, has miR-155 as a gene regulator influenced the evolution of its targets? Are there genes that have non-functional or cryptic miR-155 target sites, or are there examples throughout phylogeny where genes have acquired miR-155 target sites, thus altering their fine-tuned expression profiles? Furthermore, how has the host-virus interaction influenced the evolution of miR-155-like miRNAs in viruses, particularly keeping in mind that viral

miRNAs are usually very poorly conserved in sequence (Gottwein & Cullen 2008)?

Given the association of miR-155 expression with diverse cancers, the therapeutic potential of miR-155 is clear. Is there an anti-cancer miR-155 inhibitor therapeutic in our future? Conversely, can we use our knowledge of miR-155 targets to treat cancer?

It is astounding that the tiny miR-155 molecule—a mere handful of ribonucleotides—can shape and reshape the physiological environment in a diverse range of tissues. This miRNA is but one of hundreds that, far from being evolutionary relics of the archaic RNA world, continue to play indispensable roles in the complex network we call gene regulation.

F.N.P. is supported by grants from the Keck Foundation and the National Institutes of Health (CA098495). G.T. is supported by a National Institutes of Health National Research Service Award training grant (GM066699).

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