Minireview

A small RNA makes a *Bic* difference

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Abstract

The first highly specific knockouts of a microRNA, *miR155*, in mice result in multiple defects in adaptive immunity, and also show the feasibility of investigating at least some microRNAs by gene knockout.

MicroRNAs (miRNAs) are endogenous, small noncoding RNAs that are critical for setting the precise tempo of gene expression for numerous cellular processes in virtually every eukaryotic organism. A common theme in miRNA function across multicellular organisms is that they affect developmental transitions and cell-specific functions. There are more than 500 miRNAs in humans and 450 miRs in mice [1]. Computational methods predict that miRNAs could post-transcriptionally regulate more than one third of all protein-coding genes [2,3], implying that they regulate enormous genetic regulatory circuits. The importance of miRNA-mediated regulation of gene networks is highlighted in mice lacking the enzyme Dicer. Knocking out this enzyme, which is essential for the production of mature, functional 21-23-nucleotide miRNAs from long precursor transcripts, proves lethal in the embryo [4]. The Dicer knockout underscores the importance of miRNAs in development, but it does not help illuminate the regulatory circuits affected by individual miRNAs. The highly specific gene knockouts of an immunologically important miRNA reported recently by Rodriguez *et al.* [5] and Thai *et al.* [6], who have independently produced knockout mice for *miR155*, begin to shed light on the complex molecular circuitry of individual miRNAs. Here we review some of their findings and some of the reasons for their success.

Advantages of *miR155* as a target for gene knockout

From a genomic perspective, *miR155* was an appealing choice. Many miRNAs have multiple copies in the genome, or share seed-region homology with other miRNAs. The seed region, nucleotides 2 to 8 relative to the 5' end of the miRNA, is a critical determinant of miRNA targeting of mRNAs. Perfectly complementary base-pairing in the seed region is the most important determinant of miRNA repression of target mRNA translation, and miRNAs with identical seed regions are predicted to have overlapping regulatory roles. Thus, a full phenotypic analysis would require the knockout of multiple genomic loci. To make matters even more complicated, increased base-pairing in the 3' end of a miRNA with its target mRNA can partially compensate for translational repression for miRNAs with nucleotide mismatches in the seed region of the miRNA [7]. The *miR155* gene is present in only one copy, and miR155 does not share significant sequence with other reported miRNAs. Therefore, a single knockout will eliminate a distinct subtype of regulation.

Another attractive property of *miR155* for gene knockout is its gene architecture. Most miRNA genes resemble typical protein-coding genes, although miRNAs derived from RNA polymerase III promoters were described recently [8]. Most miRNA genes contain a TATA box in the core promoter and cell-specific transcriptional regulatory elements affecting miRNA expression. Some miRNAs, however, are processed from transcripts with a second function, either from introns in a protein-coding gene, or as a multicistronic unit containing multiple miRNAs. Interestingly, miRNAs from a common cluster are not necessarily processed to the same degree [9,10], suggesting post-transcriptional control of miRNA expression. These multifunctional transcripts complicate the

specific targeting of an individual miRNA. In contrast, *miR155* is contained in an exon of a noncoding RNA gene called *Bic*, which does not contain other miRNAs, and which does not have any other conserved RNA sequence. Thus, *miR155* can be easily targeted for disruption without interfering with the expression of a protein-coding gene or a second transcriptionally linked miRNA.

miR155 was also an attractive target from a functional perspective. MicroRNAs and RNA-based gene regulation are known to have roles in immune-system function [reviewed in 11,12], and *miR155* is uniquely expressed in activated cells of the immune system [13-15]. In addition, this miRNA is highly expressed in Hodgkin's lymphoma and in diffuse large B cell lymphomas [16] and ectopic overexpression of *miR155* indicates that it is an oncogene [17]. Despite its immune-restricted expression, neither the *miR155*-null mice of Rodriguez *et al.* [5] nor those of Thai *et al.* [6] demonstrated major defects in hematopoiesis. Unlike previous experiments using dominant expression [18-20] or dominant repression [19] of miRNAs expressed in the immune system, the *miR155*-null mice did not demonstrate lineage biasing of normal hematopoiesis. In contrast, ectopic expression of another miRNA, *miR181*, increased the ratio of circulating B cells to T cells, although without the loss of one lineage entirely in favor of another lineage. These results suggest that miRNAs act as modulators rather than switches. Although no significant developmental defects were seen, both groups [5,6] observed that the *miR155* null mice had serious defects in immune function, a phenotype consistent with the expression of *miR155* primarily in activated lymphoid and myeloid cells.

miR155-null mice display defects in adaptive and innate immunity

In their knockout mice, Rodriguez *et al.* [5] deleted the *miR155*-containing portion of exon 2 of the *Bic* gene. Multiple aspects of protective immunity were seriously compromised in these mice. Most dramatically, vaccination of *miR155*-null mice with live attenuated vaccine against *Salmonella typhimurium* failed to protect them against challenge with virulent *Salmonella*. Rodriguez *et al.* found defects in all aspects of adaptive immunity. B cells from *miR155*-null mice secreted lower levels of IgM and had fewer class-switched antibodies after immunization compared with normal mice. Dendritic cells from the *miR155*-null mice did not present antigen efficiently and activate T cells. T cells from these mice activated *in vitro* displayed an increased predilection to differentiate into the Th2 T-cell lineage, as indicated by Th2-type cytokine production. mRNA expression profiling indicated that predicted targets of *miR155* were upregulated in the *miR155*-null, activated T cells. Rodriguez *et al.* [5] suggest that production of the transcription factor c-Maf is targeted by *miR155* during T-cell activation, and that dysregulation of c-Maf may be responsible for the altered T-cell cytokine production in the *miR155*-null mice. In addition to the deficiency in adaptive immunity, the authors also observed autoimmune phenotypes in the lungs of *miR155*-null mice. The increased airway remodeling and leukocyte invasion suggested that *miR155* plays a role in regulating the response of the immune system to self-antigens.

Thai *et al.* [6] engineered two transgenic mouse strains. In the *miR155* knockout mouse, they replaced exon 2 of *Bic* with a LacZ reporter gene, which allowed them easily to detect which cells activated gene expression from this locus. Thai *et al.* [6] also engineered a mouse that conditionally coexpressed *miR155* and the enhanced green fluorescent protein (GFP) in mature B cells. These two mice were used in combination to examine the effect of *miR155* on adaptive B-cell responses to antigen in germinal centers (GC). Germinal centers are microscopically visible areas that form in immune tissues such as lymph nodes in response to antigenic challenge. They consist of interacting dendritic cells, T cells and B cells and serve as foci for B-cell switching to produce different classes of antibodies, affinity maturation (the production of antibodies with progressively higher affinity for the antigen) and the generation of memory cells. In their *miR155*-null mice, Thai *et al.* [6] observed fewer and smaller germinal centers in response to antigenic challenge compared with control mice. Consistent with these observations, *miR155*-null mice were deficient in the production of class-switched and affinity-matured antibody. In contrast, mice ectopically expressing *miR155* produced more and larger germinal centers, and marginally more class-switched antibody. Thai *et al.* [6] attribute the changes in germinal center formation to deficiencies in the production of the germinal center-promoting chemokines lymphotoxin-α and tumor necrosis factor by *miR155*-null B cells. In addition, they also observed the Th2-biased T-cell chemokine production found by Rodriguez *et al* [5].

These two studies [5,6] provide considerable insights into the role of *miR155* in adaptive immunity. Perhaps more importantly, they show that a subset of miRNAs is amenable to analysis through genetic manipulation. But, despite these advances in interfering with miRNA-based regulation of immune activation, further analysis of *miR155*-null mice is required. Multiple interacting genetic networks in multiple immune cell types are regulated by *miR155*. For example, deletion of *miR155* affects both the ability of a dendritic cell to activate T cells and the subsequent response of the T cells to activation. To decipher the genetic networks in their proper cellular context, hematopoietic lineage-specific knockouts of *miR155* would be useful. In addition, such crosses could help to order the genes in a miRNA-regulated network, as complementation crosses have done in other eukaryotes. Alternatively, adoptive transfer of specific cell lineages between *miR155*-null and wild-type mice could illuminate the roles of *miR155* in specific cell types.

Approximately one-third of all miRs demonstrate the properties of miR155. These miRs are not contained within a protein-coding transcript and are expressed from single copy genes without redundant family members [1,21]. To elucidate the functional roles of the remaining miRs through homologous recombination of its gene or genes, new techniques are required, such as targeting very small genomic regions that contain multi-cistronic genes whose expression depends upon RNA secondary structure. Another technical advance that would facilitate phenotyping redundant miR families is rapid engineering of knockout mice altered at multiple redundant miR gene loci. Such gene inactivation through homologous recombination of several miR loci may help decipher the genetic regulatory networks governed through redundant miR activities.

Another intriguing possibility is that previous knockout mice may have inadvertently altered intronic miRNA gene expression. To investigate this possibility, we searched known mouse knockout databases against known databases of annotated miRNA genes. Examples of knockouts of protein-coding genes containing intronic miRNA include the calcitonin receptor gene *CalcR* [22] and the α-myosin heavy chain gene *α-MHC* [23]. The *CalcR* knockout did not delete intronic *miR489* and the *αMHC* knockout did not delete intronic *miR208*. Deletion of portions of the *CalcR* gene may have affected *miR489* expression and the deletion of portions of the *αMHC* gene may have affected *miR208* expression by disrupting miRNA processing from their host protein coding transcripts. Consistent with this possibility, ablation of the *αMHC* gene leads to dose-dependent phenotypes. Homozygous *αMHC* knockout mice are embryonic lethal whereas heterozygous *αMHC* knockout mice display severe impairment of contractility and alterations in sarcomere structure. The same issue of *Science* that contains the reports of the intronic *miR155* knockout mice [5,6] also contains a report on the intronic *miR208* knockout mouse [24]. The *miR208* knockout led to partially overlapping phenotypes with the heterozygous *αMHC* mice, especially alterations in contractility and sarcomere structure, portending the possibility that some phenotypes observed in *αMHC* heterozygous mice may be due to altered expression of intronic miRNAs. It is thus important to consider the existence and potential roles of intragenic miRNAs when making transgenic mice. As the numbers of identified miRNAs and knockout mice increases, it becomes increasingly probable that knockout mice may inadvertently affect miRNA gene expression. In these cases, phenotypes must be carefully analyzed for effects due to loss of miRNA function relative to loss of the host gene function.

It is likely that other miRNA knockout mice are under construction. However, it may be some time before the next mouse with a deletion of a single miRNA gene is described. MicroRNA knockouts may yield only subtle phenotypes, possibly due to multiple related miRNAs with sequence similarity, especially in the seed region. The general notion in the miRNA field is that the effect of any one miRNA on any one gene may be small in degree. Indeed, it is likely that miRNAs gain their power from cooperative activity in gene silencing. Either multiple miRNAs act upon one gene or one miRNA acts upon multiple genes in a particular pathway to effect large changes in gene networks. As our knowledge of epigenetic control of gene expression continues to expand, the *miR155* knockout mice made by Rodriguez *et al.* [5] and Thai *et al.* [6] are an important step in deciphering the multiple genetic networks regulated by miRNA function.

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