Leading Edge
Review
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miRiad Roles for the miR-17-92 Cluster in Development and Disease

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MicroRNAs (miRNAs) encoded by the miR-17-92 cluster and its paralogs are known to act as oncogenes. Expression of these miRNAs promotes cell proliferation, suppresses apoptosis of cancer cells, and induces tumor angiogenesis. New work reveals essential functions for these miRNAs not only in tumor formation but also during normal development of the heart, lungs, and immune system.

Over the last decade, a fundamental role for small RNA-guided posttranscriptional regulation of gene expression has been uncovered. In particular, the microRNA (miRNA) family of small RNA regulators has attracted attention due to its increasingly appreciated importance in development and disease. MicroRNAs are 18–24 nucleotide single-stranded RNAs that together with a complex of associated proteins known as the RNA-induced silencing complex (RISC) bind to sites of complementarity in the 3′ untranslated regions of messenger RNAs. Targeted mRNAs are subsequently translated less efficiently and undergo accelerated turnover (reviewed in Stefani and Slack, 2008). MicroRNAs represent an ancient form of gene regulation and are present in all multicellular eukaryotes studied so far and even some unicellular eukaryotes. An explosion of high-throughput sequencing efforts have uncovered over 500 miRNA genes in the human genome, many of which have been conserved throughout the vertebrate radiation. Elucidation of miRNA functions, requiring traditional loss- and gain-of-function experiments in cells and animals, has lagged behind these discovery efforts. But as these types of studies have been undertaken, the potent phenotypes induced by miRNA perturbations have placed these molecules at the center of critical cellular and developmental pathways. A series of recent papers in *Cell*, *Nature Immunology*, and *Cancer Cell* establish essential roles for the miR-17-92 cluster of miRNAs in development of the heart, lungs, and immune system (Koralov et al., 2008; Ventura et al., 2008; Xiao et al., 2008) and provide mechanistic insights into the role of these miRNAs in tumor formation (Petrocca et al., 2008).

Genomic Organization of the miR-17-92 Cluster

In animals, miRNAs are frequently transcribed together as polycistronic primary transcripts that are processed into multiple individual mature miRNAs (Stefani and Slack, 2008). The genomic organization of these miRNA clusters is often highly conserved, suggesting an important role for coordinated regulation and function. The miR-17-92 cluster is a prototypical example of a polycistronic miRNA gene. In the human genome, the miR-17-92 cluster encodes six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1), which are tightly grouped within an 800 base-pair region of human chromosome 13 (Figure 1A). Both the sequences of these mature miRNAs and their organization are highly conserved in all vertebrates. The human miR-17-92 cluster is located in the third intron of a ~7 kb primary transcript known as *C13orf25* (Ota et al., 2004). Despite the extreme conservation of the miRNA sequences, the exonic sequences of *C13orf25* are not measurably constrained between species, suggesting that the sole function of this transcript is to produce these miRNAs.

Ancient gene duplications have given rise to two miR-17-92 cluster paralogs in mammals: the miR-106b-25 cluster (located on human chromosome 7) and the miR-106a-363 cluster (located on the X chromosome) (Figure 1A). The miR-106b-25 cluster is located within the 13th intron of the protein-coding gene *MCM7*. Unlike the miR-17-92 and miR-106b-25 clusters, which are both abundantly expressed across many tissues and cell types, the miR-106a-363 cluster is undetectable or expressed at trace levels in all settings that have been examined (Ventura et al., 2008). Perhaps this miRNA polycistron provides a very specialized function in a cell type yet to be studied or represents a nonfunctional pseudogene.

The miR-17-92 Cluster in Cancer

The miR-17-92 cluster first attracted attention following a series of observations linking these miRNAs to cancer pathogenesis. The human genomic locus encoding these miRNAs, 13q31.3, undergoes amplification in several types of lymphoma and solid tumors. Ota et al. (2004) defined a minimal amplicon in this region in B cell lymphoma and demonstrated that *C13orf25* (the miR-17-92 primary transcript) was within this interval. *C13orf25* expression levels closely mirror the amplification status across numerous lymphoma cell lines and tissue samples from lymphoma patients. Building on these observations, the Hannon and Hammond laboratories provided direct experimental evidence that the miR-17-92 cluster has oncogenic activity (He et al., 2005). Enforced expression of these miRNAs in the well-studied *Eμ-myc* transgenic mouse model of B cell lymphoma dramatically accelerated disease onset and

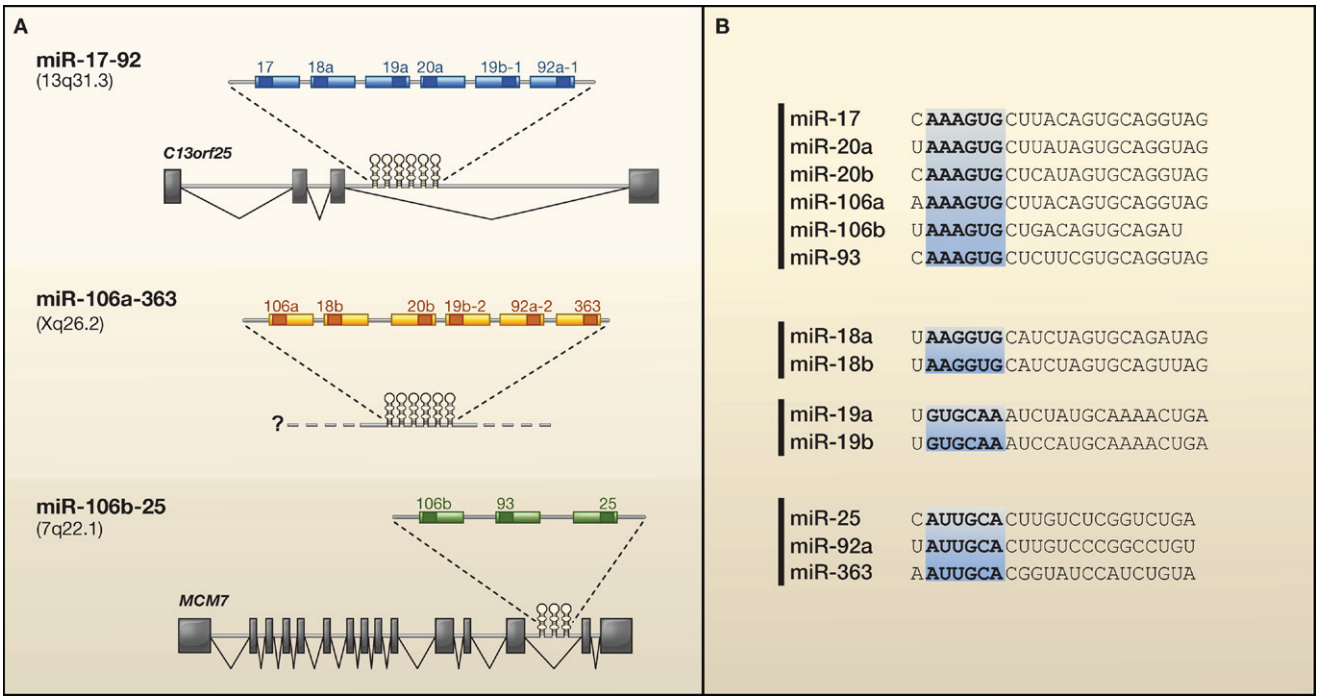

Figure 1. Organization of the miR-17-92 Cluster and Its Paralogs

(A) The genomic organization and primary transcript structures of the human miR-17-92, miR-106a-363, and miR-106b-25 clusters. The miR-106a-363 primary transcript has not been characterized.

(B) Based on their seed sequences—which are the regions considered most important for target selection (nucleotides 2–7; shown in blue)—the miRNAs of these clusters can be grouped into four families: the miR-17 family (miR-17, miR-20a/b, miR-106a/b, and miR-93); the miR-18 family (miR-18a/b); the miR-19 family (miR-19a/b); and the miR-25 family (miR-25, miR-92a, and miR-363). MicroRNAs of the miR-17-92 cluster and its paralogs have been implicated in normal development of the heart, lungs, and immune system as well as in tumorigenesis.

progression. Importantly, lymphomas with enforced expression of these miRNAs lacked the high degree of apoptosis that usually typifies these tumors in *Eμ-myc* mice. Simultaneously, we reported that transcription of the miR-17-92 cluster is directly transactivated by c-Myc (O'Donnell et al., 2005), a transcription factor that is frequently hyperactive in cancer cells. This finding provided one of the first demonstrations that miRNAs are functionally integrated into oncogenic pathways central to cancer development.

Several independent lines of evidence have further solidified the case that the miR-17-92 cluster and its paralogs can act as bona fide oncogenes. Expression profiling studies have revealed widespread overexpression of these miRNAs in diverse tumor subtypes including both hematopoietic malignancies and solid tumors such as those derived from breast, colon, lung, pancreas, prostate, and stomach (Petrocca et al., 2008; Volinia et al., 2006). Functional screens have also highlighted the importance of these miRNAs. One unbiased and powerful strategy for identifying new cancer genes relies upon the cloning of retroviral integration sites in murine malignancies. Given that retroviral insertion can lead to ectopic activation of nearby genes, multiple integration events at a given locus in independent tumors is often a signature of a neighboring oncogene. The locus encoding the miR-17-92 cluster is a common insertion site in multiple types of retrovirally induced murine leukemias (Cui et al., 2007; Wang et al., 2006). Interestingly, retroviral insertions have been observed at the miR-106a-363 locus in murine T cell leukemias (Landais et al., 2007). Although expression of the miR-106a-363 cluster is normally extremely low, this finding demonstrates that when ectopically transcribed, these miRNAs are functional and may act in a similar manner to the miR-17-92 cluster.

Establishment of an important role for the miR-17-92 cluster and its paralogs in the regulatory circuitry that controls cellular life and death decisions further illuminates the contributions of these miRNAs to tumorigenesis. In particular, these miRNAs seem to be tightly linked to the functions of the E2F family of transcription factors, which are critical regulators of the cell cycle and apoptosis. E2F1, E2F2, and E2F3—activating E2Fs that induce expression of genes that drive progression from G_1 into S phase—were among the first experimentally verified targets of the miR-17-92 cluster (O'Donnell et al., 2005; Sylvestre et al., 2007; Woods et al., 2007). Moreover, both E2F1 and E2F3 can directly activate transcription of these miRNAs, establishing a negative feedback loop. This circuitry also involves the miR-106b-25 cluster as these miRNAs are similarly upregulated by and repress translation of E2F1 (Petrocca et al., 2008). Given that high levels of E2F proteins, especially E2F1, can induce apoptosis, this negative feedback may dampen E2F activity following a physiologic proliferative signal, thereby promoting cell division rather than cell death.

Targeting of the cyclin-dependent kinase inhibitor CDKN1A (p21), a potent negative regulator of the G_1-S checkpoint, has recently emerged as an additional mechanism through which

miR-17 and related miRNAs (miR-20a, miR-106b, and miR-93) influence cell-cycle progression (Ivanovska et al., 2008; Petrocca et al., 2008). In some cell lines, overexpression or inhibition of this family of miRNAs is sufficient to promote or delay the entry of cells into S phase, respectively (Ivanovska et al., 2008). Similarly, high expression of these miRNA can impair cell-cycle arrest induced by DNA damage. Inhibition of p21 by this family of miRNAs contributes significantly to these phenotypes. Signaling via the growth factor TGFβ is another potent inducer of p21 and cell-cycle arrest at the G_1-S checkpoint. As Petrocca and colleagues (2008) report in a recent issue of *Cancer Cell*, overexpression of miR-106b or miR-93 renders gastric cancer cells insensitive to TGFβ-mediated cell-cycle arrest. Conversely, inhibition of these miRNAs increases sensitivity to TGFβ. Again, these effects are at least partly attributable to regulation of p21 by these miRNAs.

A cluster of papers in *Cell* and *Nature Immunology* as well as *Cancer Cell* have also identified the proapoptotic gene *BCL2L11/BIM* as a direct target of multiple members of the miR-17-92 cluster and related miRNAs (Koralov et al., 2008; Petrocca et al., 2008; Ventura et al., 2008; Xiao et al., 2008). Bim is a proapoptotic protein that regulates cell death in a variety of settings through its ability to antagonize antiapoptotic proteins such as Bcl2. Interestingly, haploinsufficiency for Bim accelerates lymphomagenesis in *Eμ-myc* transgenic mice (Egle et al., 2004). Downregulation of this protein by the miR-17-92 cluster therefore may contribute to the ability of these miRNAs to exacerbate disease progression in this mouse model. The antiapoptotic activity of the miR-17-92 cluster and related miRNAs in other settings, including TGFβ-stimulated gastric cancer cells, may similarly involve downregulation of Bim (Petrocca et al., 2008). An important role for regulation of Bim by these miRNAs during normal B cell development has also been reported (Koralov et al., 2008; Xiao et al., 2008; discussed below).

The pro-tumorigenic activity of the miR-17-92 cluster additionally involves cell-nonautonomous functions including induction of angiogenesis in solid tumors. The *c-Myc* oncogene is a potent inducer of tumor angiogenesis, and its activation results in downregulation of antiangiogenic proteins such as thrombospondin-1 (Tsp1) and connective tissue growth factor (CTGF) (Dews et al., 2006). Using a mouse model of colon cancer, Dews et al. demonstrated that the angiogenic activity of c-Myc is at least in part due to downstream activation of the miR-17-92 cluster. Both Tsp1 and CTGF are negatively regulated by these miRNAs, which are potently induced by c-Myc in this model. Robust vascularization of tumors can be induced either by expression of c-Myc or the miR-17-92 cluster.

Although the data overwhelmingly support a dominant role for the miR-17-92 cluster in promoting tumorigenesis, there is some evidence suggesting that loss-of-function of these miRNAs might be advantageous for cancer cells in certain settings. Loss-of-heterozygosity at the 13q31.3 locus has been observed in multiple tumor types, and a recent genome-wide analysis of copy number alterations in cancer revealed that the miR-17-92 cluster was deleted in 16.5% of ovarian cancers, 21.9% of breast cancers, and 20% of melanomas (Zhang et al., 2006). Consistent with these observations, introduction of miR-17 into breast cancer cell lines reduced proliferation of the cancer cells (Hossain et al., 2006). This effect was due in part to inhibition of the *amplified in breast cancer 1* (*AIB1)* gene, which encodes a transcriptional coactivator of the estrogen receptor and E2F1. Although a tumor suppressor role for the miR-17-92 cluster remains to be established experimentally in vivo, both pro- and antitumorigenic activities of these miRNAs are not mutually exclusive as their functions will be dictated by which targets are expressed in a given setting.

The miR-17-92 Cluster in Development

Despite a well-established role for the miR-17-92 cluster in tumorigenesis, the physiological functions of these miRNAs have remained unclear. Now, new work, published in *Cell* and *Nature Immunology*, from the Jacks and Rajewsky laboratories has begun to illuminate the roles of these miRNAs in normal development (Ventura et al., 2008; Koralov et al., 2008; Xiao et al., 2008). Ventura and colleagues (2008) documented the consequences of deletion of the miR-17-92 cluster as well as the paralogous miR-106a-363 and miR-106b-25 clusters in mice. Importantly, targeting of the miR-106b-25 cluster was performed very carefully so as not to disrupt the function of its essential protein-coding host transcript, *Mcm7*. Underscoring the importance of careful design of miRNA knockout studies, the placement of a neomycin resistance (*Neo)* cassette within the intron of *Mcm7* where the miRNAs are located was found to be incompatible with embryonic development. Only removal of *Neo* by further recombination allowed recovery of knockout animals. Absence of the miR-106a-363 and miR-106b-25 clusters, either alone or in combination, did not result in any obvious phenotype. In contrast, loss-of-function of the miR-17-92 cluster resulted in smaller embryos and immediate postnatal death of all animals. This was likely due to severely hypoplastic lungs and ventricular septal defects in the hearts of mice lacking miR-17-92. Some functional redundancy of the miR-17-92 and miR-106b-25 clusters does exist as loss of both clusters results in embryonic death associated with severe cardiac defects and widespread apoptosis. Consistent with its trace expression, additional deletion of the miR-106a-363 cluster does not cause any further developmental abnormalities.

The precise roles of the miR-17-92 cluster in heart and lung development remain unclear. The observations described above are nevertheless consistent with the earlier demonstration that these miRNAs are normally highly expressed in embryonic lung and decrease as mice reach maturity (Lu et al., 2007). Moreover, transgenic expression of these miRNAs specifically in lung epithelium results in severe developmental defects with enhanced proliferation and inhibition of differentiation of epithelial cells. Additional studies will be necessary to elucidate the critical targets underlying these phenotypes.

Because of the documented role for the miR-17-92 cluster in B cell lymphomagenesis, B cell development was examined in detail in knockout animals. During normal development of B cells, both the immunoglobulin heavy (IgH) and light (IgL) chain genomic loci undergo somatic gene rearrangements (VDJ and VJ recombination, respectively), which contributes to the diverse antibody repertoire of the mature immune system.

Only a subset of cells that enter this pathway successfully perform these rearrangements, and multiple checkpoints exist to ensure that all mature B cells express a functional membrane-bound antibody, the B cell receptor. Successful assembly of the IgH locus in progenitor B (pro-B) cells provides a survival signal that allows their progression to the precursor B (pre-B) cell stage. Subsequent productive assembly of the IgL loci signals furthers development to the immature B cell stage. A final test prior to maturation of these cells ensures that the successfully assembled B cell receptor does not react with endogenous antigens.

Hematopoiesis in mid-gestation embryos lacking the miR-17-92 cluster is characterized by a striking deficiency of pre-B cells, but not of earlier B cell progenitors (Ventura et al., 2008), and a marked increase in apoptosis of developing B cells in these animals. By reconstituting the hematopoietic system of adult wild-type mice with fetal liver cells from miR-17-92-deficient embryos, the authors were able to examine the role of these miRNAs in adult hematopoiesis. Consistent with the effects observed in embryos, there were almost normal numbers of pro-B cells but marked depletion of later stage pre-B cells and mature B cells, accompanied by increased apoptosis specifically of pro-B cells. Based on these data, the authors propose that the miR-17-92 cluster is essential for the survival signal enabling progression from the pro-B to pre-B cell stage. This survival signal likely involves downregulation of the proapoptotic gene *Bcl2l11*/*Bim*, which was shown by these authors and others to be a direct target of multiple members of the miR-17-92 cluster.

Notably, mouse hematopoiesis occurring in the absence of miR-17-92 leads to an isolated defect in B cell development. Other hematopoietic cells such as red blood cells, granulocytes, monocytes, and T cells are largely unaffected by loss of these miRNAs. This contrasts with the in vitro differentiation of human $CD34^+$ hematopoietic progenitor cells. In this system, members of the miR-17-92 cluster are downregulated during monocyte differentiation (Fontana et al., 2007). This results in upregulation of the transcription factor AML1, a direct target of miR-17 and related miRNAs that is essential for monocyte differentiation. Accordingly, overexpression of these miRNAs delays terminal differentiation of monocytes, whereas their inhibition accelerates differentiation. Further investigation will be necessary to determine whether differences between these observations and those of Ventura et al. reflect distinctions between human and mouse monocytopoiesis or indicate that in vitro differentiation of hematopoietic progenitor cells does not fully recapitulate the normal physiology of this process.

Using a different approach, Koralov et al. (2008) also uncovered a role for the miR-17-92 cluster during B cell development. In their study, the *Dicer* gene, which encodes an enzyme that is essential for miRNA biogenesis, was deleted specifically in developing B cells. The resulting phenotype was similar to the effect of miR-17-92 deletion: accumulation of pro-B cells with a marked reduction of pre-B cells and more mature cells of this lineage. Notably, *Dicer* deficiency induced apoptosis of pre-B cells rather than of pro-B cells as observed in the miR-17-92-deficient animals. These distinct effects indicate that the phenotype due to *Dicer* deletion cannot be wholly explained by loss of miR-17-92. Nevertheless, gene expression analyses revealed that the 3′ UTRs of genes upregulated upon *Dicer* deletion in pro-B cells are significantly enriched for heptamer motifs that are complementary to the seed sequences of multiple miRNAs from the miR-17-92 cluster and miR-142-3p. A high-scoring gene in these analyses, with multiple miR-17-92 miRNA-binding sites, was the proapoptotic gene *Bim*. Cells lacking *Dicer* show increased *Bim* mRNA expression and Bim protein production. Consistent with a critical role for aberrant expression of this protein in mice lacking *Dicer*, B cell development can be partially rescued in these animals by homozygous deletion of *Bim*. It is also possible, however, that this rescue is due to a general lowering of the apoptotic threshold in cells lacking *Bim* rather than a specific effect related to regulation of *Bim* by the miR-17-92 cluster. Indeed, the authors also demonstrate that overexpression of the antiapoptotic protein *Bcl2* similarly rescues B cell development.

The ability of *Bim* loss-of-function or *Bcl2* gain-of-function to overcome this block in B cell development allowed the impact of *Dicer* deletion on later steps in this developmental pathway to be examined. Recombination of IgH and IgL loci occurred normally in the absence of *Dicer*, but there were some aberrant effects. Specifically, an unusual spectrum of gene segments was incorporated during VDJ recombination, suggesting altered chromatin structure at the IgH locus. Given that Dicer regulates chromatin structure in fission yeast, this observation, as well as the previous demonstration that both sense and antisense noncoding RNAs are produced from the IgH locus, raises the intriguing possibility that a *Dicer*-dependent small RNA-guided chromatin regulatory pathway exists in mammalian cells (Chakraborty et al., 2007). Alternatively, these effects may be secondary to loss of miRNA-mediated regulation of a chromatin regulatory protein. *Dicer* deletion was also associated with abnormally sustained transcription of terminal deoxynucleotidyl transferase (TdT), the enzyme that adds nontemplated nucleotides to the recombining gene segments to increase antibody diversity, although the mechanism of this phenomenon is unclear.

In a complementary study, this group used a gain-of-function approach to further investigate the role of the miR-17-92 cluster in development of the immune system (Xiao et al., 2008). They engineered mice to modestly overexpress these miRNAs specifically in the B and T cell lineages. Importantly, expression levels of these miRNAs in transgenic animals were comparable to levels observed in human lymphoma cell lines. Enforced expression of the miR-17-92 cluster resulted in premature death of transgenic animals associated with lymphoproliferative disease and autoimmunity. Both the B and especially the T cell compartments were expanded in the peripheral immune system. In vitro, B and T cells from transgenic animals exhibited increased proliferation and survival following activation. Based on these findings, the authors propose that pathological amplification of the miR-17-92 cluster, as found in human lymphoma cells, leads to expansion of cells harboring this lesion, increasing the likelihood of accruing additional mutations leading to malignancy.

These investigators (Xiao et al., 2008) highlight not only the potential role of *Bim* regulation by the miR-17-92 cluster in these phenotypes but also the role of *Pten*, a tumor suppressor and additional validated target of these miRNAs (Lewis et al., 2003). Combined haploinsufficiency of *Bim* and *Pten* partially mimics the phenotype of miR-17-92 overexpression, highlighting the importance of these proteins but also indicating that additional targets must contribute to these effects. Regulation of E2F transcription factors by these miRNAs might also play an important role in miR-17-92 gain-of-function phenotypes given that deficiency of these proteins similarly leads to hematopoietic malignancies and autoimmunity (Zhu et al., 2001).

Future Directions

The available evidence places the miR-17-92 cluster and its paralogs at the nexus of critical pathways that regulate cellular life and death decisions during normal development and in malignancy. But clearly many questions remain. Of particular importance is the continued elucidation of the full network of targets regulated by these miRNAs. Given that each miRNA perhaps regulates hundreds of mRNAs, this will be a daunting task. Further complicating matters, the diverse functions of the miR-17-92 cluster and its paralogs in different physiological contexts likely involves different subsets of targets. More broadly, an emerging issue relates to whether the functions of a given miRNA can be attributed to strong regulation of a select few dominant targets or, alternatively, more subtle regulation of many targets simultaneously. It is likely that miRNAs act along a spectrum of these possibilities, with simple miRNA:target relationships dictating some phenotypes and complex networks of gene expression changes underlying others. Elucidating where along this spectrum the miR-17-92 cluster operates represents a major challenge for future investigations.

The organization of the miR-17-92 cluster and its paralogs raises interesting questions regarding their redundancy. Clearly, there is functional synergy between the miR-17-92 and miR-106b-25 clusters, as evidenced by the severity of the combined knockout phenotype (Ventura et al., 2008). But the severe phenotype associated with miR-17-92 deletion suggests that unique functions are attributable to these miRNAs. Notably, only the miR-17-92 and miR-106a-363 clusters encode miR-18 and miR-19 family members (Figure 1A). Given that the miR-106a-363 cluster appears to be rarely expressed, absence of miR-18 and miR-19 might contribute to the phenotypic consequences of miR-17-92 loss of function. Genetic rescue strategies would allow direct testing of this hypothesis. Another interesting issue relates to intracluster redundancy. Specifically, the miR-17-92 cluster contains multiple miRNAs of the miR-17 and miR-19 families (Figure 1B). According to prevailing target prediction models, these miRNAs should be redundant. Yet this organization is highly conserved, suggesting that each miRNA in the cluster has functional importance. Perhaps this arrangement simply allows more expression of a given miRNA family from a single transcription unit. Alternatively, each miRNA in the cluster could provide distinct functions. Indeed, related miRNAs can have different target specificities and may be subject to unique posttranscriptional regulation. Again, rescue experiments could be designed to address these issues.

Further investigation of the functions of the miR-17-92 cluster is important beyond contributing to a more complete molecular understanding of the pathways regulated by these miRNAs. Due to their potent effects on cellular proliferation and apoptosis, these miRNAs may be attractive targets for cancer therapy. MicroRNAs are readily inhibited using antisense oligonucleotides and efforts are underway in both academia and industry to develop efficient methods to deliver these agents. If miRNA-based therapeutics indeed become a reality, the miR-17-92 cluster and related miRNAs will undoubtedly be among the first to be targeted.

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