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Functional Interactions among Members of the miR-17~92 Cluster in Lymphocyte Development, Differentiation and Malignant Transformation

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MicroRNAs (miRNAs) are small (~22 nucleotides) single-stranded, non-coding RNA molecules that are critical regulators of gene expression (Ambros, 2004; Bartel, 2004; Bushati and Cohen, 2007). Binding of miRNAs to the target sequence in messenger RNAs (mRNAs), in the context of RNA-induced silencing complex (RISC), leads to translational repression or mRNA destruction (Huntzinger and Izaurralde, 2011). Thanks to the short sequence length of miRNAs and the imperfect complementarity between miRNA and mRNA, each miRNA can target multiple mRNAs, and one single mRNA can harbor binding sites for multiple miRNAs (Bartel, 2009). Many miRNAs and miRNA binding sites on mRNAs are well conserved during evolution, implying functional importance of this posttranscriptional regulation mechanism. Indeed, genetic studies have shown that miRNAs are critical regulators of a wide range of developmental, physiological and pathological processes. In recent years, emerging evidence has demonstrated that deregulation of several miRNAs greatly impacts lymphocyte development and functions, and causes immune disorders such as autoimmunity and lymphoma, underscoring functional significance of miRNA control in the immune system (O'Connell et al., 2010; Xiao and Rajewsky, 2009).

A mature miRNA is processed from its hairpin precursor of ~70 nucleotides (pre-miRNA), which is the product of a much longer primary transcript (pri-miRNA) (Bartel, 2004; Krol et al., 2010). Genomic analysis of the human primary transcripts of miRNAs indicates that approximately half of miRNAs reside in the introns of protein-coding genes, while the other half derive from independent transcription units (Saini et al., 2007). Remarkably, 25–40% miRNA precursors are found to be located within close proximity (<10kb) of other miRNA precursors, forming miRNA clusters (Altuvia et al., 2005; Griffiths-Jones et al., 2008; Saini et al., 2007). The majority of miRNA clusters are transcribed into single polycistronic primary transcripts, giving rise to multiple mature miRNAs under the same transcriptional control. This prevailing feature of polycistronic miRNA assembly is evolutionarily stable and conserved across species (Altuvia et al., 2005; Marco et al., 2013; Thatcher et al., 2008), yet its functional significance remains to be unveiled. Based on the sequence homology of

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the seed region (a region of 6–8 nucleotides at the 5' end of miRNA that governs mRNA targeting) (Bartel, 2009), miRNAs can be categorized into different families, and miRNAs of the same family are thought to regulate the same group of target genes and may have similar functions. Thus, the same-family miRNAs within a cluster likely offer an increase in miRNA gene dosage. A majority of miRNA clusters consist of miRNAs of different families. Conceivably, such organization would expand the capacity and complexity of the gene regulation network. How the interactions of these co-transcribed miRNAs and their regulated targets determine functional consequences is still largely unknown.

The miR-17~92 cluster, composed of miRNAs of the same and different families, offers arguably the best opportunity for studying functional interactions of intra-cluster miRNAs, as this cluster exhibits highly regulated expression patterns and is involved in a myriad of cellular signaling and processes (Mendell, 2008). The six miRNAs encoded by miR-17~92 belong to four families: miR-17 family (miR-17 and 20), miR-18 family (miR-18), miR-19 family (miR-19a and miR-19b), and miR-92 family (miR-92a) (Figure 1). The genomic organization of miR-17~92 is conserved and specific in vertebrates, with miR-92 being the only member found in both vertebrates and invertebrates (Tanzer and Stadler, 2004). In addition, two homologous clusters, miR-106a~363 and miR-106b~25, exist on other chromosomes. While both miR-17~92 and miR-106a~363 contain members from all four families, miR-106b~25 lacks miR-18 and miR-19 family members (Figure 1). These three clusters probably originated from the same ancestral cluster. Gene amplification and overexpression of miR-17~92 are frequently found in lymphomas and solid cancers (Conkrite et al., 2011; Hayashita et al., 2005; Lu et al., 2005; Mestdagh et al., 2010; Ota et al., 2004; Tagawa and Seto, 2005). Conversely, miR-17~92 haploinsufficiency due to microdeletion of the gene causes skeletal defects found in Feingold syndrome (de Pontual et al., 2011). Recent deep sequencing analyses of miRNAs in lymphoid lineages at different developmental stages and in various subsets showed that miR-17~92 expression is tightly regulated during lymphocyte development and immune response (Kuchen et al., 2010; Spierings et al., 2011). Here we review current knowledge of miR-17~92 in regulating lymphocyte development, function and lymphomagenesis, from studies in mouse and human, and focus on functional interactions among members of the miR-17~92 cluster.

Roles of miR-17~92 in Regulating B Cell Development, Tolerance and Differentiation

B cell development is a highly ordered process that generates a diverse B cell repertoire for protection from pathogens while tolerant to self-tissues (Clark et al., 2014; Hardy et al., 2007; Schlissel, 2003; Shlomchik, 2008). In the bone marrow, this process navigates from pro-B, pre-B to immature B cells, featuring sequential immunoglobulin gene recombination and crucial checkpoints to ensure production of signaling-competent pre-BCR and BCR and removal of self-reactivity. Interestingly, miR-17~92 miRNAs display a highly regulated expression pattern during B cell development: they are relatively abundant in progenitor cells, but their expression decreases greatly when developing B cells transition from pre-B to immature B cells (Kuchen et al., 2010; Spierings et al., 2011).

To study its physiological functions, Ventura and colleagues generated and examined mice harboring germline deletion of miR-17~92. These mice suffered from a profound block of B cell development at the pro- to pre-B transition, along with lethal defects in heart and lung development. They also analyzed mice with compound deletion of miR-17~92 and miR-106b~25 clusters, and found a more severe B cell developmental block and more profound abnormalities in the central nervous system, fetal liver and heart, demonstrating functional cooperation between these two homologous clusters (Ventura et al., 2008). To circumvent the limitation of perinatal lethality caused by germline knockout of the miR-17~92 gene and potential functional redundancy of these homologous clusters, we generated mutant mice with B cell-specific deletion of the miR-17~92 family miRNAs, and identified a cell-intrinsic role of these miRNAs in controlling early B cell development at the transition from late pro-B to large pre-B cell (Xiao laboratory, unpublished results).

While the miR-17~92 cluster is generally thought to be pro-survival and pro-proliferation, it might be necessary to keep its expression level low during tolerance induction, which is essential for keeping detrimental autoimmunity at bay. Xiao and colleagues generated transgenic mice expressing the human miR-17~92 cluster in both B and T lymphocytes, and observed lymphoproliferative and autoimmune disease-like phenotypes as a result of the elevated miR-17~92 expression (Xiao et al., 2008). Although the follow-up study showed similar immunopathology and lethality induced by T cell-restricted miR-17~92 overexpression, the much weaker phenotypes and delayed disease onset suggest pathological contribution by deregulated miR-17~92 in B cells (Kang et al., 2013). Supporting this hypothesis, approximately 20% of mice with B cell-specific miR-17~92 transgene died of lymphoproliferative diseases, while the other 80% died of lymphomas (Jin et al., 2013) (see lymphoma section below). Whether unharnessed miR-17~92 expression compromises the tolerance checkpoints warrants further investigation.

Studies of these transgenic mice, prior to disease onset, also revealed other processes that miR-17~92 regulates during B cell development and differentiation. Elevated miR-17~92 expression expanded B1 cell population in the spleen and peritoneal cavity, while greatly reduced the number of marginal zone B cells. These mice also showed higher frequency of germinal center and activated B cells as they aged (Jin et al., 2013; Xiao et al., 2008). Many of these phenotypes were reminiscent of those displayed by conditional deletion of Pten, a miR-17~92 target, in B cells, except that Pten deficiency led to expansion, instead of reduction, of marginal zone B cells (Anzelon et al., 2003).

To investigate the role of individual miRNAs of miR-17~92, Shan et al. generated mice overexpressing the transgene harboring four tandem copies of miR-17. Intriguingly, these miR-17 transgenic mice showed smaller organs and overall growth retardation. Also unexpectedly, miR-17 overexpression inhibited B-lymphopoiesis (Shan et al., 2009). It is not clear whether this effect is B cell-intrinsic or results from non-physiological levels of miR-17 expression in the stroma cells of the bone marrow. Further delineation of individual miRNA functions in a B lineage-specific manner will help solve the puzzle.

The MiR-17~92 Cluster as a Pleiotropic Regulator of T Cell Differentiation and Effector Functions

MiR-17~92 expression is markedly upregulated in CD4⁺ T cells from patients with lupus and multiple sclerosis, airway-infiltrating T cells from patients with asthma, and splenic T cells of MRL-lpr mice, a commonly used lupus mouse model (Dai et al., 2010; Lindberg et al., 2010; Qin et al., 2013; Simpson et al., 2014). Much work has been published lately, shedding light on regulation of T-cell differentiation and effector functions by the miR-17~92 miRNAs. Apart from promoting proliferation and survival of T cells, miR-17~92 was shown to be important in modulating the T_H1 response and essential for anti-tumor immunity. Functional dissection concluded that miR-19b (targeting Pten) and miR-17 (targeting TGFβ receptor II (Tgfr2) and transcription factor Creb1) are responsible for this regulation and suggested that miR-18 might antagonize this function (Jiang et al., 2011). Whereas miR-17~92 is not required for the development and homeostasis of natural regulatory T cells (nTregs), overexpression of miR-19b and miR-17 inhibited inducible Tregs (iTregs) differentiation *in vitro* (Jiang et al., 2011). In a later study, miR-17~92 was shown to be critical for normal Treg cell accumulation and function. Treg-specific deletion of miR-17~92 cluster greatly reduced the frequency of IL-10-producing effector Tregs and exacerbated experimental autoimmune encephalitis (EAE) *in vivo* (de Kouchkovsky et al., 2013). Notably, miR-17~92 deletion in CD4⁺ T cells, on the contrary, mitigated EAE progression. This was attributed to the role of miR-19b (by targeting Pten) and miR-17 (by targeting Ikaros zinc finger transcription factor Ikzf4) in promoting T_H17 cell differentiation (Liu et al., 2014). Recently, miR-17~92 was shown to be critical for augmenting the T_H2 response, and implicated in asthma pathogenesis. Particularly intriguing, miR-19a is the only member of the miR-17~92 cluster that is preferentially upregulated in asthma airway T cells, and its promotion of T_H2 cytokine production was mediated by coordinated repression on negative regulators of signaling including Pten (PI3K pathway), Socs1 (Jak-STAT pathway) and A20 (NFκB pathway) (Simpson et al., 2014).

We have recently reported that the abundance of miRNAs of the miR-17~92 family is dynamically regulated during T_{FH} differentiation. The mutant mice in which miR-17~92 is conditional deleted in the T cell lineage while its homologous clusters miR-106a-363 and miR-106b-25 are deleted in germline showed severely compromised follicular helper T cell (T_{FH}) differentiation, along with defects in germinal center formation and antibody production, and failed to control chronic virus infection. Conversely, T cell-specific miR-17~92 transgenic mice spontaneously accumulated T_{FH} cells and developed fatal immunopathology. We further demonstrated that miR-17~92 controlled the migration of CD4⁺ T cells into B cell follicles, a critical step of the T_{FH} differentiation program, at least in part by suppressing Pten and Phlpp2 expression and thus regulating ICOS-PI3K-Akt signaling (Kang et al., 2013). Work carried out by UCSF research groups independently discovered the essential role of miR-17~92 in controlling the T_{FH} differentiation program. Besides Pten, they also identified Rora as another functional relevant target, and their results suggested that the miR-17~92:Rora axis acts in favor of pre-T_{FH} differentiation while repressing the non-T_{FH} differentiation path (Baumjohann et al., 2013). The question of

whether miR-19 and/or other members of the miR-17~92 cluster play distinct roles in this regulation is yet to be answered.

Intricate Balance: Cell Context-Dependent Cooperation and Antagonism between MiR-17~92 Members in Hematopoietic Malignancies

Overexpression of miR-17~92 occurs in a broad spectrum of human cancers, including lymphomas, leukemias and solid cancers. This is mainly caused by gene amplification and Myc-mediated transcriptional upregulation (O'Donnell et al., 2005; Ota et al., 2004). Lin He et al. showed that vertebrate-specific portion of miR-17~92 (miR-17~19b) (Figure 1) accelerated tumor development in the Eμ-Myc B-cell lymphoma model, and therefore miR-17~92 was coined as the first “oncomiR” (He et al., 2005). MiR-17~92 was also reported to cooperate with Notch1 in promoting T-cell acute lymphoblastic leukemia (T-ALL) (Mavrakis et al., 2010). The following questions became prominent to understand the importance of the miR-17~92 cluster miRNAs in lymphoma and other cancers: (i) Does miR-17~92 play a causative role in lymphomagenesis? (ii) To what degree does this Myc-induced cluster contribute to Burkitt's lymphoma where Myc:miR-17~92 activation is an overwhelming feature? (iii) Is there oncomiR addiction in tumors featuring miR-17~92 gene amplification/overexpression? (iv) How do individual miRNA members and their relevant targets contribute to tumor onset and maintenance?

To determine whether deregulated miR-17~92 expression itself is sufficient to predispose cancer, we investigated mice expressing miR-17~92 transgene restrictively in B cells. These mutant mice developed monoclonal, transplantable lymphomas (mostly diffuse large B cell lymphoma) at high penetrance and died before one year of age. We further uncovered that miR-17~92 coordinates multiple oncogenic pathways, including activation of PI3K and NFκB pathways and inhibition of apoptosis, in causing lymphomagenesis, and thus establishing miR-17~92 as a *bona fide* cancer driver gene in the cancer genome landscape (Jin et al., 2013).

Recent analysis of a large collection of lymphoma biopsies revealed that the Myc:miR-17~92 axis operates in 100% of Burkitt lymphoma cases (Schmitz et al., 2012). To investigate the role of endogenously expressed miR-17~92 in Myc-induced lymphoma, Mu et al. utilized a conditional knockout allele of miR-17~92, and showed that withdrawing miR-17~92 compromised the survival and transplantability of Eμ-Myc lymphoma cells (Mu et al., 2009). In order to dissect the contribution of miR-17~92 in *de novo* tumor formation induced by Myc oncogene activation, we introduced CD19Cre-mediated deletion of endogenous miR-17~92 alleles into λ-Myc Burkitt lymphoma model. Strikingly, lymphomas developed in these mice could only originate from cells that avoided miR-17~92 loss, predominantly early B cell precursors prior to CD19 expression whereby Myc activation is opportunistic (Jin et al., 2013). This finding demonstrated the absolute requirement of miR-17~92 miRNAs for Myc-induced lymphomagenesis. This functional cooperation between c-Myc and miR-17~92 is also important to other Myc family members, such as MYCN, as shown in the Feingold syndrome and in neuroblastoma (de Pontual et al., 2011; Loven et al., 2010; Northcott et al., 2009; Schulte et al., 2008).

Understanding individual contribution of miR-17~92 miRNAs in malignant transformation is of clinical importance. MiR-19 plays distinct roles in lymphocyte survival and in promoting Myc-induced B cell lymphoma and Notch-induced T-ALL, in a large part, by targeting Pten to suppress apoptosis (Mavrakis et al., 2010; Mu et al., 2009; Olive et al., 2009). Deemed as the primary oncogenic determinant of this oncomiR cluster, miR-19 might offer targeting opportunity for anti-cancer therapeutics. Functional cooperation of other cluster members contributes to oncogenesis in various cancer contexts. MiR-17 and miR-20 repress p21 in conferring resistance to Ras-induced senescence (Hong et al., 2010). Another report established a non-cell-autonomous function of miR-17~92 in promoting tumor angiogenesis, involving repression of anti-angiogenic proteins thrombospondin-1 (Tsp1) and connective tissue growth factor (Ctgf) by miR-19 and miR-18, respectively (Dews et al., 2006). Olive et al. provided some interesting findings regarding miR-92, the longest existing component of the cluster in evolution. They demonstrated complex, cell context-dependent oncogenic roles of miR-92. In the presence of wild-type p53, miR-92 counteracts the oncogenic effects of miR-19, by promoting apoptosis as a result of repression on its target Fbw7 and thus stabilization of Myc. They suggested that it is the loss of the intricate balance between miR-19 and miR-92 levels, as well as acquisition of p53 mutation, that promotes the Myc tumor formation (Olive et al., 2013). Another example of functional antagonism between miR-17~92 components came with a study showing that erythroleukemia development induced by retroviral overexpression of miR-92a was abrogated by co-overexpressing miR-17 (Li et al., 2012). While co-expressed miR-17~92 miRNAs seem to work in concert to achieve functional consequences in many cases, depending on specific cell contexts, intra-cluster miRNA antagonism might exist as a self-regulatory mechanism.

The Smoking Guns: Changes in Mature MiRNA Abundance and Targetomes of the Cluster

The studies of the miR-17~92 cluster shed some light on how members of heterogeneous miRNA clusters work together to accomplish their functions (Table 1). The genomic organization and mature miRNA sequences of miRNA clusters are often evolutionary conserved, and the expression of different members of a miRNA cluster is frequently co-regulated. These observations suggest that miRNAs in a heterologous miRNA cluster cooperate with each other to achieve common functions. Consistent with this idea, bioinformatics studies have shown that members of a miRNA cluster tend to target the same gene or genes involved in the same pathway or in the same functional network (Yuan et al., 2009). However, functional dissection of the miR-17~92 cluster in the aforementioned studies highlighted the complexity of this issue (Table 1). First, there are differential contributions of members of a miRNA cluster to its function in different physiological and pathological contexts. For example, miR-19a is critical for regulating differentiation of T_H2 cell, while miR-17 plays a central role in inhibiting oncogene-induced senescence. Second, co-expressed cluster miRNAs might cooperate or counteract with each other. In Myc-induced oncogenesis, miR-92 antagonizes with miR-19 when p53 pathway is intact; derailing of Myc expression by miR-92, in the absence of p53, coordinates with miR-19-

regulated program in promoting the formation and growth of tumors. These themes can be attributed to cell context-specific miRNA abundance and targetomes.

Although clustered miRNAs are transcribed as a single unit, their processing, maturation, and stability are under differential post-transcriptional control. A good example is the disproportionately high expression of miR-19a in the airway T cells in asthma pathogenesis (Simpson et al., 2014). MiR-18, while barely detectable in normal lymphocytes, is found highly expressed in lymphoma cells (Jin et al., 2013). During Myc-induced lymphoma development, the differential regulation of miR-19 and miR-92 determined the overall oncogenic activity of miR-17~92 (Olive et al., 2013). Specific miRNA-binding proteins could cause differential miRNA biogenesis and/or stability (Krol et al., 2010). RNA-binding protein hnRNP A1 was shown to specifically bind to pre-miR-18 and regulate processing of miR-18 in a context-dependent manner (Guil and Caceres, 2007).

It is conceivable that different members of a heterologous miRNA cluster regulate distinct, yet overlapping, sets of target genes, and may have different effects on the molecular pathways under the control of the cluster. It is also conceivable that at different developmental stages of a single cell lineage, or in other cellular contexts, the functional relevance and importance of these molecular pathways may differ to a large degree. This explains the differential contribution of members of a miRNA cluster to its function in different cellular contexts. For example, targeting of Pten by miR-19 is critically involved in many reported processes, yet Pten does not contribute to the pro- to pre-B transition block as a consequence of miR-17~92 deficiency (Xiao laboratory, unpublished results). Similarly, functional interaction among members of miRNA clusters relies, indeed, on the interaction of their targetomes. Future work on miRNA cluster genes warrants further investigation of dynamic changes of intra-cluster miRNA member-target interactomes and their functional contributions. This is pivotal for translating our knowledge of miRNAs into clinical applications.

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Figure 1.

The genomic organization of miR-17~92 (A) and homologous clusters (B). The sequential processing from the primary transcript (pri-miRNA) to precursor miRNAs (pre-miRNAs, indicated as rectangular boxes) and to mature miRNAs was shown for miR-17~92. MiRNAs of the same family have identical seed regions (nucleotides 2–7), and their precursors were marked with the same color.

Table 1

Functional dissection of the miR-17~92 cluster in lymphocyte development, differentiation and malignant transformation

Cellular context/Process	Key member	Known functional targets	References
Pro- to pre-B transition	unknown	unknown	Ventura et al. 2008; Xiao laboratory, unpublished results
B1 expansion	unknown	Pten	Xiao et al. 2008; Jin et al. 2013
Marginal zone differentiation	unknown	unknown	Xiao et al. 2008; Jin et al. 2013
B cell activation	unknown	Pten, Bim	Xiao et al. 2008; Jin et al. 2013
T _H 1 differentiation	miR-19b	Pten	Jiang et al. 2011
miR-17	Tgfbr2 and Creb1		
miR-18 (antagonizing)	unknown		
T _H 2 differentiation	miR-19a	Pten, Socs1 and A20	Simpson et al. 2014
T _H 17 differentiation	miR-19b	Pten	Liu et al. 2014
miR-17	Ikzf4		
Treg effector function	unknown	unknown	de Kouchkovsky et al. 2013
T _{FH} differentiation	unknown	Pten, Phlpp2 and Rora	Kang et al. 2013; Baumjohann et al. 2013
Myc-induced lymphoma development	miR-19 family	Pten and Bim	Olive et al. 2009; Mu et al. 2009
miR-92 (antagonizing)	Fbw7	Olive et al. 2013	
Notch-induced leukemia development	miR-19 family	Pten, Bim, Prkaa1 and Ppp2r5e	Mavrakis et al. 2010
miR-17~92-induced lymphoma development	unknown	Pten, Phlpp2, Bim, Cyld and A20 complex	Jin et al. 2013