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MicroRNAs in the Midst of Myeloid Signal Transduction

SARA L. STOFFERS¹, SARA E. MEYER¹, and H. LEIGHTON GRIMES^{1,2,*}

¹Division of Immunobiology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio

²Division of Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio

Abstract

MicroRNA (miRNA) play important roles in the development and physiological function of hematopoietic stem/progenitor and mature cell lineages. In addition, deregulated miRNA expression and subsequent gene expression changes are associated with hematologic diseases including myelodysplastic syndromes and acute myeloid leukemia. This review focuses on myelopoiesis as a model to highlight the involvement of miRNA in the regulation of normal and malignant cellular signaling pathways. By incorporating miRNA regulation into well-established myeloid signal transduction pathways, we hope to shed light on targetable factors both upstream and downstream of miRNA signaling. These pathway-specific miRNA functions suggest scenarios wherein miRNA-based therapeutics may be beneficial either alone or in combination with current therapies.

Discovered in 1993, microRNA (miRNA, or miR) comprise a class of small non-protein coding RNA molecules that regulate post-transcriptional gene expression (Lee et al., 1993; Wightman et al., 1993). miRNA are highly conserved across species and are involved in a broad range of normal developmental, cellular, and disease processes, including embryogenesis, developmental timing, organogenesis, differentiation, proliferation, apoptosis, and tumorigenesis (Reinhart et al., 2000; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Ruvkun, 2001; Calin et al., 2004; Chen et al., 2004; Alvarez-Garcia and Miska, 2005; Georganta et al., 2007; Chivukula and Mendell, 2008; Hu et al., 2010). Currently, there are 1,048 human miRNA recorded in the Sanger miRNA registry (Ambros et al., 2003; Griffiths-Jones, 2004; Griffiths-Jones et al., 2006, 2008). In mammals, a significant proportion of miRNA transcripts are encoded within introns of protein-coding genes (~80%), while a smaller proportion are encoded in intergenic regions (~20%) and some are co-expressed as unique polycistronic clusters (Lagos-Quintana et al., 2001; Lau et al., 2001; Rodriguez et al., 2004; Baskerville and Bartel, 2005). In general, miRNA repress protein expression through sequence-directed binding to complementary sites within the 3' untranslated region (UTR) of messenger RNA (mRNA) targets, resulting in mRNA destabilization and degradation or inhibition of translation (Bartel, 2009; Winter et al., 2009). Recently, however, evidence suggests that miRNA may also regulate mRNA targets through less stringent mechanisms, such as by binding to "seedless" or non-complementary

*Correspondence to: H. Leighton Grimes, Cincinnati Children's Hospital Medical Center, Division of Immunobiology—MLC 7038, 3333 Burnet Avenue, Room S5.224, Cincinnati, OH 45229-3039., lee.grimes@cchmc.org.

regions, or by binding to sites located within the coding regions of transcripts (Lal et al., 2009; Lee et al., 2009). Overall, the breadth of miRNA target gene regulation is extensive. Perceiving miRNA as members of signal transduction pathways should further our understanding of how molecular signaling events are relayed and regulated in previously well-characterized pathways, and may point to novel therapeutic approaches.

MicroRNA Biogenesis, Target Recognition, and Regulation

In mammalian cells, miRNA loci are transcribed by RNA polymerase II into 5'-capped and 3'-polyadenylated primary transcripts, called pri-miRNA (Cai et al., 2004; Lee et al., 2004). Concurrent with transcription, the first round of pri-miRNA processing is carried out by the RNase III endoribonuclease Drosha-DGCR8 microprocessor complex and results in a ~70–120 nucleotide hairpin precursor, or pre-miRNA (Denli et al., 2004; Han et al., 2004; Lee et al., 2004; Morlando et al., 2008). The pre-miRNA is transported from the nucleus to the cytoplasm by Exportin-5 where another endonuclease, Dicer, cleaves the stem-loop to yield a ~19–24 nucleotide mature miRNA/passenger strand RNA-duplex (Hutvagner et al., 2001; Yi et al., 2003; Bohnsack et al., 2004; Chendrimada et al., 2005). Next, the mature miRNA strand is incorporated into the RNA-induced silencing complex (RISC), comprised of an Argonaute (Ago) core, that guides the miRNA to bind target mRNA transcripts (Khvorova et al., 2003; Meister et al., 2004; Okamura et al., 2004). In contrast to conventional miRNA processing, many alternative and context-dependent mechanisms of miRNA biogenesis and target recognition have been identified, thus there is ongoing investigation into the novel and unique properties of miRNA signaling. For example, a novel miRNA biogenesis pathway was recently identified in which erythroid-specific miR-451 was generated by Ago2 catalysis in a Dicer-independent manner (Cifuentes et al., 2010). In addition, the post-transcriptional processing of pri-miR-21 into pre-miR-21 is enhanced by transforming growth factor- β (TGF- β) and bone morphogenic protein (BMP)-induced SMAD binding to the Drosha microprocessor complex resulting in increased mature miR-21 expression and target gene regulation (Davis et al., 2008). While these and other unique mechanisms of miRNA genesis may also occur in other tissues, such as the bone marrow, further research is necessary to unravel such complexities of miRNA processing in response to specific signaling events.

The miRNA/RISC complex recognizes target mRNA 3'UTR sequences that are complementary to a “seed” region (nucleotides 2–8) in the 5' end of the mature miRNA molecule, resulting in post-transcriptional mechanisms of gene regulation including mRNA destabilization and translational inhibition (Lewis et al., 2005; Filipowicz et al., 2008; Chekulaeva and Filipowicz, 2009). The complementarity of the miRNA to different target 3'UTR sequences is variable and may contribute to observed differences in target mRNA regulation and the difficulty of target identification (Doench et al., 2003; Zeng et al., 2003; Thomas et al., 2010). Although rare, perfect or near perfect base-pairing of the mature miRNA with mRNA targets has been described and results in transcript degradation by an Ago-mediated endonucleolytic mechanism, which is similarly employed by siRNA (Yekta et al., 2004). Alternatively, miR-24 directly represses cell cycle regulatory gene transcripts by binding to “seedless” sites in the 3'UTR of target mRNA (Lal et al., 2009). In contrast, miRNA signaling has also been shown to increase translation of specific mRNAs by two

independent mechanisms, one in which miRNA-target binding promotes translation during cell cycle arrest (Vasudevan et al., 2007) and another in which the miRNA outcompetes mRNA for binding to a translational repressor protein during leukemic progression (Eiring et al., 2010). Its likely that the full extent of miRNA-mediated post-transcriptional mechanisms of gene regulation remain to be elucidated.

MicroRNAs in Hematopoiesis

In the bone marrow, multipotent, self-renewing hematopoietic stem cells (HSC) give rise to lymphoid and myeloid progenitors that differentiate into the mature cells of the blood system (Fig. 1A). This process of adult hematopoiesis is tightly regulated by both extrinsic (extracellular) and intrinsic (intracellular) factors (Orkin, 2000; Zhu and Emerson, 2002; Orkin and Zon, 2008). Extrinsic stimuli from the bone marrow microenvironment, or niche, including cell adhesion molecules, growth factors, and cytokines, provide essential external cues to direct HSC fate decisions under conditions of homeostasis and stress. In addition, cell-intrinsic networks of signaling molecules and transcription factors act to coordinate HSC self-renewal and lineage commitment decisions (Calvi et al., 2003; Zhang et al., 2003). miRNA add yet another layer of regulation to the determination of hematopoietic cell fate and function (Chen et al., 2004) by targeting the expression of cell surface receptors and ligands (Felli et al., 2005; Wang et al., 2008; Girardot et al., 2010), downstream signaling molecules, and transcription factors (Yekta et al., 2004; Tanzer et al., 2005; Shen et al., 2008; Lund, 2010) to fine tune and integrate the cellular response to both extrinsic and intrinsic stimuli. Thereby, miRNA play a role in early HSC outcomes such as self-renewal potential (Han et al., 2010), apoptosis (Guo et al., 2010), and balance of lymphoid and myeloid subsets within the multipotent progenitor fraction (Ooi et al., 2010).

In the niche, cell surface receptor/ligand interactions initiate the bridge between extracellular cytokines and growth factors and intracellular signaling components to coordinate hematopoiesis. Although miRNA target sites are under-represented in 3'UTRs of cytokine and chemokine ligands and receptors relative to the genome as a whole (Asirvatham et al., 2009), these molecules are not exempt from regulation. Cytokine transcripts such as IL-2 and IL-10 are targeted by miR-181c and miR-106a, respectively, and may result in differential states of immune activation (Sharma et al., 2009; Xue et al., 2011). In addition, miR-24, miR-221, and miR-222 have been shown to negatively regulate erythropoiesis by targeting TGF β family activin type I receptor (ALK4) and stem cell factor receptor (c-Kit), respectively (Fig. 1B, red and purple) (Felli et al., 2005; Wang et al., 2008). In the megakaryoid lineage, miR-28 has been shown to inhibit differentiation by targeting the thrombopoietin receptor (TpoR) (Fig. 1B, orange) and is overexpressed in platelets in a fraction of MPN patients (Girardot et al., 2010). miRNA also target central transcription factors, such as the highly conserved homeobox (Hox) genes, to either enhance or disrupt intracellular networks. miR-10, miR-196, and miR-126 have been shown to directly target different Hox gene transcripts (Yekta et al., 2004; Tanzer et al., 2005; Shen et al., 2008; Lund, 2010), which are temporally expressed during hematopoiesis in a cell-type specific manner. CCAAT enhancer binding protein α (C/EBP α)-regulated miR-34a targets members of the E2F family of transcription factors to block myeloid proliferation and drive granulopoiesis. miR-34a expression is deregulated in AML patients with CEBPA mutations

(Pulikkan et al., 2010b). miRNA-transcription factor signaling also participates in mutual feedback loops during hematopoiesis. To control monocytopenia, miR-17-5p, miR-20a, and miR-106a translationally repress the transcription factor Runx1 (AML1), while Runx1 transcriptionally represses miR-17-5p-92 and 106a-92 clusters (Fontana et al., 2007). In addition, C/EBP α -regulated miR-223 targets the cell-cycle regulator E2F1 to control granulocytic differentiation. Furthermore, E2F1 binds to the miR-223 promoter in AML blast cells and inhibits miR-223 transcription, revealing a molecular network between miRNA and transcription factors that not only controls the granulocytic differentiation program, but is also deregulated in AML (Pulikkan et al., 2010a).

To summarize, numerous miRNA play critical and pleiotropic roles in hematopoietic cell subsets operating in the stem/progenitor (Georganta et al., 2007; Guo et al., 2010; Han et al., 2010), lymphoid (Neilson et al., 2007; Xiao et al., 2007; Ventura et al., 2008), myeloid (Fontana et al., 2007; Johnnidis et al., 2008; Velu et al., 2009), erythroid (Felli et al., 2005), and megakaryocytic (Garzon et al., 2006; Lu et al., 2008) branches of the hematopoietic system, as well as exhibiting defined roles in the immune function of all these cell lineages (O'Connell et al., 2010). Furthermore, miRNA expression profiles have been resolved in most hematopoietic lineages. Integration of hematopoietic miRNA expression profiles with mRNA target predictions in silico revealed a model for miRNA-mediated control of hematopoiesis in which many of the genes specifying hematopoietic differentiation, such as HOXA9, HOXA10, HOXB5, HOXB7, AML1/RUNX1, and C/EBP β , are expressed in the stem/progenitor compartment, but are held in check by miRNAs until differentiation occurs (Georganta et al., 2007). miRNA expression profiles have also been characterized in leukemic subtypes exhibiting cell-specific expression patterns, which have been reviewed recently (Baltimore et al., 2008; Garzon and Croce, 2008; Havelange and Garzon, 2010). Thus, it is plausible that these biased expression patterns may also be exploited to treat hematologic diseases. This review will focus on the dynamic regulation of gene expression by miRNA at the interface of the extrinsic and intrinsic cues in key hematopoietic signaling pathways that govern stem cell maintenance, differentiation, and mature cell function, providing specific examples of such signaling cascades that may be exploited therapeutically.

MicroRNA in Signal Transduction Networks

Highlighting the critical impact of proper miRNA signaling in the hematopoietic system, accumulating evidence has shown aberrant miRNA expression and function in hematopoietic malignancies such as leukemia, hematopoietic cell deficiencies including severe congenital neutropenia (SCN), pre-leukemic syndromes like myelodysplastic syndrome (MDS), and autoimmune disorders such as rheumatoid arthritis (RA). Identifying which miRNA are deregulated in disease, as well as delineating their upstream and downstream signaling partners, is crucial to improving the efficacy of current therapies and may also identify novel miRNA or combinatorial therapeutic avenues. Figure 1B summarizes miRNA regulation and deregulation of myeloid-specific differentiation signaling through the centralized Ras/MAPK signaling cascades and Figure 2 summarizes miRNA regulation of myeloid immune responses mediated by Toll receptor signaling.

Myeloid lineage development and AML: Ras, MAPK, transcription factors, and miRNA

Severe congenital neutropenia is characterized by maturation arrest and accumulation of promyelocytes in the bone marrow. Current treatment of recombinant granulocyte-colony stimulating factor (G-CSF) stimulates granulopoiesis in many, but not all, patients, and results in functionally impaired peripheral blood neutrophils (Donini et al., 2007). SCN patients often develop MDS or AML, which may be accelerated by G-CSF therapy (Rosenberg et al., 2006). Mutation of the growth factor independent-1 (GFI1) gene, which encodes a transcription factor repressor protein whose expression is induced during the transition from common myeloid progenitor (CMP) to granulocyte-monocyte progenitor (GMP) (Velu et al., 2009), is one underlying cause of SCN (Person et al., 2003). Gfi1 loss-of-function mutations in mice arrest myeloid differentiation (Karsunky et al., 2002; Hock et al., 2003; Person et al., 2003; Zarebski et al., 2008). G-CSF treatment of human and murine cells mutant for GFI1 does not induce granulopoiesis (Person et al., 2003; Zarebski et al., 2008). Recent work in our laboratory revealed that Gfi1 represses the expression of miR-21 and miR-196b during normal myelopoiesis (Fig. 1B), and both Gfi1^{-/-} mice and GFI1-mutant (GFI1N382S) SCN patients display aberrantly increased expression of miR-21 and miR-196b in the bone marrow (Velu et al., 2009). In vitro, co-expression of miR-21 and miR-196b in wild-type bone marrow is sufficient to recapitulate a Gfi1-mutant block in neutrophil formation. This synergistic action of the miR suggests that miR-21 and -196b are responsible, at least in part, for the block in granulopoiesis downstream of Gfi1. Interestingly, G-CSF stimulated Gfi1^{-/-} cells are unable to activate the Ras pathway as evidenced by reduced MEK1/2 and Erk1/2 mitogen-activated protein kinase (MAPK) phosphorylation, which is necessary to induce neutrophil differentiation downstream of G-CSFR signaling (Kamezaki et al., 2005; de la Luz Sierra et al., 2010). The forced expression of RasGRP1, a Ras guanine nucleotide-exchange factor was reported to partially correct this signaling defect resulting in some neutrophil differentiation (de la Luz Sierra et al., 2010). However, Erk1/2 phosphorylation is not completely restored and myeloid colony formation is not fully rescued. We note that RasGRP1 is a predicted target of miR-21 and miR-196b. Together, this suggests that additional missing links, such as miRNA signaling, between Gfi1 and Erk remain to be elucidated. While these examples do not resolve why G-CSF-induced neutrophils in GFI1-normal SCN patients do not have fully functional neutrophils, it does underscore the importance of miRNA signaling in granulopoiesis and perhaps other SCN patients should be examined for aberrant miRNA expression.

The expression of miR-125b blocks G-CSF-induced granulocytic differentiation and enhances proliferation of 32D cells, reminiscent of SCN. However, the validity of the 32D model of granulopoiesis is in question, because expression of miR-125b in transplanted lineage negative mouse bone marrow progenitor cells resulted in increased myelopoiesis (Surdziel et al., 2011). Another report demonstrated that overexpression of miR-125b in transplanted HSCs resulted in expansion of lymphoid-biased HSC subsets and lymphoid-skewing of the peripheral blood constituency (Ooi et al., 2010). Both studies suggested that miR-125b targeting of Bak1 is involved in progenitor/HSC survival of both myeloid and lymphoid lineage progenitors. This is corroborated by a third study showing increased

survival upon miR-125a expression in stem/progenitor cells, which was also mediated by repression of Bak-1 (Guo et al., 2010). Reduced expression of Stat3 and Bak1 was observed when miR-125b was overexpressed in primary lineage negative cells, however, neither alone nor in combination were sufficient to recapitulate the miR-125b block to G-CSF-differentiation (Surdziel et al., 2011). Together these studies suggest that miR-125a/b are inhibitors of hematopoietic differentiation. Furthermore, miR-125b overexpression enforces this phenotype by targeting the STAT3 cofactors c-Jun and JunD, also mediators of MAPK signaling, and thereby highlighting a central role for miRNA in the regulation of multiple myeloid signaling pathways during differentiation.

Upstream of MAPK signaling, the regulation of Ras activation in response to hematopoietic cytokines, growth factors, and cell-stress signals is central to normal hematopoiesis. Gain-of-function Ras mutations and resultant signaling aberrations are commonly associated with hematologic malignancies, including pre-leukemic states such as MPD (Hirai et al., 1987; Liu et al., 1987), as well as a wide variety of neoplasms including JMML and AML (Bos et al., 1987; Farr et al., 1988). Three major families of MAPKs including Erk, JNK, and p38 can be activated by a plethora of hematopoietic cytokines. Interestingly, miRNA that play important roles in hematopoietic cell generation and function also have defined targets in MAPK signaling networks (Fig. 1B). For example, let-7 is a known regulator of Ras expression in *Caenorhabditis elegans*, and is highly enriched in the HSC and progenitor compartment in humans (Petriv et al., 2010). All three human Ras genes possess let-7 binding sites in their 3'UTRs and have been suggested, though immunohistochemical analyses, as targets of let-7-mediated regulation (Johnson et al., 2005). Further downstream, miR-34a has been shown to target the Ras effector MEK1 in the Erk/MAPK cascade to inhibit cell proliferation during megakaryocyte differentiation (Ichimura et al., 2010). In contrast, the AP-1 driven expression of the myeloid-enriched miR-21 amplifies Ras signaling by targeting several direct antagonists of the Ras/MEK/Erk pathway such as SPRY1, SPRY2, and BTG2 (Hatley et al., 2010). miR-21 also targets several indirect antagonists of Ras signaling crosstalk with the PI3K/Akt pathway, including PTEN and PDCD4 (Meng et al., 2007; Hatley et al., 2010). Overall, this regulation imposed by miR-21 culminates in the propagation of Ras signaling and enhanced tumorigenesis (Buganim et al., 2010; Hatley et al., 2010). Of note, Ras genes are the most common targets for somatic gain-of-function mutations in human cancer and Ras gene mutations are found in 30% of myeloid leukemias (Bos, 1989; Schubbert et al., 2007). miRNA-based therapeutics thus have the potential to generate several novel avenues for the difficult-to-target Ras-based hematologic malignancies such as AML.

The JNK and p38 MAPK signaling cascades are also targeted by miRNA. Recently it was found that miR-24 represses MAPK phosphatase-7 (MKP-7), a negative regulator upstream of JNK and p38. When overexpressed in myeloid progenitor cells, miR-24 drives enhanced MAPK phosphorylation, IL-3-independent growth, and a developmental block in granulocytic differentiation (Zaidi et al., 2009). Runx1/AML1 transcriptionally represses the miR-24-23-27 cluster in myeloid progenitor cells (Zaidi et al., 2009); however, abrogation of AML1 nuclear localization and disruption of transcription factor function occurs frequently in myeloid leukemias due to chromosomal rearrangements, such as t(8;21) that fuses Runx1 to ETO. The resultant AML1-ETO fusion protein leads to de-repression of the miR-24 locus

and other transcriptional targets. Interestingly, both mouse genetic models and human AML patients with the t(8;21) translocation indicate that this rearrangement alone is not sufficient to initiate disease and requires one or more additional mutations for leukemogenesis (Yergeau et al., 1997; Yuan et al., 2001). Thus, these data suggest that miR-24 may be an important effector downstream of AML1 in myeloid cell development and as well as in the progression and maintenance of disease. Overall, the analysis and exploitation of miRNA in the context of MAPK signaling provides a unique opportunity for the design and development of far reaching therapeutic agents in the treatment of hematologic malignancies.

Myeloid lineage immune function: TLR, MAPK, and miRNA

The MAPK cascade also has defined roles in mature hematopoietic cell function, including the early production of pro-inflammatory immune mediators TNF- α , IL-1 β , and IL-6 in response to Toll-like receptor (TLR) ligands known as pathogen associated molecular patterns (PAMP) (Fig. 2). TLRs comprise a family of both extracellular and intracellular receptors expressed on a variety of cell types, including B lymphocytes (Pasare and Medzhitov, 2005), mast cells (Supajatura et al., 2002), and organ-specific epithelial cells (Abreu et al., 2003), although the scope of this review extends only to cells within the stem/progenitor and differentiated myeloid compartments. Originally identified in *Drosophila*, TLR family members are characterized by conserved Toll/IL-1 receptor (TIR) and leucine-rich repeat (LRR) domains (Rock et al., 1998). Most TLRs are believed to form homodimers, although TLR2 forms heterodimers with TLR1 and TLR6 (Takeuchi et al., 2001, 2002). After binding to PAMPs, such as bacterial lipopolysaccharide (LPS), subsequent downstream TLR signaling results in myeloid cell activation and initiation of the innate immune response (Medzhitov et al., 1997; Poltorak et al., 1998). Classically, TLR stimulation by LPS potentiates the activation of two signaling pathways, either myeloid differentiation primary-response protein 88 (MyD88)-dependent or MyD88-independent (Kawai et al., 1999). The MyD88-dependent pathway results in the early activation of NF- κ B transcription factors as well as MAPK and PI3K/Akt signaling pathways, leading to the production of proinflammatory cytokines (Takeda and Akira, 2004). In contrast, MyD88-independent pathways utilize alternative adaptor proteins such as TIRAP (Horng et al., 2001), TRIF (Yamamoto et al., 2002; Fitzgerald et al., 2003), or TRAM (Fitzgerald et al., 2003; Yamamoto et al., 2003) to mediate the late-phase activation of NF- κ B, as well as interferon (IFN)-regulatory factor 3 (IRF3), both of which result in the production of IFN- β and IFN-inducible gene products (Kawai et al., 2001; Hoshino et al., 2002). To resolve inflammation and restore homeostasis following the clearance of infection, TLR signaling must be turned off, and is therefore tightly controlled. The expression of several miRNA increases following TLR activation and have emerged as important regulators of the TLR pathway as reviewed recently (O'Neill et al., 2011). In addition, even though different TLR family members share common downstream signaling molecules and pathways, unique gene expression patterns are observed following stimulation of each receptor, thus further suggesting that miRNA may play a role in directing the specificity of the immune response.

In the myeloid branch of hematopoiesis, monocytes, macrophages, dendritic cells (DC), and granulocytes are the key players of the innate immune response (Fig. 1A, bottom part). A

host of miRNA that act to enhance or antagonize inflammation are upregulated in these cell types following TLR activation, including miR-147 (Liu et al., 2009), miR-155 (Taganov et al., 2006; O'Connell et al., 2007, 2009; Androulidaki et al., 2009; Ceppi et al., 2009), miR-146a/b (Taganov et al., 2006; Hou et al., 2009), and miR-101 (Zhu et al., 2010) (Fig. 2). miR-147 has been shown to negatively regulate TLR signaling in macrophages, resulting in reduced macrophage-mediated inflammation post-stimulation by multiple TLR ligands (Liu et al., 2009). This has been postulated to occur through the targeting of upstream TLR effectors such as MyD88 and TRIF, although targets have not yet been validated. In addition, miR-155 expression is upregulated in monocytes, macrophages, and DCs following TLR activation and mediates contrasting effects by targeting the pro-inflammatory signaling molecule TAB2 (Ceppi et al., 2009), and the TLR antagonists SHIP1 (O'Connell et al., 2009) and SOCS1 (Androulidaki et al., 2009). NF- κ B-dependent induction of miR-146a/b occurs rapidly in monocytes and macrophages following LPS or vesicular stomatitis virus (VSV) exposure. miR-146 targets the pro-inflammatory signaling molecules TRAF6, IRAK1, and IRAK2 to potentiate the negative regulation of both extracellular TLR signaling and cytoplasmic retinoic acid-inducible gene I (RIG-I)-like helicase signaling to return immune function back to baseline steady state (Taganov et al., 2006; Hou et al., 2009). Of note, an earlier report suggested that intracellular pattern recognition receptors specific for viral nucleic acids, such as the RIG-I-like helicases, have little effect on miR-146 expression, thus more studies need to be done to determine the miRNA that are induced/repressed upon viral infection in this VSV model (Taganov et al., 2006). Downstream of TRAF6, miR-101, which is induced in response to multiple TLR ligands including LPS, directly targets the MAPK antagonist MKP-1 resulting in MAPK activation, increased production of pro-inflammatory mediators, and enhanced innate immune response (Zhu et al., 2010).

TLRs are also expressed on HSCs and drive myeloid-biased proliferation and differentiation in response to stimulation with the microbial ligands LPS and Pam₃CSK₄ (Nagai et al., 2006). The aberrant overexpression of miR-155 in the bone marrow progenitor compartment leads to the development of a myeloproliferative disease in a SHIP1-dependent mechanism (O'Connell et al., 2008, 2009). In contrast, the overexpression of miR-146 results in a transient myeloid progenitor expansion, decreased erythropoiesis, impaired lymphopoiesis, and impaired bone marrow reconstitution (Starczynowski et al., 2011). Conversely, miR-146 and miR-145 are deleted in 5q-syndrome, a subtype of MDS, and the loss of these miRNA in hematopoietic stem and progenitor cells results in abnormal repression of the TLR effector molecules TIRAP and TRAF6, which leads to inappropriate activation of innate immune signals (Starczynowski et al., 2010). The roles of other known TLR-inducible miRNA in the stem/progenitor compartment remain to be characterized, and may prove to be instrumental in further unraveling the mechanisms of myeloproliferative and myelodysplastic hematopoietic diseases.

Excessive immune responses are detrimental to the host. The dysregulation of TLR signaling often occurs in chronic auto-inflammatory disorders, such as RA. Interestingly, patients with RA also exhibit altered expression of miR-155 and miR-146, further suggesting a link between these miRNA and the TLR signaling pathway (Pauley et al., 2008, 2009). However, several mechanisms outside of the canonical NF- κ B TLR signaling axis are in place to

minimize inflammation and injury. The PI3K/Akt signaling cascade is one such pathway that has been reported to negatively regulate the inflammatory response upon LPS exposure by promoting the production of IL-10 (Guha and Mackman, 2002; Schabbauer et al., 2004). Deletion of the PI3K pathway antagonist, PTEN, in macrophages results in constitutively active PI3K signaling and enhanced Akt phosphorylation. In response to TLR stimulation by bacterial infection and LPS treatment, PTEN-deficient macrophages demonstrate reduced MAPK activation and produce reduced levels of inflammatory cytokines (Gunzl et al., 2010). The normal macrophage inflammatory response results in robust upregulation of miR-21 by AP-1, which has been shown to target PTEN in hepatocellular carcinoma (Meng et al., 2007). Thus it is plausible that miR-21 may function in immunoregulation to repress PTEN in macrophages leading to resolution of inflammation, although this connection remains to be elucidated experimentally. All together, these examples provide several unique potential therapeutic avenues for the treatment of chronic autoinflammatory conditions such as RA.

Discussion

Despite the complexities and controversies surrounding miRNA genesis and targeting, it has become clear that, because of their critical role in gene regulation, miRNA may prove in the near future to be excellent therapeutic targets for a variety of developmental and tumor disease phenotypes. The pharmaceutical industry is becoming increasingly engaged in the discovery of RNAi-based therapeutics, which encompasses siRNA (protein coding gene-specific inhibition), anti-miRNA (miRNA inhibition), and miRNA replacement (miRNA mimic) designs. In general, results of pre-clinical and clinical trials have demonstrated successes over sequence design barriers ("GU" rich regions, deoxy modifications, 2'-O-me modifications, modified phosphorothioate backbone, cholesterol modifications) that help increase the ability of the siRNA/miRNA therapies to aide in cellular uptake and increase stability (Krutzfeldt et al., 2005; Esau, 2008; Davis et al., 2009), but delivery mechanisms that do not elicit an immune response or other off-target effects remain a challenge for siRNA and miRNA mimic approaches (Sledz et al., 2003; Baker, 2010; Jackson and Linsley, 2010b). Unlike siRNA or RNAi-based therapies, single target sequence-directed specificity does not play a central role in the design or implementation of anti-miRNA or miRNA mimic therapies. Rather, one miRNA can regulate many genes that have been selected by evolution to lie in pathways that can produce a given phenotype and thus a strong therapeutic effect (Jackson and Linsley, 2010b). Because anti-miRNA therapeutics are single stranded oligonucleotides that have favorable pharmacokinetic properties making formulation unnecessary (Geary, 2009), these compounds have and will continue to enter the clinic before miRNA mimic therapeutics that require additional processing for effective delivery (<http://clinicaltrials.gov/ct2/show/NCT01200420>) (Jackson and Linsley, 2010a). However, different modifications and sequences of anti-miRNAs vary in their ability to result in miRNA degradation, thus measurement of anti-miR activity has been difficult (Krutzfeldt et al., 2005; Chan et al., 2010).

Recent reports have demonstrated that miRNA may serve as predictive or prognostic biomarkers of primary and therapy-resistant disease (Sarasin-Filipowicz et al., 2009; Heneghan et al., 2010; Hwang et al., 2010; Ju, 2010; Ranade et al., 2010; Zhang et al.,

2011), as mechanisms of therapeutic resistance themselves (Miller et al., 2008; Kojima et al., 2010; Sarkar et al., 2010), and a novel concept is to combine miRNA therapeutics with the current standard of care therapy for a given disease.

Lastly, leaders in industry with ongoing clinical trials specifically for miRNA-based therapeutics as of March 2011 include Regulus Therapeutics (four anti-miRs in pre-clinical phases for HCV, fibrosis, cancer, and cardiovascular disease), miRagen Therapeutics (two anti-miR in pre-clinical phases for heart diseases), Mirna Therapeutics (several “miRNA replacement therapies” close to pre-clinical testing for non-small cell lung cancer and prostate cancer), Rosetta Genomics (developing miRNA diagnostic tests), and Santaris Pharma a/s (in Phase II with miravirsin targeting miR-122 for treatment and prevention against Hepatitis C). While the robust implementation of miRNA based therapeutics remains a future goal, these industry efforts should provide a proof of principle to move the field forward.

This review integrates miRNA signaling into key myeloid signal transduction pathways from points of regulation at extracellular stimuli to intracellular signaling cascades. This interpretation of miRNA signaling was designed to help specify novel potential combinatorial therapeutic strategies for the treatment of hematologic malignancies and disorders, identify new areas of research where such advances may be possible in the future, and illustrate the complex nature of miRNA signaling and regulation that may be exploited for treatment of myeloid malignancies and other human diseases.

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Fig. 1. miRNA pathways in myeloid differentiation and myeloid-derived malignancies. A: Schematic of adult definitive hematopoiesis. Multipotent, self-renewing HSCs (yellow background) give rise to lymphoid and myeloid multi-potent progenitors (orange background), which differentiate into mature blood cells (red background). B: Schematic of miR-regulated signaling cascades in myeloid differentiation. Cytokines and growth factors bind to their respective receptors to stimulate the activation of downstream signaling cascades. G-CSF signals primarily through the Ras/MEK/Erk MAPK pathway to induce granulocytic differentiation (blue arrows). Aberrations in Ras signaling, via gain-of-function mutations or otherwise, are known causes of MPD and AML (red knob-endline). All human Ras mRNAs possess let-7 binding sites in their 3' UTRs, subjecting the initiation of this cascade to let-7-mediated regulation. miR-34a targets MEK1 to inhibit cell proliferation during megakaryocyte differentiation. Expression of the transcriptional repressor Gfi1 is induced during the transition from CMP to GMP. Gfi1 negatively regulates the expression of miR-21 and miR-196b. Gfi1 loss-of-function mutations in mice and humans arrest myeloid development, and offer one underlying cause of SCN. Co-expression of miR-21 and miR-196b in wild-type bone marrow is sufficient to recapitulate a Gfi1-mutant block in neutrophil formation. In contrast, the AP-1 driven expression of the myeloid-enriched miR-21 amplifies Ras signaling by targeting several direct antagonists of the Ras/MEK/Erk pathway such as BTG2. miR-21 also targets several indirect antagonists of Ras signaling crosstalk within the PI3K/Akt pathway, including PTEN and PDCD4. The JNK and p38 MAPK signaling cascades are also regulated by miRNA. miR-24 represses MKP-7, a negative regulator upstream of JNK and p38 phosphorylation. Additionally, miRNAs play marked roles in the regulation of other lineages by targeting the expression of essential cell-surface receptors. miR-28 targets the thrombopoietin receptor to inhibit megakaryocyte differentiation (orange arrow). Overexpression of miR-28 is associated with MPN (red knob-end line). miR-24 targets the type I activin receptor ALK4 to inhibit erythropoiesis (maroon arrow). miR-221 and miR-222 also inhibit erythropoiesis by targeting the SCF receptor kit. ACT, activin; ALK4, activin type I receptor; AML, acute myeloid leukemia; AP-1, activator protein 1; B, B-cell; BTG2, B-cell translocation gene 2; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; DC, dendritic cell; Er, erythrocytes; Erk, extracellular-signal-regulated kinases; G-CSF, granulocyte colony-stimulating factor; G-CSFR,

granulocyte colony-stimulating factor receptor; Gfi1, growth factor-independent 1; GMP, granulocyte-monocyte progenitor; Gr, granulocytes/neutrophils; JNK, c-Jun N-terminal kinase; LT-HSC, long-term hematopoietic stem cell; MΦ, monocytes/macrophages; MEK, mitogen-activated protein kinase kinase; MEP, megakaryoid-erythroid progenitor; miR, microRNA; Mk, megakaryocytes; MKP-7, MAPK phosphatase-7; MPD, myeloproliferative disease; MPN, myeloproliferative neoplasm; MPP, multipotent progenitor; mTOR, mammalian target of rapamycin; NK, natural killer cell; PDCD4, programmed cell death protein 4; PI3K, phosphatidylinositol 3-kinases; PTEN, phosphatase and tensin homolog; SCF, stem cell factor; SCN, severe congenital neutropenia; ST-HSC, short-term hematopoietic stem cell; T, T-cell; Tpo, thrombopoietin; TpoR, thrombopoietin receptor. Black, blue, orange, and maroon solid arrows represent normal hematopoietic signaling, solid red lines indicate abnormal signaling, arrowhead represents causal mechanism, ⊥-ended dashed lines represent inhibitory mechanism, knob-end represents aberrant expression associated with abnormal signaling/phenotype.

Fig. 2.

miRNA pathways in mature immune cell function. Schematic of TLR signaling in response to pathogenic stimuli. Following TLR activation, subsequent downstream signaling through MyD88/TRAF (shown) or TRAM, TRIF, and TIRAP (not shown) results in NFκB nuclear localization, which promotes the expression of several miRNAs, including miR-21, miR-101, miR-147, miR-146a/b, and miR-155. MAPK signaling also enhances the early inflammatory response by inducing the nuclear translocation and activation of AP-1, resulting in the production of early pro-inflammatory mediators such as TNF-α and IL-1. miR-155 enhances inflammation by targeting the TLR antagonists SHIP1 and SOCS1. Conversely, miR-147 antagonizes TLR activation, potentially by targeting upstream signaling molecules such as MyD88. miR-146a/b also negatively regulate TLRs by targeting the pro-inflammatory molecules IRAK1, IRAK2, and TRAF6. Deregulation of TLR signaling occurs in chronic auto-inflammatory disorders, such as RA. Patients with RA also exhibit altered expression of miR-155 and miR-146 (red knob-end line), further suggesting a link between these miRNA and the TLR signaling pathway. TLRs are also expressed on HSCs and drive myeloid-biased proliferation and differentiation in response to stimulation with microbial ligands. The aberrant overexpression of miR-155 in the bone marrow progenitor compartment leads to the development of an MPD in a SHIP1-dependent mechanism (red knob-end line). Conversely, miR-146a (and miR-145—not pictured) are deleted in 5q-Syndrome, a subtype of MDS. The MAPK and PI3K/Akt signaling cascades also play an important role in the regulation of TLR signaling. MAPK signaling is associated with early, NFκB-independent inflammation and the production of pro-inflammatory cytokines TNFα, IL-1β, and IL-6 via AP-1. miR-101 enhances the inflammatory response by targeting MKP-1, an antagonist of both JNK and p38 MAPKs. In

contrast, PI3K/Akt signaling has been reported to negatively regulate the inflammatory response via the production of IL-10. miR-21 is robustly upregulated in response to TLR stimulation, and may target the PI3K inhibitor PTEN to drive enhanced PI3K/Akt signaling and the resolution of inflammation by enhanced IL-10 production. AP-1, activator protein 1; IRAK1/2, IL-1R-associated kinase 1 and 2; MAPK, mitogen-activated protein kinase; MDS, myelodysplastic syndrome; miR, microRNA; MPK1, map kinase phosphatase 1; MPD, myeloproliferative disease; MyD88, myeloid differentiation primary response gene 88; NF κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; PAMP, pathogen-associated molecular pattern; PI3K, phosphatidylinositol 3-kinases; PTEN, phosphatase and tensin homolog; RA, rheumatoid arthritis; SHIP1, SH2-domain-containing inositol-5-phosphatase 1; SOCS1, suppressor of cytokine signaling 1; TLR, Toll-like receptor; TRAF6, TNF-receptor-associated factor 6. Black arrows represent normal TLR signaling, red lines indicate abnormal signaling, arrowhead represents causal mechanism, $\perp$ -ended dashed lines represent inhibitory mechanism, knob-end represents aberrant expression associated with abnormal signaling/phenotype.