THE ROLE OF MICRORNAS IN PROMOTING FLT3-ITD-INDUCED MYELOID MALIGNANCY

by

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ABSTRACT

MicroRNA expression is dysregulated in many human cancers, including hematopoietic malignancies. Among hematopoietic malignancies, acute myeloid leukemia (AML) carries a particularly poor prognosis, leading to over 10,000 deaths each year in the US alone. The most common genetic aberration in AML is a gain-of-function mutation in the FMS-like tyrosine kinase 3 (FLT3) receptor. FLT3 internal tandem duplication (ITD) occurs in ~30% of all AML cases, and confers a negative prognosis. MicroRNA expression has been shown to be highly dysregulated in FLT3-ITD+ AML; however, the functional relevance of many of these microRNAs on leukemic phenotypes remains unclear. We performed a genome-wide CRISPR-Cas9 screen to identify which microRNAs, and which of their putative mRNA targets, regulate FLT3-ITD+ AML cell growth. Our screen identified a number of microRNAs that function to suppress or promote FLT3-ITD+ AML cell growth, revealing that microRNAs are extensively integrated into the molecular networks that control tumor cell physiology. We also performed anticorrelation functional profiling to predict relevant microRNA-mRNA target pairs in this context, and identified miR-150 targeting of p53 as a critical relationship governing the growth of these cells. We validated one of our targets, miR-155, as a critical regulator of FLT3-ITD+ AML cell growth in vitro, where miR-155-deficient cells displayed a competitive growth disadvantage compared to cells with miR-155 intact. We extended these findings into an in vivo model of FLT3-ITD-driven

myeloid malignancy, where mice containing a FLT3-ITD mutation but lacking miR-155 exhibited decreased myeloid expansion in the bone marrow, spleen, and blood compared to their FLT3-ITD miR-155+/+ counterparts. This phenotype was attributed to miR-155's role in promoting proliferation of the hematopoietic stem cell and myeloid progenitor cell compartments in the bone marrow. Further analysis revealed that miR-155 likely exerts these effects by regulating multiple pathways involved in cellular proliferation, including repressing the interferon response through targeting Cebpb, and activating AKT signaling through targeting of Ship1. These findings correlated with human AML data from The Cancer Genome Atlas dataset, where we found that FLT3-ITD+ AML samples had a decreased interferon signature and lower levels of Cebpb and Ship1 compared to FLT3-WT AML samples. Finally, we treated FLT3-ITD+ AML primary patient samples with a miR-155 inhibitor and observed decreased colony forming potential and increased apoptosis in these cells. These results suggest that miR-155 inhibition could be a novel therapeutic approach in FLT3-ITD+ AML.

To my family who made it possible:

Craig, Dianna, Ashleigh, Ryan, Dylan, Kirsten

and to my amazing wife,

Annie

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LIST OF ABBREVIATIONS

AML.....Acute myeloid leukemia

CRISPR.....Clustered regularly interspaced short palindromic repeats

FLT3.....FMS-like tyrosine kinase 3

FLT3L.....FLT3 ligand

GM.....Granulocyte-monocyte

HSC.....Hematopoietic stem cell

HSPC.....Hematopoietic stem and progenitor cell

ITD.....Internal tandem duplication

miRNA.....MicroRNA

mRNA.....Messenger RNA

MPD.....Myeloproliferative disease

NPMc.....Nucleophosmin1 mutation

PDGF.....Platelet-derived growth factor

RISC.....RNA-induced silencing complex

PDGFR.....Platelet-derived growth factor receptor

RISC.....RNA-induced silencing complex

TKI.....Tyrosine kinase inhibitor

UTR.....Untranslated region

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CHAPTER 1

INTRODUCTION

MicroRNAs are key regulators of hematopoiesis

Hematopoiesis is a dynamic biological process giving rise to cells of the blood that carry out a variety of essential functions, such as oxygen delivery to tissues and defense against infectious microbes. Careful balance during hematopoiesis is required to both generate mature blood cells through a hierarchical differentiation process and preserve stem cell compartments to ensure a long-term source of blood cell production in animals with long life spans. Over the past several decades, we have learned a tremendous amount regarding the genes and signaling pathways governing various aspects of blood cell development. These include genes that regulate responses to inflammatory stress, and others that become dysregulated in pathological conditions of the hematopoietic system, including various hematologic malignancies. Among these genes important for blood cell development are those that produce noncoding RNAs, including the microRNA (miRNA) family.

First discovered in *C. elegans* in the early 1990s (1), miRNAs are now recognized to be a novel class of small noncoding RNAs that are evolutionarily conserved across nearly all species, from plants to mammals (2). It is now estimated there may be more than 6000 miRNAs in the human genome (3), each with a potentially unique biological function. miRNAs are typically transcribed from intergenic or intronic regions of the genome into the primary miRNA transcript, which then undergoes a number of processing steps in the nucleus and cytoplasm leading to the production of the mature miRNA, a short RNA molecule 20-24 nucleotides in length. The mature miRNA is then loaded into the RNA-induced silencing complex (RISC), which binds to the 3' untranslated region (UTR) of its messenger RNA (mRNA) targets by Watson-Crick base

pair complementarity (4). The binding of the miRNA-RISC complex leads to decreased target protein production through mechanisms involving decreased mRNA stability and reduced translation (5).

The past two decades of research have established miRNAs as playing a vital role in posttranscriptional gene regulation. This reduction is typically within the range of 2- to 4-fold, indicating that miRNA-mediated repression does not serve as an on-off switch, but instead as a rheostat for biological processes requiring fine-tuning of gene expression (6). Hematopoiesis is one such biological process, where miRNAs are critical for achieving proper lineage specification and differentiation by ensuring proper expression of genes in complex, regulatory networks and signaling pathways (7,8).

miRNA expression patterns, function, and target gene regulation have been extensively studied throughout the process of normal hematopoietic development, starting with hematopoietic stem cells (HSCs). HSCs are characterized by their ability to both self-renew and differentiate into all hematopoietic lineages. Further, it is clear that long-term HSCs persist largely in a state of quiescence, while differentiating stem cell populations cycle at an accelerated rate. Several studies have found that this balance is carefully regulated by miRNAs. A subset of miRNAs are enriched in mouse and human HSCs compared to mature hematopoietic cells (9), and some of these miRNAs are critical for properly managing hematopoietic output (10). HSC-specific deletion of Dicer1, an essential enzyme in the miRNA biogenesis pathway, leads to loss of function and significantly increased cell death in this compartment (11), thus suggesting miRNAs are critical for maintaining a functional pool of HSCs. For example, one study identified miR-125a as being specifically enriched in long-term HSCs, and overexpression of miR-

125a led to expansion of HSC cell numbers by preventing apoptosis (11). Another independent study found that miR-125a overexpression conferred a competitive reconstitution advantage (12). Other examples of miRNAs regulating HSC function include miR-29a, which represses the methyltransferase enzyme Dnmt3a leading to increased HSC self-renewal capacity and quiescence (13), and miR-126, whose expression prevents HSC expansion by repressing multiple targets in the proproliferative PI3K/AKT pathway (14). It is also becoming clear that miRNAs regulate HSC function across the lifespan of mammals. The increased expression of the let-7 family of miRNAs in HSCs signifies a transition from fetal hematopoiesis to adult HSCs with decreased self-renewal potential (15), whereas the miRNA-212/132 cluster is critical for HSC maintenance in aging mice through inhibition of Foxo3 expression (16).

Not only do miRNAs clearly regulate hematopoiesis at the level of the HSC, but they also regulate hematopoietic development throughout maturation in both myeloid and lymphoid lineages. The myeloid lineage gives rise to a variety of mature blood cell types with a broad range of form and function. This includes cells of the granulocyte-monocyte (GM) lineage, recognized for their essential role in innate immunity. Deletion of Dicer1 specifically in myeloid progenitors leads to neutrophil dysplasia and blocks monocytic differentiation in mice (17), thus suggesting miRNAs are critical for the development of mature myeloid cells from the GM lineage. miR-155 and miR-146a are two well-characterized miRNAs that have been shown to counter-regulate GM cell expansion in the bone marrow through their opposing roles in regulating inflammatory pathways (18,19). Interestingly, several miRNAs, including miR-155 and miR-146a, have been found to be critical for myeloid cell function, regulating key innate immune pathways,

and cytokine production in macrophages and dendritic cells (18,19).

Beyond GM cells, several studies have identified specific miRNAs that have evolved to regulate both megakaryocyte and erythroid development (20). RNA expression profiling of the megakaryocyte lineage has identified distinct miRNA expression patterns during their development (21,22). miR-150 is one example of a miRNA that is enriched in mature megakaryocytes. Overexpression of miR-150 in megakaryocyte-erythrocyte progenitors favors megakaryocyte production at the expense of erythropoiesis through the repression of c-Myb (23). Conversely, miR-486-5p is critical for erythroid differentiation and survival in both in vitro and in vivo studies (24).

miRNAs have also been extensively studied in the stetting of lymphocyte development and function. The lymphoid lineage gives rise to cells of adaptive immunity, including T, B, and natural killer (NK) cells. In some cases, a specific miRNA may have a broad impact on lymphoid formation. miR-142 was recently found to be critical for proper lymphopoieis, as mice with a genetic deletion of the miR-142 locus fail to develop both T and B1 B cells, leading to severe immunodeficiency (25). However, most studies to date have focused on the effects of individual miRNAs on specific lymphoid lineages.

miRNAs are widely accepted as playing a critical role in B cell development and effector function. The importance of miRNAs in B cell development was convincingly demonstrated when deletion of Dicer1 in the B cell lineage of mice led to a developmental block in the transition from pro- to pre-B cells (26). Additionally, the first study to recognize the importance of miRNAs in hematopoietic lineage differentiation identified miR-181 as a driver of B cell development (27). Further studies have identified distinct patterns of miRNA expression across developmental B cell stages in humans,

including naïve B cells, germinal center B cells, plasma cells, and memory B cells (28). These patterns suggest that proper miRNA expression is critical for promoting specific steps in mature B cell formation. A variety of miRNAs have now been found to affect B cell development and differentiation (29), where genetic deletion of these miRNAs leads to an increased or decreased number of mature B cells. Other miRNAs seem to govern different aspects of mature B cells function, including differentiation into plasma cells, high affinity antibody production, and enhancing the germinal center reaction (30-33). These studies lend credence to the idea the miRNAs are deeply rooted into all aspects of B cell biology.

Much attention has been paid to the role of miRNAs in T cells, as T cells are key orchestrators of adaptive immune responses and have been the focus of many immunotherapeutic strategies. Initial studies based on global disruption of miRNAs via Dicer1 deletion in developing T cells showed decreased proliferation and increased apoptosis leading to fewer mature T cells (34), thus suggesting miRNAs are critical for T cell production. Indeed, miRNAs are important for all levels of T cell development, starting with thymopoiesis, where miR-181a was found to affect T cell signaling through the T cell antigen receptor, and thus thymic selection, through downregulation of multiple phosphatases (35). Many studies have shown the importance of miRNAs in the differentiation of naïve T cells into the different CD4+ Th cell subsets (36). For example, miR-182 is induced by IL-2 following naïve CD4+ T cell activation, and it promotes clonal expansion through inhibition of Foxo1, leading to the production of Th1, Th2 and Th17 cells (37). The miR-17-92 cluster is critical for the differentiation of Th1 and Tfh cells, and also inhibits inducible Treg differentiation (38). miRNAs are also known to

play critical roles in CD8+ T cell development and responses (39). For example, the miR-17-92 cluster promotes short-lived effector CD8+ T cell expansion at the suspense of CD8+ memory T cell formation (40), whereas miR-139 and miR-150 block the differentiation of activated CD8+ T cells into cytotoxic T lymphocytes (41).

miRNAs are also recognized as critical regulators of both NK cell development and function (42). Much attention is being paid to NKT cells, because they have been recognized as critical to developing appropriate antitumor responses. Interestingly, miR-150 has been identified as a pivotal branch point between NK versus NKT development, where high levels of miR-150 expression favors NK cells, but a reduction of NKT cells in the thymus (43). Let-7 miRNAs are also important for the development of NKT cells, as their increased expression leads to NKT cells differentiating into IFNγ producing NKT1 cells (44).

miRNA dysregulation in hematopoietic malignancies

The above section highlights the importance of miRNAs in hematopoiesis, as the proper expression and processing of miRNAs is critical in normal hematopoietic development and function. However, it is now appreciated that dysregulation of miRNA levels can also have severe consequences. Early studies discovered that the miRNA profiles of cancerous cells differ greatly from the profiles of normal cells (45). Certain miRNA signatures have been identified that correlate not only with diagnosis, but also with staging, prognosis, and response to treatment in a variety of cancers, including hematopoietic malignancies (46). Several miRNAs, referred to as oncomiRs, can promote cancer if their expression is elevated. Other miRNAs can act as tumor suppressors, where

their deletion or downregulation can promote cancer progression (47).

In one of the first studies identifying miRNA dysregulation as an important step in tumorigenesis, Calin et al. found that miR-15a and miR-16-1 deletions or downregulation in chronic lymphocytic leukemia promoted cell survival through increased expression of the antiapoptotic protein BCL2 (48). Since these seminal findings, many other miRNAs have been found to drive malignancies of lymphoid origin, such as the miR-17-92 polycistron in B cell malignancies. Overexpression of this miRNA cluster occurs due to its genomic proximity to regions often amplified in B cell lymphomas (49). Increased expression of miR-17-92 in lymphocytes triggers a lymphoproliferative disorder (50), while aberrant expression of miR-17-92 from a B cell-specific promoter leads to a lymphoid malignancy (51). miR-21 is another miRNA with broad potential as an oncomiR. Overexpression of miR-21 leads to a pre-B cell malignancy that regresses when miR-21 expression returns to endogenous levels (52). This supports the concept that targeting aberrantly expressed miRNAs could be an effective strategy for combating hematologic malignancies.

Many miRNAs have also been implicated in the development and prognosis of myeloid malignancies (53). The overexpression of a number of miRNAs is sufficient to induce myeloid malignancies in mice. For example, overexpression of miR-155 leads to the development of a myeloproliferative disease (MPD) in mice, resembling a chronic myeloid leukemia characterized by the overproduction of mature myeloid cells (54). Overexpression of other miRNAs including miR-125b and miR-29a results in an MPD that progresses to an acute leukemia (10,55). An improved understanding of the roles miRNAs play in promoting or preventing hematopoietic malignancies is critical to

unlocking the therapeutic potential of miRNA biology.

Acute myeloid leukemia

Acute myeloid leukemia (AML) is an aggressive hematologic malignancy that carries a poor prognosis. According to the American Cancer Society, nearly 20,000 new cases of AML will be diagnosed this year, and over 10,000 patients die from this disease annually. AML is characterized by the overproduction of immature myeloid cells, known as blasts, which disrupts normal hematopoiesis and eventually leads to bone marrow failure. Patients often present to the clinic with anemia, infection, or chronic bleeding resulting from this disruption in normal blood cell production (56). One of the primary challenges facing the treatment of AML is the high rate of relapse due to the aggressive nature of the disease, as well as the development of drug resistant forms of malignancy (56). This high rate of recurrence illustrates the need for research to elucidate the cellular mechanisms driving AML formation and pathways leading to drug resistance. This knowledge would allow for novel therapies to more effectively combat this deadly disease.

The mutational landscape in AML varies dramatically from patient to patient, and a plethora of genetic abnormalities have been found at the root of this malignancy. A number of chromosomal rearrangements have been identified that drive disease progression in AML, including 5q and 7q deletions, t(15;17), t(8;21), and inv(16), among others. (57). However, many AML cases are cytogenetically normal, where somatic mutations are the key drivers of disease.

FLT3-ITD mutations in AML

Among the most common mutations occurring in AML are gain-of-function mutations in the FMS-like tyrosine kinase 3 (FLT3) receptor (57). FLT3 is a member of the platelet-derived growth factor receptor (PDGFR) subfamily (class III) of tyrosine kinases and is selectively expressed on the cell surface of hematopoietic stem and progenitor cells (HSPCs) (58). FLT3 normally responds to FLT3 ligand (FLT3L) and activates downstream signaling pathways, including STAT5, AKT, NFkB, and MAPK to promote proliferation and survival of this critical stem cell compartment (59). In 1996, Nakoa et al. observed genetic duplications in the juxtamembrane domain of the FLT3 gene specifically in AML samples, which they referred to as internal tandem duplications of the FLT3 gene, or FLT3-ITD (60). FLT3-ITD leads to ligand-independent signaling and confers a growth and survival advantage to cells harboring this mutation (61). FLT3-ITD occurs in ~30% of AML diagnoses and confers a poor prognosis to patients (62), thus highlighting the importance of further research on FLT3-ITD biology to identify novel therapeutic targets.

miRNA dysregulation in FLT3-ITD+ AML

miRNA expression is dysregulated in a number of hematologic malignancies, including AML (63,64). An extensive review on our current understanding of the role miRNAs are playing in AML will be covered in Chapter 2, with a specific emphasis on therapeutic implications and emerging concepts. Through sequencing of clinical samples, a number of groups have found that FLT3-ITD+ AML has a distinct miRNA expression profile compared to FLT3-WT AML (65,66). However, the significance of this

dysregulated miRNA profile, and whether specific miRNAs could be affecting disease progression, remained unclear. To answer this question, we performed a genome-wide CRISPR-Cas9 screen to identify which miRNAs could be affecting cell growth in a FLT3-ITD+ AML cell line. This work is further characterized in Chapter 3.

miR-155 was one of the top miRNA candidates identified by our CRISPR-Cas9 screen as being a critical promoter of FLT3-ITD+ AML cell growth in vitro. This finding was particularly interesting because miR-155 had previously been identified as the most highly overexpressed miRNA in FLT3-ITD+ AML through microarrays or RNA-sequencing of clinical AML samples (66-68). miR-155 has also previously been shown to play a critical role in the development of hematopoietic cells, where its highest level of expression is observed in HSPCs and myeloid progenitor cells (54). Interestingly, overexpression of miR-155 in the bone marrow is sufficient to cause a MPD in mice, a precursor step to AML (54). miR-155 has also been identified to have oncogenic potential in a number of other cancers (69), including other models of AML (70,71).

Based on these observations and findings, we hypothesized that miR-155 is critical for disease development in FLT3-ITD-driven myeloid malignancy. We obtained mice containing a human FLT3-ITD mutation knocked-in to the endogenous mouse locus (61), and crossed this strain to miR-155-deficient mice to determine whether miR-155 is necessary for FLT3-ITD-driven disease development. This novel mouse strain was also used to determine the downstream effects of miR-155 overexpression in the context of FLT3-ITD. The results of this study will be discussed at length in Chapter 4.

Dissertation summary

This dissertation investigates the importance of miRNA dysregulation, specifically in FLT3-ITD+ AML. Through the use of a genome-wide CRISPR-Cas9 screen, we identified a number of miRNAs and their relevant mRNA targets that regulate FLT3-ITD+ AML cell line growth in vitro. miR-150 was our top miRNA candidate promoting cell growth, which we attributed to its repression of the well-known tumor suppressor p53. Our screen also identified miR-155 as a key regulator of FLT3-ITD+ AML cell growth, which has previously been identified as the most significantly overexpressed miRNA in FLT3-ITD+ AML. Utilizing a mouse model of FLT3-ITD-induced myeloid malignancy, we found that miR-155 was critical for myeloid expansion in the bone marrow, spleen, and blood of these animals, leading to decreased disease severity in miR-155-deficient FLT3-ITD animals. We found that miR-155 acts at the HSPC and myeloid progenitor levels to increase proliferation of these cell compartments through a multitarget mechanism affecting downstream pathways, including interferon and AKT signaling. These findings were extended into human FLT3-ITD+ AML through analysis of The Cancer Genome Atlas (TCGA) AML dataset and through inhibition of miR-155 in both FLT3-ITD+ AML cell lines and primary human samples. These studies highlight the importance of miRNAs, and specifically miR-155, in FLT3-ITD+ AML. They also suggest that miR-155 inhibition may warrant consideration as a novel therapeutic strategy in FLT3-ITD+ AML.

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CHAPTER 2

MICRORNAS AND ACUTE MYELOID LEUKEMIA: THERAPEUTIC IMPLICATIONS AND EMERGING CONCEPTS

Wallace, J.A., and R.M. O'Connell. 2017. MicroRNAs and acute myeloid leukemia: therapeutic implications and emerging concepts. *Blood* 130: 1290-1301.

Review Article

blood

MicroRNAs and acute myeloid leukemia: therapeutic implications and emerging concepts

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Acute myeloid leukemia (AML) is a deadly hematologic malignancy characterized by the uncontrolled growth of immature myeloid cells. Over the past several decades, we have learned a tremendous amount regarding the genetic aberrations that govern disease development in AML. Among these are genes that encode noncoding RNAs, including the microRNA (miRNA) family. miRNAs are evolutionarily conserved small noncoding RNAs that display important physiological effects through their posttranscriptional regulation of messenger RNA targets. Over the past decade, studies have identified miRNAs as playing a role in nearly all aspects of AML disease development, including cellular proliferation, survival, and differentiation. These observations have led to the study of miRNAs as biomarkers of disease, and efforts to therapeutically manipulate miRNAs to improve disease outcome in AML are ongoing. Although much has been learned regarding the importance of miRNAs in AML disease initiation and progression, there are many unanswered questions and emerging facets of miRNA biology that add complexity to their roles in AML. Moving forward, answers to these questions will provide a greater level of understanding of miRNA biology and critical insights into the many translational applications for these small regulatory RNAs in AML. (*Blood*. 2017;130(11):1290-1301)

Introduction

MicroRNA (miRNAs) are small noncoding RNAs (~20-24 nucleotides) that play vital roles in posttranscriptional gene regulation through repression of target messenger RNAs (mRNAs).[1] miRNA-encoding genes in the nucleus are transcribed into primary miRNA transcripts, which then undergo a number of processing steps in the nucleus and cytoplasm to generate the mature miRNA molecule. The mature miRNA is loaded into the RNA-induced silencing complex (RISC), and this miRNA-RISC complex targets the 3′ untranslated region (UTR) of specific mRNAs on the basis of sequence complementarity, resulting in reduced protein outputs through mechanisms involving decreased mRNA stability and reduced translation.[2]

miRNAs are now recognized to play roles in nearly all physiological processes and have been implicated in a number of human diseases including cancer, where miRNAs can act as either oncogenes (oncomiRs) or tumor suppressors.[3] Hematologic malignancies are no exception, as dysregulated miRNA expression contributes to blood cancers from many different hematopoietic lineages.[4] This review will focus on recent advances in understanding the role of miRNAs in a hematologic malignancy with a particularly high rate of mortality, acute myeloid leukemia (AML).

miRNAs in AML: background

AML is a heterogeneous disease characterized by the increased proliferation and survival of immature myeloid cells and is the result of a number of genetic abnormalities, including mutations and chromosomal rearrangements.[5] Early studies characterizing the role of miRNAs in AML focused on identifying AML-specific miRNA expression patterns. Distinctive miRNA profiles were identified for many cytogenetic subtypes of AML,[6-8] as well as for several specific mutations in cytogenetically normal AML, including mutations in *NPM1*, *FLT3*, and *CEBPA*.[9-13] miRNA expression profiles also correlate with prognosis,[12,14] highlighting the potential importance of miRNAs in this disease. However, although miRNAs are enriched in leukemia-associated genomic alterations, only ~100 are expressed above background level,[15] suggesting that only a subset of miRNAs have functional effects in AML.

Beyond dysregulated miRNA expression profiles, it is now well accepted that miRNAs can function as either oncomiRs or tumor suppressors in many subtypes of AML, affecting a broad range of leukemic processes, including proliferation, survival, differentiation, self-renewal, epigenetic regulation, in vivo disease progression, and chemotherapy resistance[8,15-57] (Table 1). miRNAs impact leukemic development and progression through collaboration with known oncogenes or tumor suppressors, either by directly targeting them on the mRNA level or by working in concert with these proteins to promote malignancy. To illustrate these concepts, we have summarized findings for selected miRNAs consistently found to play a role in AML in Table 1, and we discuss some of the more novel aspects of miRNA biology in AML below, as a greater understanding of miRNA biology will enable more strategic design of therapies in the future.

Mechanisms of dysregulated miRNA expression in AML

Alterations in miRNA expression can occur through a variety of mechanisms in AML (Figure 1). Copy number alterations (CNAs), which include deletions[22,25] and amplifications,[20] can drastically alter miRNA expression. However, acquired CNAs specifically targeting miRNAs may be relatively rare in AML. By using a combination of comparative genomic hybridization and whole-genome sequencing,

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Table 1. Selected miRNAs involved in AML pathogenesis

miRNA	Expression change	Mechanism of dysregulation	Confirmed targets	Functional effect of expression		References
				OncomiR	Tumor suppressor	
miR-9	↑ MLL-rearranged AML, ↓ t(8;21), ↓ EVI1-induced AML	Promoter targeted by MLL-fusion proteins; EVI1 hypermethylates promoter	*RHOH*, *RYBP*, *HMGA2*, *LIN28B*, *FOXO1*, *FOXO3*	↑ Proliferation, ↑ survival, ↑ leukemogenesis in mice (*MLL* rearranged)	↓ Proliferation, ↑ monocytic differentiation (t(8;21) and EVI1+)	16-18
miR-17-92 cluster	↑ In LSCs in MLL-associated AML	Activated by MYC; epigenetically activated by MLL-fusion proteins; genomic amplification in MLL-rearranged AML	*P21*	↑ Proliferation, ↑ survival, ↓ differentiation, ↑ self-renewal, ↑ colony-forming capacity, ↑ leukemogenesis in mice		19, 20
miR-22	↑ MDS/MDS-derived AML, ↓ de novo AML	Downregulated via TET1/GFI1/EZH2/SIN3A-mediated epigenetic repression and DNA copy-number loss; increased with loss of PU.1	*TET2*, *CRTC1*, *FLT3*, *MYCBP*	↑ Proliferation, ↑ survival, ↓ differentiation, ↑ self-renewal, overexpression leads to myeloid malignancy in mice (MDS/MDS-derived AML)	↓ AML blast cell growth, ↑ differentiation, ↓ leukemic progression in mice (de novo AML)	21-23
miR-29b	↓ Various subtypes of AML	Downregulated via loss of *CEBPA*; chromosome 7q deletions; MYC represses; NF-kB represses	*MCL-1*, *CXXC6*, *CDK6*, *AKT2*, *CCND2*, *SP1*, *DNMT3A*, *DNMT3B*		↓ Cell growth, ↑ apoptosis, ↓ leukemic progression in vivo, ↓ KIT activation, prevents global DNA hypermethylation	24-27, 56
miR-125b	↑ t(2;11)(p21;q23) AML, ↑ MDS/MDS-derived AML, ↑ pediatric AML	Increased by t(2;11)(p21;q23) translocation	*LIN28A*, *IRF4*	↑ Proliferation, ↑ production of myeloid progenitors, ↑ self-renewal, overexpression leads to AML in mice		28-32
miR-126	↑ t(8;21) AML, ↑ in LSCs of CN-AML	Promoter demethylated in t(8;21) AML	*PLK2*, *ADAM9*, *ILK*, *GOLPH3*, *CDK3*, *TOM1*, *ERRFI1*, *SPRED1*, *FZD7*	↑ Proliferation, ↑ survival, ↓ differentiation, ↑ colony-forming ability, ↑ LSC maintenance and self-renewal, ↓ cell cycling, ↑ quiescence, ↑ chemotherapy resistance		8, 33-36
miR-146a	↓ Various subtypes of AML, ↓ 5q syndrome MDS/MDS-derived AML	Deletion in del(5q) MDS/MDS-derived AML	*TRAF6*, *IRAK1*, *TIRAP*		↓ Proliferation, ↓ survival, ↓ NF-kB activation, deletion leads to myeloproliferation in mice	15, 37-41
miR-155	↑ CN-AML (highest in FLT3-ITD$^+$ AML)	Targeted by STAT5 and NF-kB in FLT3-ITD$^+$ AML; upregulated by MLL-fusion genes via MEIS1	*CEBPB*, *SHIP1*, *PU.1*	↑ Proliferation, ↑ survival, overexpression leads to myeloproliferative neoplasm in mice, confers negative prognosis in CN-AML, no effect in MLL-AF9 mouse model of leukemia		42-46
miR-193a	↓ Various subtypes of AML (lowest in t(8;21) AML)	Epigenetically silenced by AML1/ETO	*AML1/ETO*, *DNMT3A*, *HDAC3*, *KIT*, *CCND1*, *MDM2*		↓ Cell growth, ↑ apoptosis, ↑ differentiation, ↓ cell cycling, ↓ KIT expression	47, 48
miR-196b	↑ MLL-associated AML	Activated by MLL-fusion proteins; co-expressed with *HOXA9* in MLL-rearranged leukemia	*HOXA9*, *MEIS1*, *FAS*	↑ Proliferation, ↑ survival, ↓ differentiation, ↓ replating potential, ↑ MLL-AF9–induced leukemic progression in mice		49, 50
miR-223	↓ t(8;21) AML, ↓ various subtypes of AML	Targeted by transcription factors CEBPA (activates) and E2F1 (represses); epigenetically silenced by AML1/ETO	*E2F1*, *MEF2C*, *FBXW7*		↓ Proliferation, ↑ apoptosis, ↑ differentiation/granulopoiesis	51-54

Table represents selected miRNAs involved in AML disease progression, with a focus on miRNAs with clinical evidence of dysregulation, as well as in vivo and in vitro functional evidence of role in leukemogenesis.
CN, cytogenetically normal; LSC, leukemic stem cell.

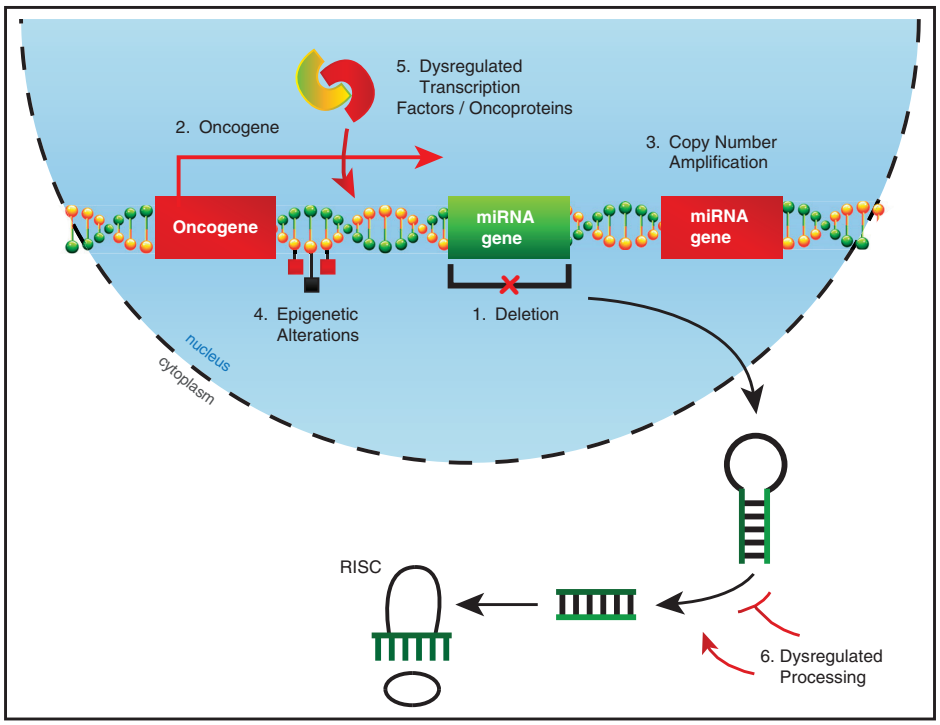

Figure 1. Mechanisms of dysregulated miRNA expression in AML. miRNA dysregulation can contribute to the development of AML. Thus far, numerous mechanisms by which miRNAs become dysregulated in AML have been identified, including (1) deletions leading to decreased miRNA expression, (2) improper expression because of close proximity to an oncogenic genomic region created as a result of either a translocation event or overexpression of a neighboring protein-coding gene, (3) copy number amplifications leading to increased miRNA expression, (4) epigenetic alterations affecting miRNA expression, (5) miRNA promoter regions being aberrantly targeted by dysregulated transcription factors or oncoproteins, and (6) dysregulated miRNA processing leading to altered levels of mature miRNAs.

researchers found that 18% of patients had CNAs involving miRNA genes, with a single CNA affecting up to 121 miRNAs.[58] However, these CNAs always contained one or more protein-coding genes, suggesting that the miRNA genes involved in these CNAs may be passenger alterations. miRNAs may also be aberrantly expressed when located in oncogenic genomic locations, which occurs through chromosomal translocations[28] or overexpression of nearby protein-coding genes.[49]

The most common mechanisms by which miRNA expression becomes dysregulated in AML are epigenetic alterations and via targeting by dysregulated transcription factors or oncogenic fusion proteins. These two mechanisms are not always distinct, as epigenetic alterations to miRNA loci often occur via dysregulated transcription factors or oncoproteins.[18,47] There is some evidence that alterations in miRNA expression in cancer can be the result of dysregulated miRNA processing[59,60]; however, it is unclear whether this occurs in AML.

Although mutations in the mature miRNA sequence would likely have no effect on expression levels, these mutations could change mRNA target specificity and dramatically alter phenotypic effects in AML. In perhaps the most thorough AML sequencing effort to date, The Cancer Genome Atlas group reported that miR-142-3p was the only miRNA bearing recurrent somatic single nucleotide variants in its mature strand that could alter binding to targets (4 of 187).[13,61] Only 7 miRNA single nucleotide variant mutations were discovered in the 187 samples analyzed, indicating that these are rare events. However, while mutations in the miRNA sequence itself are uncommon, polymorphisms in the mRNA 3′ UTR miRNA binding site may happen more frequently and could predispose patients to AML by altering miRNA regulation of specific genes.[62] Taken together, it seems that aberrant miRNA levels that are observed in AML are largely driven by altered transcription of miRNA primary transcripts, which suggests that targeting of key transcription factors or epigenetic regulators may be one way to restore proper miRNA expression in AML.

Translational aspects of miRNA biology

It is now well established that miRNAs play a variety of critical roles in AML, in which they can either promote or inhibit tumor cell biology. However, these advances have yet to make a clinical impact. Here we will highlight the efforts being made toward moving miRNA research in AML to the clinic and focus on the potential for using miRNAs as disease biomarkers, as well as advances in miRNA-targeting therapeutic strategies in AML.

miRNAs as AML biomarkers

Perhaps the most encouraging clinical application of miRNA research to date is the potential use of miRNAs as disease biomarkers in AML.[63] When a patient initially presents with leukemia, proper classification is critical to determining the correct treatment. However, a small number of leukemias are difficult to identify as myeloid or lymphoid, thus making treatment decisions challenging. miRNA expression profiling can help classify acute leukemias of ambiguous lineage as either AML or acute lymphoblastic leukemia,[64] with 1 group claiming that as few as 2 miRNAs can be used to discriminate between acute lymphoblastic leukemia and AML at an accuracy of >95%.[65] As mentioned earlier, specific subtypes and mutant drivers of AML are associated with distinctive miRNA expression profiles, again suggesting that miRNAs could be useful in the initial classification of disease.

Beyond classification, miRNA expression profiles may provide important prognostic information. Several groups have reported that miRNA expression at diagnosis adds relevant prognostic information in patients with AML and can even predict survival in some cases.[66-68] It was also recently reported that miRNA expression can predict progression of myelodysplastic syndrome (MDS) to AML.[69]

A major issue with patients receiving treatment for AML is the persistence of a small number of leukemic blasts in the bone marrow after intensive chemotherapy known as minimal residual disease (MRD), which can eventually give rise to leukemia relapse. Because the appearance of leukemic blasts in circulation often occurs late in the relapse process, a number of highly sensitive polymerase chain reaction–and flow cytometry–based methods for detection of blast nucleic acid or protein products have been developed for monitoring MRD,[70] as recognizing MRD before patient relapse could allow for preemptive therapy.[71] A number of groups have proposed screening circulating miRNAs as an inexpensive, noninvasive, and sensitive option to monitor for MRD, because the serum expression of miRNAs changes after standard chemotherapy,[72] and patients with AML have a distinctive serum miRNA expression profile compared with healthy controls.[73,74] These early results are promising, because a specific AML-associated miRNA serum profile could not only be used to track MRD after chemotherapy, but could also potentially provide an important screening tool for early detection of de novo AML in the clinic, as alterations in serum miRNA profiles may precede the entry of leukemic blasts into the periphery. However, there has been a lack of concordance between these individual studies, suggesting that more work is needed on larger AML cohorts with more rigorous study design to validate these initial findings.

Advances in miRNA-based therapeutics

As the list of miRNAs and their mRNA targets that are relevant in AML disease progression continues to grow, therapeutic manipulation of these miRNAs becomes more enticing. It is easy to imagine delivering locked nucleic acid (LNA) oligonucleotide inhibitors to target known oncomiRs in AML or delivery of synthetic miRNA mimics that act as tumor suppressors. These approaches have exciting therapeutic potential, because miRNAs are endogenous molecules that often repress multiple targets, either in the same pathway or by affecting a common biological process. Thus, resistance to miRNA-based therapies through target site mutation would be unlikely.

A good example of the effectiveness of an miRNA-based therapeutic in AML was recently demonstrated with targeted delivery of miR-29b via transferrin-conjugated lipid nanoparticles both in vitro and in mice engrafted with human AML cell lines.[75] Delivery of miR-29b led to decreased leukemic cell growth and improved survival in the AML xenograft mouse model, which was attributed to miR-29b downregulating *CDK6*, *SP1*, *FLT3*, *DNMTs*, and *KIT*, either directly or indirectly. These target genes affect a variety of cellular processes in AML, and this study highlights the ability of 1 miRNA-based treatment to target many pathways simultaneously. Several studies involving the use of miR-based therapeutics have shown encouraging results in preclinical in vitro and animal models,[22,33,75-77] the results of which are summarized in Table 2.

Another underexplored area of miRNA-based therapy is the possibility of repurposing existing drugs known to influence miRNA levels by targeting the pathways that regulate miRNA expression. MLN4924 (Pevonedistat), a drug known to reduce nuclear factor κB (NF-κB) activation that is currently being evaluated in clinical trials, was recently shown to decrease the levels of oncogenic miR-155 in FLT3-ITD$^+$ AML cell lines, leading to decreased leukemic phenotypes both in vitro and in vivo.[78] miRNA-based therapeutics may also be efficacious when used in combination with existing chemotherapeutics. Manipulation of miRNA expression levels can increase AML responsiveness to standard chemotherapeutic regimens.[34,75,79-81]

Although several studies have implicated miRNAs and their putative targets as being clinically actionable, the vast majority of these studies have yet to achieve clinical relevance, with the first therapies targeting miRNAs just entering clinical trials within the last few years.[82,83] One miRNA-based therapy in clinical trials that shows promise is treatment of hepatitis C virus by miR-122, in which researchers found that patients treated with an LNA inhibitor of mature miR-122 have reductions in hepatitis C virus RNA levels in a dose-dependent manner.[84] This study provides proof of principle that should encourage future endeavors of this kind in the cancer arena. Although the list of miRNA-based therapeutics entering clinical trials continues to grow, to the best of our knowledge, no miRNA-based therapies have made their way to clinical trials specifically for the treatment of AML.

A major barrier preventing the development of miRNA-based therapies is the lack of more efficient and specific delivery methods, because synthetic miRNAs or oligonucleotide inhibitors are degraded rapidly in circulation and have limited cellular uptake and specificity. To further complicate matters, delivery of drugs to the bone marrow is difficult, and higher doses are often required to elicit a therapeutic effect. This highlights the importance of developing novel targeting techniques for more effective delivery. Consequently, there are many new approaches being explored for improved delivery of miRNA-based therapies, including liposomes, nanoparticles, LNAs with increased stability, peptide-based inhibitors, and several other creative approaches,[85] some of which are highlighted in Table 2. Efficient and specific delivery of miRNA mimics or antagonists to the proper cell types in vivo is a key step toward unlocking the therapeutic potential of manipulating miRNA function to combat AML.

Emerging concepts

Going forward, there remain several aspects of miRNA biology that need further investigation to fully grasp how miRNAs function within AML cells. This includes a continued effort to better answer certain questions that have been raised in the past and work in novel areas that have recently emerged. Several of these areas and how they relate to AML are described below.

Improper regulation of inflammatory pathways leads to AML

A newly appreciated mechanism by which miRNAs promote malignancy is through their impacts on classical inflammatory pathways. It has long been known that there is a strong link between inflammation

Table 2. Preclinical studies of miRNA-based therapeutics

Therapy	Delivery method	Targets	Efficacy of delivery	In vitro results	In vivo results	References
miR-22 mimic	G7 poly(amidoamine) dendrimer nanoparticles	*CRTC1, FLT3, MYCBP*	N/A	N/A	↑ Survival in MV4-11 xenotransplantation mouse models; ↑ Survival (40% cure rate) in primary mouse AML (MLL-AF9 and AE9a) transplant models	22
miR-29b mimic	Transferrin-conjugated anionic lipid-based nanoparticle	*DNMT3A, DNMT3B, DNMT1, SP1, CDK6, FLT3, KIT*	>100-fold ↑ in vitro 20-fold ↑ in vivo	↓ Cell proliferation and ↓ colony formation in Kasumi-1, OCI-AML3, and MV4-11 cells ↓ Cell viability in primary AML samples; pretreatment ↑ efficacy of decitabine treatment	↑ Survival and ↓ splenomegaly in NSG mice xenografted with MV4-11 cells Pretreatment ↑ efficacy of decitabine treatment	69
miR-21/miR-196b antagomiRs	Naked antagomiR delivered via implanted osmotic pumps	N/A	60%-80% ↓ of miRNAs in peripheral WBCs in vivo	↓ Colony formation in HOXA9, Nup98-HOXA9, or MLL-AF9–transduced mouse Lin^- BM cells	Cured MLL-AF9 transplanted primary mouse AML; ↑ survival in combination with induction chemotherapy in MLL-AF9 xenotransplantation model	71
miR-126 antagomiR	Transferrin or CD45.2- conjugated anionic lipid-based nanoparticle	*MMP7, CHD7, JAG1*	80% ↓ in primary AML blasts in vitro; 50% ↓ in BM and spleen in vivo	↓ Long-term colony formation, ↓ self-renewal capacity, and ↓ quiescence of LSCs	↑ Survival in NSG mice engrafted with human AML primary blasts and MLL FLT3-ITD mouse model	33
miR-181a mimic	Transferrin-conjugated anionic lipid-based nanoparticle	*KRAS, NRAS, MAPK1*	2.6-fold ↑ in BM and 35-fold ↑ in spleen in vivo	↓ Cell proliferation and ↓ colony forming in KG1a, MV4-11, OCI-AML3 cell lines; ↑ apoptosis in primary AML blasts	↑ Survival and ↓ splenomegaly in NSG mice xenografted with MV4-11 cells	70

This table summarizes the results from selected pre-clinical miRNA-based therapies, including information such as method of delivery, miRNA targets (direct and indirect), efficacy of delivery, and in vivo/in vitro results.
BM, bone marrow; N/A, not applicable; WBC, white blood cell.

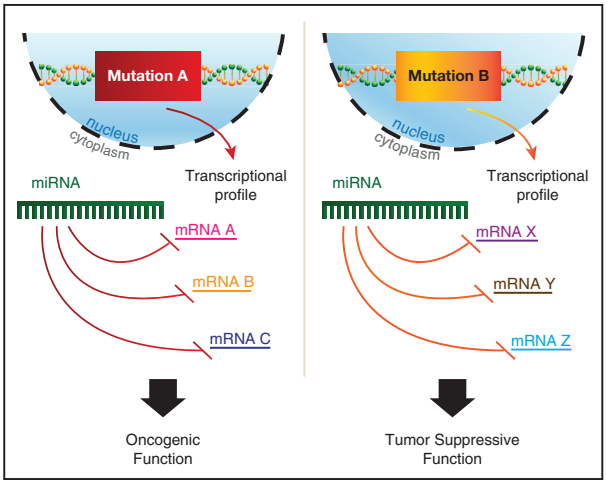

Figure 2. miRNAs play context-dependent roles in AML. A model for context-dependent effects of a specific miRNA given different transcriptional backgrounds between 2 distinct AML-driver mutations, mutation A and mutation B. Mutation A leads to the transcription of mRNA A, B, and C, whereas mutation B drives the transcription of mRNA X, Y, and Z. All mRNAs have predicted targeting by the example miRNA. The miRNA depicted in mutation A and mutation B is the same hypothetical miRNA.

and cancer; however, the mechanisms governing this association are still largely unclear. NF-κB is critical in initiating inflammatory responses, and dysregulation of this critical transcription factor is heavily integrated into cancer biology because of its role in promoting proliferation and survival.[86]

Several groups have shown that dysregulation of certain miRNAs can disrupt normal NF-κB signaling that results in cancerous transformation,[87,88] including in myeloid malignancies.[39] Because miR-146a is a negative regulator of NF-κB signaling, and overactivation of NF-κB is involved in malignant transformation, one could predict that loss of miR-146a might lead to the development of hematopoietic cancers. Indeed, a miR-146a deficiency has been shown to result in the development of both lymphoid and myeloid malignancies in an age-dependent manner.[39,41] Chromosome 5q deletions, which are common in MDS progressing to AML, leads to loss of miR-145 and miR-146a because they are both encoded on the long arm of chromosome 5.[37] The loss of these miRNAs leads to myeloproliferation and eventual progression to AML in mice as a result of increased NF-κB signaling,[37,40] as miR-145 and miR-146a target *TIRAP*, *IRAK1*, and *TRAF6*, known activators of NF-κB. Targeted inhibition of IRAK1 has significant activity against MDS/AML cells in vitro and in xenograft mouse models,[89] suggesting that targeting of these traditional innate immune pathways may have clinical efficacy.

Not only does miRNA regulation of NF-κB signaling seem to be important in AML progression, but there is also evidence that NF-κB activates miRNA expression to promote leukemic phenotypes.[90] Additionally, there could be some contribution from the bone marrow microenvironment. Activation of inflammatory signaling in mesenchymal cells was recently found to drive development of an MDS preleukemic condition in mice.[91] A further understanding of how alterations to these miRNA-regulated classical inflammatory pathways can promote AML progression will be an interesting new area of miRNA research in the future.

miRNAs play context-dependent roles in AML

An interesting aspect of miRNA biology in AML is that a miRNA can have opposing roles, depending on the disease context. For example, miR-9 was identified as being specifically upregulated in MLL-rearranged AML, in which it plays an oncogenic role in promoting leukemogenesis in the presence of MLL-AF9.[16] However, other studies have found miR-9 to play a tumor suppressive role in AML, including in pediatric AML with t(8;21), in which miR-9 overexpression reduced leukemic growth and induced monocytic differentiation in human AML cells and in xenotransplantation mouse models,[17] as well as in EVI1-induced AML, in which miR-9 is epigenetically silenced leading to decreased apoptosis and myelopoiesis.[18]

There are many other examples of miRNAs displaying context-dependent roles in AML. miR-155 seems to have no phenotypic effect in MLL-rearranged AML,[44] but it is consistently found to play an oncogenic role in FLT3-ITD–driven AML pathogenesis.[42,45] miR-126 plays different roles and regulates different targets in normal vs malignant hematopoietic stem cells[35] and, interestingly, both overexpression and knockout of miR-126 promote leukemogenesis.[34] These studies highlight how the influence of an miRNA in AML can be dependent on the underlying genetic abnormalities that drive disease or cell type of expression.

A variety of potential explanations for context-dependent discrepancies have been proposed, including RNA-binding protein regulation of miRNA binding to 3′ UTRs or differential splicing to include or exclude a given 3′ UTR.[92] In addition, recent findings suggest that mutant proteins in AML can alter miRNA-mRNA interactions.[93] Perhaps the most plausible explanation would be differences in the mRNA target availability for the miRNA, because AML driven by independent mutations would have distinct transcriptional profiles (Figure 2). However, most studies that find context-specific roles for miRNAs in AML do not go on to explore the mechanistic basis underlying this phenomenon, and more work in this area is needed to better understand context-dependent miRNA functions.

Varying levels of miRNA expression may have opposing effects

One potential explanation for the context-dependent effects observed for miRNA dysregulation in AML is that different levels of miRNA expression may have vastly different effects on host-cell phenotypes.

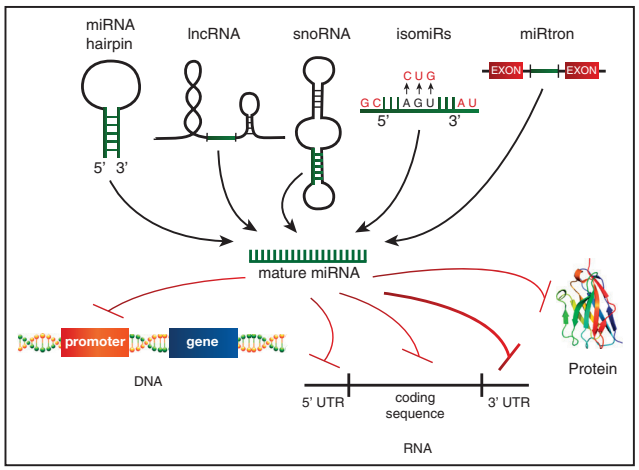

Figure 3. Alternate miRNA sources and noncanonical targeting. A schematic depicting the varying sources identified for production of mature miRNAs, including the 5′ or 3′ strand of the traditional miRNA hairpin structure, processing of other noncoding RNAs, such as lncRNAs, small nucleolar RNAs (snoRNAs), miRNAs transcribed from the same gene but having different mature miRNA sequences (isomiRs), and miRNA spliced from introns (miRtrons). Nontraditional miRNA targets beyond the 3′ UTR are also depicted, including promoter regions of protein-coding genes, the 5′ UTR, the RNA coding sequence, and protein.

miR-125b is one of the more interesting known oncomiRs because it plays a role in promoting both myeloid and lymphoid malignancies. Overexpression in mice has been shown to cause both lymphoproliferative and myeloproliferative disorders and, ultimately, a frank malignancy in these compartments.[29,30,94,95] Recently, some light was shed on the dual nature of miR-125b in promoting hematologic malignancy in which miR-125b was found to selectively induce either myeloid or lymphoid leukemia based on the level and time course of miR-125b overexpression.[31]

miR-155 is another well-studied oncogenic miRNA in AML, and overexpression correlates with a poor prognosis.[43] However, evidence has emerged that miR-155 may play a role as a tumor suppressor in certain contexts.[96] A recent study examined the role of miR-155 in AML more closely to help resolve these opposing effects. The study found that when overexpressing miR-155 in 3 different murine models of AML (HoxA9/Meis1, MLL-ENL, MLL-AF9) to an intermediate level (~5- to 10-fold above control), miR-155 displayed oncogenic function, leading to increased proliferation and enhanced colony-forming potential.[97] This was in contrast to miR-155 high levels (>10-fold above control), in which miR-155 acted as a tumor suppressor by repressing colony formation and proliferation, establishing a dose-dependent effect of miR-155 in these AML mouse models. The study did confirm that the intermediate miR-155 expression levels were a better representation of what was seen in their pediatric AML data set (increased ~one- to sevenfold), values that were consistent with miR-155 expression in other AML data sets,[12,45] suggesting that miR-155 likely has a predominately oncogenic effect in human AML. The study highlights the importance of considering the level of expression in various model systems when studying miRNAs in AML.

5p vs 3p transcripts

A long-standing question regarding miRNAs is the significance of differential use of 5p vs 3p miRNA transcripts. 5p and 3p miRNAs are encoded by the same genomic region and are both contained within the initial transcript and mature miRNA duplex before 1 of them is chosen as the active or guide strand (miR) and the other as the passenger strand (miR*). The passenger strand is then typically degraded and traditionally thought not to have a functional role. Interestingly, the 2 miRNA strands each have unique seed sequences and therefore do not share the same mRNA target spectrum. This means that the biological processes and pathways being regulated by any given pri-miRNA transcript could be vastly different depending on the selection of either the 5p or 3p strand as the guide. There are several examples of 5p and 3p transcripts from the same duplex having distinct biological functions.[98,99]

In a small percentage of cases, the passenger strand can be stabilized at the same level as the active strand, and may even exhibit important physiological function in myeloid cells.[100] A recent report identified a specific passenger strand, miR-9*, that is not detectable in normal myeloid cells but is expressed in 59% of AML cases, and expression levels correlated with prognosis.[101] This group also found that miR-9* expression levels had prognostic value, because patients with high

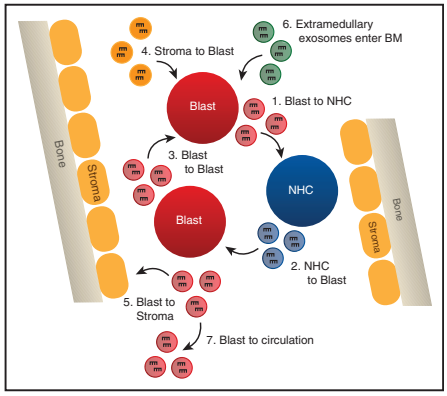

Figure 4. Exosomally transferred miRNAs alter leukemic phenotypes. A model depicting the different possibilities of exosomal miRNA transfer in the bone marrow, including (1) transfer from AML blast to normal hematopoietic cells (NHCs), (2) NHC to AML blast, (3) AML blast to AML blast, (4) bone marrow (BM) stromal cells to AML blast, (5) AML blast to BM stromal cells, (6) entrance of extramedullary produced exosomes into the hematopoietic niche, and (7) AML blast-derived exosomes leaving the BM and entering circulation.

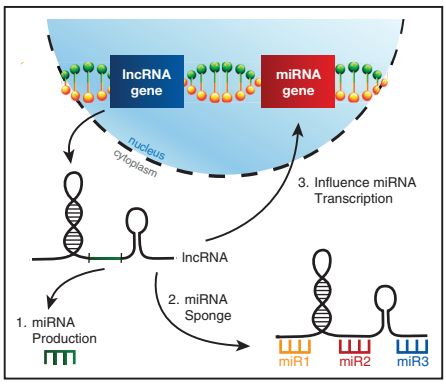

Figure 5. lncRNAs can interfere with miRNA function in AML. A picture depicting the ways lncRNAs can affect miRNA biology in AML, including (1) lncRNAs serving as a source for mature miRNA production, (2) lncRNAs acting as miRNA sponges, binding miRNAs to prevent them from repressing their target mRNAs, and (3) altering miRNA gene transcription.

miR-9* expression vs low expression were associated with positive patient outcome. Although the mechanisms behind passenger strand–retained expression are still largely unknown, there is some evidence that posttranscriptional modifications to the RNA duplex and differential expression of various RISC components could play an important role in strand selection.[102,103] Whether these processes are dysregulated in AML remains to be determined.

A variety of miRNA sources and noncanonical targeting

Although the traditional dogma of miRNA biology states that miRNAs are encoded from their own genes, go through a distinct processing pathway, and then repress mRNA targets via binding the 3′ UTR, there is mounting evidence that this canonical biogenesis pathway might not be exclusive. It is now understood that the mature miRNA can come from a variety of sources, including from the 5p or 3p transcript, long noncoding RNAs (lncRNAs),[104] small nucleolar RNAs,[105,106] or spliced from introns[107] (Figure 3). Identifying and characterizing miRNAs generated from these nontraditional sources may be challenging, but it is key to comprehensively understanding the breadth of small RNAs in AML.

Functional effects exhibited by miRNAs are often attributed to a handful of targets predicted by seed sequence complementarity in the 3′ UTR. But miRNAs can also bind and repress targets without predicted binding sites in their 3′ UTRs, referred to as noncanonical targets. Researchers found that when they pulled down the RISC complex to identify mRNA targets loaded in an miR-155–specific manner, ~40% of the targets identified were noncanonical targets.[108] This was explained, in part, by laxity in the seed matching of miRNAs and mRNA 3′UTRs but could also be explained by the concept of isomiRs, which are miRNAs transcribed from the same gene but having different mature miRNA sequences, found extensively in a murine model of leukemia.[109] These variants are a result of posttranscriptional modifications, including "errors" in miRNA processing, nucleotide addition to the 3′ end, and nucleotide substitution.[110,111] Beyond isomiRs, there is also some evidence that miRNAs can bind to promoter regions of DNA,[112] 5′ UTRs,[113] the mRNA coding sequence,[114,115] and even proteins.[116] Thus far, the evidence for noncanonical targeting playing a functional role in AML is lacking, but it could be a more prominent mode of miRNA function than we realize.

Transfer of miRNAs in exosomes alters leukemic phenotypes

Recently, miRNAs have been found within extracellular vesicles, including exosomes that are produced by the multivesicular body pathway.[117] Both primary and malignant cells can release miRNAs in exosomes, which can be taken up by certain recipient cells where they deliver their miRNA cargo in a functionally relevant manner.[118,119] Although evidence for the functional role of exosomally transferred miRNAs in AML is somewhat limited, preliminary studies indicate this could be a paradigm-shifting field of study.

Early work showed that both primary AML cells and AML cell lines do in fact release exosomes containing miRNAs.[120] Moreover, these exosomes contain an miRNA population that is compositionally distinct from the miRNA population of the host cell,[120] suggesting that there is specificity with the loading of miRNAs into exosomes. Other functional studies have revealed that miRNAs can be transferred in exosomes from AML cells to both stromal and normal hematopoietic cells and alter their function in a manner that promotes leukemic phenotypes.[121,122] Interestingly, exosomes from extramedullary tumors have been shown to alter the bone marrow niche, suggesting that miRNA-containing exosomes can home to the bone marrow and alter function independent of cell contact.[121] In a recent study,[122] authors found that exosomes containing miR-150 and miR-155 released from AML cells suppressed normal hematopoietic stem cell proliferation and differentiation through inhibition of *c-MYB*, thus perpetuating a malignant phenotype by directly altering hematopoietic stem cell biology.

Such studies provide evidence that miRNAs secreted in exosomes are a novel form of intercellular communication that may play vital regulatory roles in suppressing normal hematopoiesis and disrupting the hematopoietic niche to promote leukemic cell outgrowth (Figure 4). Analysis of the miRNA content of exosomes has even been suggested as a novel biomarker for the detection of AML, because blast-derived exosomes can be isolated from circulation before the appearance of circulating blast cells in a xenograft mouse model.[74] Further study of the machinery required for specific miRNA loading into exosomes and uptake by recipient cells is critical to manipulating this system in a manner that will better test the relevance of exosomal miRNAs in AML.

lncRNAs can interfere with miRNA function in AML

lncRNAs are a distinct class of noncoding RNAs much longer than mature miRNAs (>200 nucleotides) that have been observed to exhibit a variety of functions, but are typically involved in regulating gene expression,[123] including miRNA genes.[124] It has recently been learned that lncRNA dysregulation in AML can alter the function of specific miRNAs leading to skewed disease phenotypes (Figure 5).

HOTAIRM1 is a lncRNA located in the *HOXA* genomic region that was recently found to impact prognosis in various subtypes of AML and was associated with a distinct miRNA expression profile.[125] Additional work on lncRNA HOTAIRM1 revealed that it was acting to sequester autophagy-regulating miRNAs miR-20a, miR-106b, and miR-125b, which affected the degradation of PML-RARA in acute promyelocytic leukemia.[126] Another lncRNA, HOTAIR1, was also found to act as a miRNA sponge in AML, competitively binding miR-193a, which leads to increased c-KIT expression and ultimately confers a poor prognosis.[127] Other examples of lncRNAs acting to competitively bind miRNAs in myeloid malignancy have started to emerge.[128,129]

As mentioned above, lncRNAs can provide a nontraditional source for mature miRNA production. It was recently learned that the pri-miR-223 transcript is actually a functional lncRNA in AML that displays tumor suppressive functions by sponging oncogenic miRNAs miR-125a and miR-125b.[104] Interestingly, while miR-223 is processed out of this lncRNA, miR-223 and lncRNA-223 are expressed at different levels, and these 2 noncoding RNAs have distinct functions in the myeloid lineage. It is unclear how prevalent this phenomenon is in AML, but there is additional evidence that lncRNAs can serve as precursors for miRNAs in T-cell lymphomas.[130,131] A better understanding of the ways in which lncRNAs regulate miRNAs in AML could shed more light on their biology in this setting.

Conclusion

miRNAs are now widely regarded as playing a critical role in AML pathogenesis. Specific miRNA expression profiles can help classify subtype, determine prognosis, and predict response to treatment in AML, but the use of miRNAs as biomarkers is not yet routine practice. Therapies targeting miRNAs in AML have shown promise in preclinical models but have not made the leap to human clinical trials, which will require improvements in our delivery methods.

The continued development of advanced genomic approaches, including CRISPR-Cas9 technology, will allow us to more quickly identify and efficiently study relevant miRNAs and their targets in AML. Indeed, genome-wide CRISPR-Cas9 screening has been used to identify functionally relevant miRNA-mRNA target pairs that regulate AML cell line growth[132] and will likely be extended to additional preclinical models of AML.

Many complexities and mysteries of miRNA biology remain, but their solutions will substantially improve our understanding of how miRNAs function in AML. In some cases, novel aspects of miRNA biology have already shed additional light on how AML cells are regulated, whereas in other cases, emerging mechanisms have yet to be explored in AML despite their potential to help us understand how miRNAs influence this deadly leukemia. Addressing long-standing questions and exploring emerging concepts of miRNA biology will provide important insights into how miRNAs function in AML, and only through an improved understanding of these mechanisms can we better exploit miRNAs therapeutically to improve disease outcomes in the clinic.

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CHAPTER 3

GENOME-WIDE CRISPR-CAS9 SCREEN IDENTIFIES MICRORNAS THAT REGULATE MYELOID LEUKEMIA CELL GROWTH

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RESEARCH ARTICLE

Genome-Wide CRISPR-Cas9 Screen Identifies MicroRNAs That Regulate Myeloid Leukemia Cell Growth

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Abstract

Mammalian microRNA expression is dysregulated in human cancer. However, the functional relevance of many microRNAs in the context of tumor biology remains unclear. Using CRISPR-Cas9 technology, we performed a global loss-of-function screen to simultaneously test the functions of individual microRNAs and protein-coding genes during the growth of a myeloid leukemia cell line. This approach identified evolutionarily conserved human microRNAs that suppress or promote cell growth, revealing that microRNAs are extensively integrated into the molecular networks that control tumor cell physiology. miR-155 was identified as a top microRNA candidate promoting cellular fitness, which we confirmed with two distinct miR-155-targeting CRISPR-Cas9 lentiviral constructs. Further, we performed anti-correlation functional profiling to predict relevant microRNA-tumor suppressor gene or microRNA-oncogene interactions in these cells. This analysis identified miR-150 targeting of p53, a connection that was experimentally validated. Taken together, our study describes a powerful genetic approach by which the function of individual microRNAs can be assessed on a global level, and its use will rapidly advance our understanding of how microRNAs contribute to human disease.

Introduction

Acute Myeloid Leukemia (AML) is an aggressive hematologic malignancy that carries a poor prognosis. In AML, hematopoiesis is disrupted by the overproduction of transformed myeloid cells, leading to life-threating anemia, immunosuppression, and bleeding due to decreased normal blood cell production. A variety of genetic and epigenetic aberrations are thought to drive leukemic phenotypes, including alterations in protein-coding genes and microRNAs.

MicroRNAs (miRNAs) are small non-coding RNAs that repress their target genes by binding to cognate 3' UTR sites in their respective mRNA targets, preventing their translation and/or triggering mRNA degradation. miRNA expression is highly dysregulated in AML [1, 2], and

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certain miRNAs have been shown to modulate leukemia cell biology *in vitro* [3]. Furthermore, the overexpression of a few specific miRNAs is sufficient to induce leukemic transformation in mice [4, 5], whereas other miRNAs act as tumor suppressors via repression of known protein oncogenes in hematopoietic malignancy [6, 7]. However, while the dysregulation of a number of miRNAs has been implicated in leukemia, the functional impact of many miRNAs and their putative targets on leukemic phenotypes remains unclear.

In this study, we took an unbiased, global loss-of-function approach to determine which miRNAs, and which of their putative targets, are involved in MV4-11 cell line growth, a model of myeloid leukemia. Because of the many caveats associated with previously described methods of miRNA loss-of-function screening that limits their use, we employed CRISPR-Cas9 technology [8–10]. Using this approach, each human miRNA and protein-coding gene in MV4-11 cells was individually disrupted and the impact on cellular growth was determined. Results point to a subset of evolutionarily conserved miRNAs that regulate cellular growth, and have also determined the impact of predicted miRNA targets that mediate these effects on tumor cell proliferation and survival. Furthermore, we have validated miR-150 as a critical promoter of leukemic cell growth in our system through targeting of p53. Taken together, our study demonstrates that CRISPR-Cas9 technology can be used to identify novel, functionally relevant miRNAs in mammalian cell phenotypes, while simultaneously identifying putative target proteins with opposing function. Our dataset also provides a resource describing the effects of individual miRNAs and protein-coding genes on leukemic cell fitness.

Results

CRISPR-Cas9 screen identifies protein-coding genes that regulate AML cell line growth

In order to determine which protein-coding genes and miRNAs regulate leukemic cell growth, we utilized a genome-scale CRISPR-Cas9 library (lentiCRISPRv2 library) [11, 12] to disrupt specific genes and evaluate the impact on cellular fitness over time. The lentiCRISPRv2 library contained 3 unique single guide RNAs (sgRNAs) targeting each protein-coding gene, as well as 4 unique sgRNAs targeting each miRNA gene locus cloned into an all-in-one CRISPR-Cas9 construct (lentiCRISPRv2). MV4-11 cells, a human-derived AML cell line homozygous for the FLT3-ITD mutation [13] and positive for the fusion protein MLL-AF4 [14], were transduced with the lentiCRISPRv2 library at ~250X coverage and an MOI of 0.3 to favor single viral integrations. An initial time point (TP0) was taken two days post-infection to assess library representation. Cells were selected with puromycin (puro) for 7 days, at which point puro was removed and growth was allowed to continue for an additional 16 days before a final time point (TP23) was collected (Fig 1A). Following genomic DNA (gDNA) extraction from cells at both time points and PCR amplification of each sgRNA sequence, we performed Illumina sequencing to generate read counts for each gene-targeting lentiCRISPRv2 construct. In order to accurately determine the impact of each gene on cell fitness over the 23-day time course, we combined the normalized read counts of all lentiCRISPRv2 constructs targeting a given gene at TP23 and expressed this as log2 fold change relative to the initial abundance of constructs at TP0 using DEseq2. We calculated the average log2 fold change across three independently-performed experiments to determine whether loss-of-function of each gene expressed in MV4-11 cells led to increased, decreased, or no change in cell growth over time using a cutoff p-value of 0.05. Furthermore, MV4-11 cells were transcriptionally profiled using RNA sequencing, and only expressed genes were included in our analysis. Using this approach, we identified protein-coding genes whose deletion significantly affected MV4-11 cell growth (Fig 1B and S1 Table). Of the 19,052 protein-coding genes targeted in our screen, we found 715 genes whose deletion

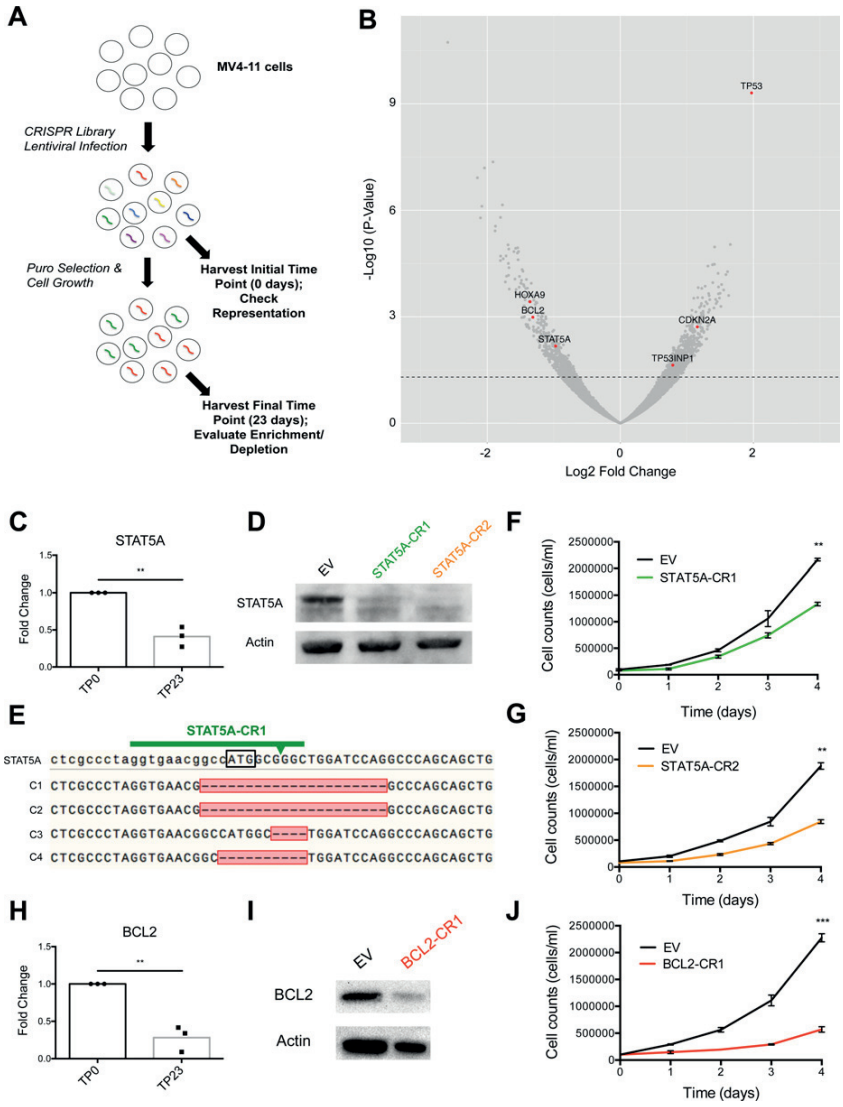

Fig 1. CRISPR-Cas9 loss-of-function screen identifies protein-coding genes, including known oncogenes STAT5A and BCL2, as important for MV4-11 cell line growth. (A) Overall experimental design of lentiCRISPRv2 library screen. (B) Log2 Fold Change of each protein-coding gene targeted in lentiCRISPRv2 library screen (x-axis) plotted against–Log10 P-Value (y-axis). Dotted line represents p-value = 0.05. (C) Fold change of STAT5A normalized read counts in lentiCRISPRv2 library screen as compared to TP0. (D) Western blot of STAT5A using cellular extract from STAT5A-CR1, STAT5A-C2, or EV

control infected MV4-11 cells with actin serving as load control. (E) DNA sequencing of the STAT5A locus from four representative STAT5A-CR1 infected MV4-11 clones (C1-C4). STAT5A represents the wild type (WT) sequence. Black box indicates translational start site. Arrow represents predicted cleavage site of Cas9 endonuclease. Red box identifies mutated region, with dashed lines indicating deleted nucleotides. (F, G) Growth curve for STAT5A-CR1 or STAT5A-CR2 infected MV4-11 cells compared to EV control. (H) Fold change of BCL2 normalized read counts in lentiCRISPRv2 library screen as compared to TP0. (I) Western blot of BCL2 in BCL2-CR1 or EV control infected MV4-11 cells with actin serving as load control. (J) Growth curve for BCL2-CR1 infected MV4-11 cells compared to EV control. (B, C, H) Represents combined data from three independently performed lentiCRISPRv2 library infections. Data represented as mean +/- SEM. P-values as indicated: *≤0.05, **≤0.01, ***≤0.001, and ns p>0.05. See also S1 Table.

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consistently resulted in increased cell numbers, which included many known tumor suppressor genes (TSGs). We also identified 516 genes whose deletion reproducibly resulted in decreased cellular growth, including a number of known oncogenes.

Signal transducer and activator of transcription 5A (STAT5A) was among the known oncogenes that our screen identified as important for cell growth (Fig 1B and 1C). STAT5 is a key signaling pathway that is inappropriately activated by FLT3-ITD mutations [15] and promotes FLT3-ITD driven growth. Thus, we tested the efficacy of two distinct STAT5A targeting lentiCRISPRv2 constructs (STAT5A-CR1 and STAT5A-CR2) through individual transduction of MV4-11 cells, and observed significantly reduced STAT5A protein levels by western blotting two weeks post-infection (Fig 1D). We sequenced the STAT5A locus in individual clones transduced with the STAT5A-CR1 vector and found that 80% of cells contained mutations at the expected Cas9 cut site (Fig 1E**).** STAT5A-CR1 and STAT5A-CR2 cells grew at a slower rate than cells transduced with a lentiCRISPRv2 empty vector (EV) control, confirming results from our screen indicating that STAT5A is a promoter of FLT3-ITD+ leukemic cell growth (Fig 1F and 1G). Similar results were obtained when we independently validated another known oncogene, BCL2 (Fig 1H–1J).

Interestingly, we also found that cells with CRISPR-Cas9-mediated depletion of Argonaute 2 (Ago2), Dicer, or Drosha, important proteins in the miRNA processing pathway, displayed reduced cell numbers over time, with p-values that trended towards, but did not reach, statistical significance (Data not shown). Because these genes are all in the miRNA biogenesis pathway, we sought to investigate this observation further. Therefore, we created Ago2 (Ago2-CR1) and Drosha (Drosha-CR1) deleted MV4-11 cell lines by using the lentiCRISPRv2 system to deliver sgRNAs against each of these protein-coding genes, and deletion was confirmed via western blotting (Fig 2A). These cell lines also demonstrated decreased cellular growth compared to EV-infected control MV4-11 cells (Fig 2B), suggesting that microRNAs were playing a net role in promoting cell growth in this context.

Critical role for specific, evolutionarily conserved miRNAs during MV4-11 cell growth

We next evaluated which individual miRNAs were playing a functional role in regulating MV4-11 cell growth. We found that 27 of the 197 evolutionarily conserved miRNAs targeted in our screen had a positive or negative influence on cell numbers over time with an average fold change p-value of less than 0.05 when the 3 biological replicate experiments were combined (Fig 2C and S2 Table). Only mature miRNAs expressed in MV4-11 cells, as assessed by RNA sequencing, were considered in our analysis. Interestingly, we found that fewer miRNAs had a functional effect during our screen than protein-coding genes. This observation could be explained by the fact that there are fewer miRNA vs protein-coding genes overall. Furthermore, this finding may also be due to the observation that miRNAs typically display only partial repression of their mRNA targets leading to modest changes in protein levels, whereas many protein-coding genes encode essential proteins that regulate core cellular processes required for growth and viability. miR-150, miR-155, and miR-182 were included in our top hits among

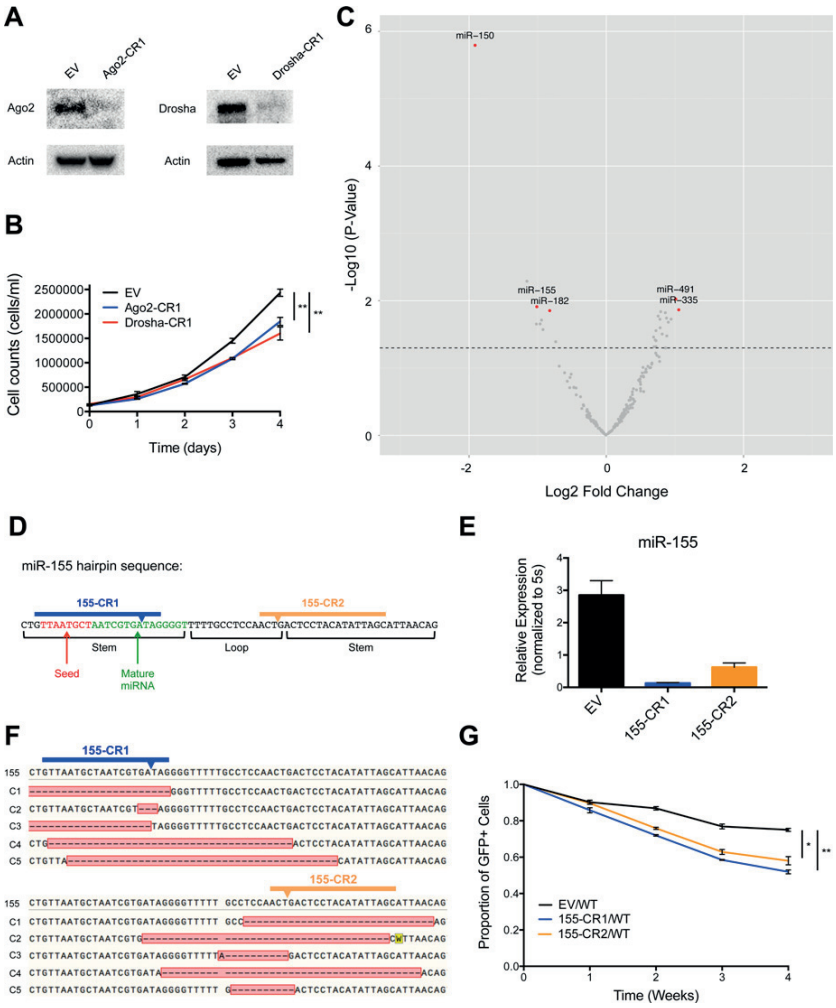

Fig 2. Identification of individual microRNAs, including miR-155, that regulate MV4-11 cell line growth. (A) Western blots of Ago2 and Drosha using cellular extract from Ago2-CR1, Drosha-CR1, and EV infected MV4-11 cell lines with actin serving as load control. (B) Growth curve for Ago2-CR1 and Drosha-CR1 infected MV4-11 cells compared to EV control. (C) Log2 Fold Change of each conserved microRNA gene targeted in lentiCRISPRv2 library screen (x-axis) plotted against–Log10 P-Value (y-axis). Dotted line represents p-value = 0.05. Represents combined data from three independently performed lentiCRISPRv2 library infections. (D) Schematic of miR-155 hairpin sequence as annotated in miRBase and sgRNA design of two independent miR-155-targeting lentiCRISPRv2 constructs (155-CR1, 155-CR2). (E) Expression levels of miR-155 in MV4-11 cells infected with EV control, 155-CR1, or 155-CR2 lentiCRISPRv2 constructs determined by qPCR. Expression normalized to 5s. (F) DNA sequencing of five representative 155-CR1 and 155-CR2 infected MV4-11 clones (C1-C5). 155 represents the WT sequence. Arrow indicates predicted cleavage site of Cas9. Red box identifies the mutated region, with dashed lines indicating deleted nucleotides. (G) Competitive growth curve of EV (GFP+), 155-CR1 (GFP+), or 155-CR2 (GFP+) infected MV4-11 cells mixed ~1:1 with WT MV4-11 cells at time point 0. Y-axis = (%GFP+ cells at indicated time point)/(%GFP+ cells initial). Data represented as mean +/- SEM. P-values as indicated: *≤0.05, **≤0.01, ***≤0.001, and ns p>0.05. See also S2 Table.

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conserved miRNAs that promote cell growth. Of relevance, all three have previously been implicated in hematopoietic malignancy [16–18]. We also identified conserved miRNAs that acted to repress cell growth in our screen, including miR-491 and miR-335.

miR-155 was identified as a top miRNA candidate that promoted FLT3-ITD+ cell growth (Fig 2C). Interestingly, miR-155 is also the most highly dysregulated miRNA in primary FLT3-ITD+ AML cells compared to FLT3-WT AML or normal CD34+ hematopoietic stem and progenitor cells [1, 2], and has been implicated in regulating the survival and growth of FLT3-ITD+ cells [19]. To independently validate miR-155, we used two distinct lentiCRISPRv2 constructs represented in our library to generate miR-155 deficient FLT3-ITD+ cell lines; one targeting the mature miRNA region of the miR-155 hairpin sequence (155-CR1), and the other targeting the loop (155-CR2) (Fig 2D). We found that cell lines carrying the 155-CR1 or 155-CR2 constructs had significantly decreased levels of mature miR-155 (Fig 2E). We further analyzed the mutations being created by 155-CR1 and 155-CR2, and found that 15/16 clones analyzed (8/8 of 155-CR1; 7/8 of 155-CR2) contained mutations at the predicted Cas9 cut site (Fig 2F). Of these 15 mutations, we observed 12 deletions and 3 insertions, indicating that NHEJ-mediated deletions were favored in these cells. 5 of the 7 clones analyzed that had been transduced with 155-CR1 contained mutations spanning the seed sequence, the critical portion of the mature miRNA that leads to target repression via complementary binding to the 3' UTR. In the case of 155-CR2, we conclude that deletion of the loop region leads to disrupted biogenesis of mature miR-155. Both 155-CR1 and 155-CR2 cells exhibited decreased competitive cell growth compared to EV control cells (Fig 2G), thus confirming our library findings.

Anti-correlation functional profiling identifies relevant microRNA-target pairs, including miR-150 and p53

We also used our dataset to identify miRNAs predicted to target known oncogenes and TSGs (S3 Table). In the case of several representative oncogenes, their promotion of cell growth inversely correlated with the impact of specific miRNAs with conserved binding sites in their 3' UTRs (Fig 3A). Similar observations were made for a subset of known TSGs, where their negative effects on cell growth inversely correlated with the impact of specific miRNAs with conserved binding sites in their 3' UTRs (Fig 3B). This approach, which we refer to as anti-correlation functional profiling, is a powerful method that can be used to globally identify miRNA-target gene pairs that may be functionally linked.

To test the ability of anti-correlation functional profiling to identify functionally relevant miRNA-target pairs in our system, we tested the top association from our miRNA-TSG plot, TP53 (p53) and miR-150. miR-150, the top miRNA hit in our lentiCRISPRv2 library screen, has a conserved binding sequence in the 3'UTR of p53 (Fig 3C), and has been shown to be directly targeted by miR-150 in luciferase reporter assays [20, 21]. We generated a lentiCRISPRv2 construct designed to cut near the mature miRNA coding sequence using one of the miR-150 sgRNAs (150-CR1) represented in the lentiCRISPRv2 library (Fig 3D), and confirmed that 150-CR1 containing MV4-11 cells displayed a significant decrease in miR-150 levels compared to EV control cells by qPCR (Fig 3E). We also generated p53 deficient cell lines (p53-CR1), again using a sgRNA from the lentiCRISPRv2 library cloned into a lentiCRISPRv2 construct. We confirmed that p53 protein levels were reduced in the p53-CR1 cells compared to EV control cells (Fig 3F), and saw an increase in p53 protein level in the 150-CR1 cells compared to EV control, indicating that miR-150 is indeed repressing p53 in MV4-11 cells. Finally, we observed that p53-CR1 cells had a competitive growth advantage compared to EV infected cells (Fig 3G), while 150-CR1 cells had a growth disadvantage. These results validate our anti-correlation functional profiling approach to finding relevant miRNA-target pairs that regulate

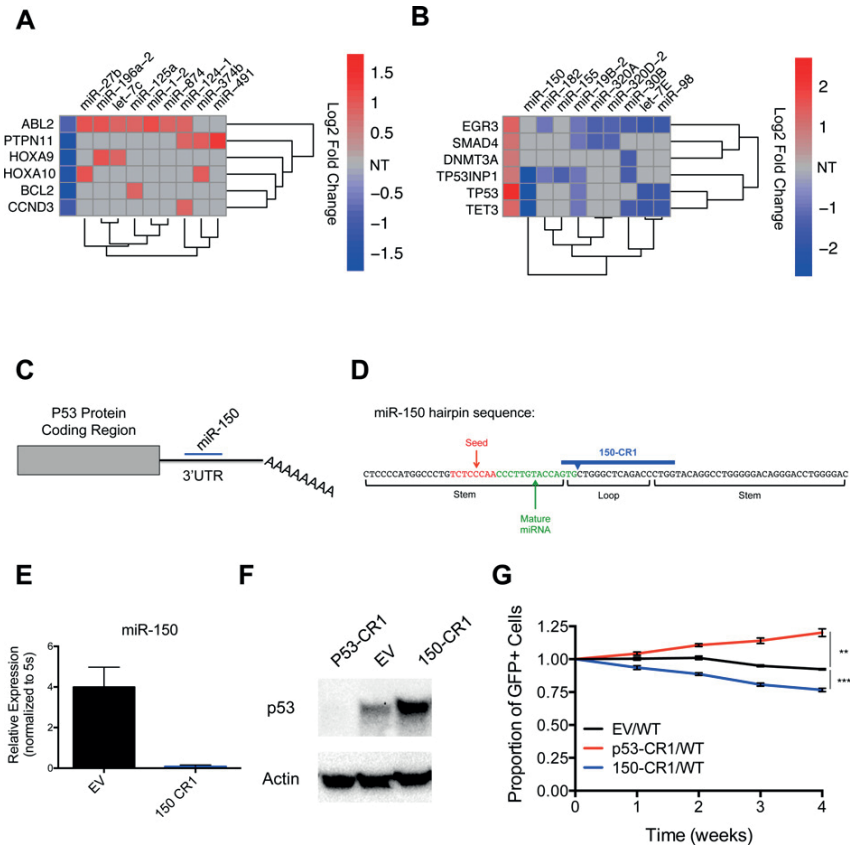

Fig 3. Anti-correlation functional profiling identifies relevant miRNA-target interactions, including miR-150 repression of p53, that regulate MV4-11 cell line growth. (A) Heat map indicating representative oncogenes whose loss leads to decreased cell growth according to Log2 Fold Change values from lentiCRISPRv2 library screen (first column), and functionally anti-correlated miRNAs that are predicted to target each oncogene. Grey boxes indicate that the miRNA is not predicted to bind the 3'UTR of the oncogene (NT = Not targeted). (B) Heat map indicating representative TSGs whose loss lead to increased cell growth according to our Log2 Fold Change values from lentiCRISPRv2 library screen (first column), and miRNAs predicted to target each TSG whose growth anti-correlated in library. Grey boxes indicates that the miRNA is not predicted to bind the 3'UTR of TSG (NT = Not targeted). (C) Schematic showing miR-150 targeting of the p53 3'UTR. (D) Schematic of the miR-150 hairpin sequence as annotated in miRBase and sgRNA design of the miR-150-targeting lentiCRISPRv2 construct (150-CR1). (E) Expression level of miR-150 in MV4-11 cells infected with EV control or 150-CR1 lentiCRISPRv2 constructs determined by qPCR. Expression normalized to 5s. (F) Western blot of p53 in p53-CR1, 150-CR1, and EV control infected MV4-11 cell lines with actin serving as load control. (G) Competitive growth curve of EV (GFP+), p53-CR1 (GFP+), or 150-CR1 (GFP+) infected MV4-11 cells mixed ~1:1 with WT MV4-11 cells at time point 0. Y-axis = (%GFP+ cells at indicated time point)/(%GFP+ cells initial). (A, B) Only expressed protein-coding genes and microRNAs with p-values <0.05 were analyzed. Data represented as mean +/- SEM. P-values as indicated: *≤0.05, **≤0.01, ***≤0.001, and ns p>0.05. See also S3 Table.

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specific cellular phenotypes, and point to miR-150 repression of p53 as a significant pro-growth and survival mechanism in at least some types of myeloid leukemias.

Discussion

There are over 1000 different miRNAs in human cells, each with the potential to be functionally relevant in diseases such as cancer. However, the use of large-scale loss-of-function screening to identify functionally relevant miRNAs has been hampered by technical limitations, including the inability of shRNAs to effectively block miRNA biogenesis and function. Most miRNAs that have been studied to date have been assessed on an individual basis. While this has provided important insights into the roles of those examined, many miRNAs have been left uncharacterized. Consequently, there is a tremendous need for high throughput approaches to identifying functionally relevant miRNAs in an unbiased manner to obtain a comprehensive list of miRNAs that impact specific phenotypes. Here, we have successfully used CRISPR-Cas9 technology to carry out a miRNA loss-of-function screen, and although further validation of individual hits is ultimately necessary in more physiologically relevant systems, our results clearly demonstrate that subsets of specific miRNAs act to positively or negatively control cellular proliferation and survival in the system under study and provide a resource to guide future work.

Because miRNAs repress protein-coding target genes, data from our screen could be used to predict functionally relevant miRNA-target gene interactions that regulate leukemic cell growth. Using this anti-correlation functional profiling approach, we identified miRNAs with functions that oppose a specific TSG or oncogene predicted to be a conserved target of each respective miRNA. Further, we confirmed that miR-150 repression of p53 is a promoter of cell growth in our system, which validates this approach. Although additional miRNA-target connections in this setting require further validation, our results underscore the potential of this approach to identify novel miRNA-target networks with relevance to cancer, and do so during a single experiment.

Our screen also identified miR-155, a miRNA that has been clinically connected to FLT3-ITD+ AML, as a promoter of FLT3-ITD+ cell proliferation. To validate this result, we confirmed that two independent sgRNAs against the human miR-155 hairpin sequence were able to dramatically reduce production of mature miR-155. While one of these sgRNAs targeted mature miR-155, including the seed sequence, the other targeted the loop region of the hairpin. These results indicate that, although miRNA hairpin sequences are short, one can find multiple CRISPR-Cas9 sites that can be used to disrupt miRNA biogenesis and subsequently validate and study loss-of-function phenotypes.

Beyond regulating tumor cell proliferation and survival, miRNAs have been implicated in other aspects of cancer, including drug resistance and metastasis [22, 23]. Our current approach has the potential to be used to identify specific miRNAs and their targets that regulate these deleterious processes, and reveal key miRNA species that represent promising therapeutic targets in these contexts. Together, these approaches will provide a systematic view of the molecular networks that coordinate malignant disease origin and subsequent outcomes with a focus on functional relevance.

Material and Methods

Cells and tissue culture

MV4-11 cells were purchased from ATCC and used for all *in vitro* experimentation. Cells were cultured in RPMI based media supplemented with 10% FBS, and kept at 37ºC with 5% CO_2. Cells were passaged every 2–3 days in order to stay within $1x10^5$-$1x10^6$ cells/ml to maintain logarithmic growth.

CRISPR-Cas9 library screen and individual LentiCRISPRv2 infections

Genome-scale CRISPR Knock-Out (GeCKO) v2.0 was purchased from Addgene for application in all lentiCRISPRv2 library screens, and performed as described in S1 Supporting Methods. In

brief, cells were infected, selected with puromycin, DNA was extracted, the integrated sgRNAs were then amplified and amplicons were subjected to DNA-Seq. Single CRISPR-Cas9 vector infections were performed using a similar approach. Unique sgRNA sequences were cloned into a lentiCRISPRv2 construct (a gift from Feng Zhang; Addgene plasmid #52961) containing either a puro resistance or GFP selection marker. Sequences can be found in our supplemental methods section. The CRISPR-Cas9 library screen data have been deposited in NCBI's Gene Expression Omnibus under GEO: GSE71544.

Growth curves and competition assays

Growth curves using STAT5A-CR1, STAT5A-CR2, Drosha-CR1, or Ago2-CR1 cells were performed in 2 ml triplicate cultures in a 6 well plate. EV infected cells were grown in parallel as a positive control. Cells were split to 100,000 cells/ml and counted daily via microscopy using a hemacytometer and trypan blue exclusion until cells reached ~$2x10^6$ cells/ml, or the point when cells no longer demonstrated logarithmic growth. Competitive growth assays were performed by mixing miR-155-CR1, miR-155-CR2, p53-CR1, miR-150-CR1, or EV control cells (all GFP+) at a 1:1 ratio with WT MV4-11 cells (GFP-), and measuring the percentage of GFP + cells over a 4-week time course via flow cytometry.

Quantitative PCR

Total RNA was isolated from MV4-11 cell lines and mouse BM cells using the miRNeasy spin column kit (Qiagen). Mature miR-155 or miR-150 was quantified using the miRCURY LNA Universal RT microRNA PCR cDNA Synthesis Kit II (Exiqon) and ExiLENT SYBR Green master mix kit (Exiqon) on a Light Cycler 480 PCR machine (Roche). Human or mouse miR-155 LNA primers, human miR-150 LNA primers, and 5S rRNA loading control primers were purchased from Exiqon.

Expression profiling

Total RNA was isolated using the miRNeasy spin column kit (Qiagen). Expression of small RNAs and long RNAs in MV4-11 cells was performed using RNA sequencing as described further in S1 Supporting Methods. Data have been deposited into GEO as GSE71544.

Western blot analysis

Total protein extracts from MV4-11 cell lines were harvested using RIPA lysis buffer with protease inhibitors, and protein concentration was determined using a Bio-Rad Protein Assay Dye Reagent kit. SDS-denatured protein was separated via gel electrophoresis and transferred onto a nitrocellulose membrane. Protein was detected via overnight antibody staining with the following antibodies: STAT5A (Santa Cruz L-20), Drosha (Cell Signaling D28B1), Ago2 (Cell Signaling C34C6), p53 (Santa Cruz FL-393), and Actin (Sigma A5441).

Statistics

Significant p-values were determined using an unpaired Student's t-test, unless otherwise noted. P-values for lentiCRISPRv2 library screen were determined using DEseq2. Quantitative data are displayed as mean +/- SEM. P-values are shown as indicated: $^{*}\leq 0.05$, $^{**}\leq 0.01$, $^{***}\leq 0.001$, and ns $p>0.05$. All statistics were performed in either GraphPad Prizm6.0 or Microsoft Excel. For calculation of p-values in growth curves, individual t-tests were performed for the final time point.

Supporting Information

S1 Supporting Methods. Supplemental experimental procedures.
(DOCX)

S1 Table. Protein-coding genes significantly affecting MV4-11 cell growth.
(XLSX)

S2 Table. MicroRNAs significantly affecting MV4-11 cell growth.
(XLSX)

S3 Table. Anti-correlation functional profiling identifies miRNA-protein coding gene (PCG) pairs that regulate MV4-11 cell growth.
(XLSX)

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Author Contributions

Conceived and designed the experiments: JW RMO. Performed the experiments: JW RH. Analyzed the data: JW JLR DSR WZS RMO. Contributed reagents/materials/analysis tools: TJD. Wrote the paper: JW RMO. Performed bioinformatics analysis: TLM.

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CHAPTER 4

MIR-155 PROMOTES FLT3-ITD-INDUCED MYELOPROLIFERATIVE DISEASE THROUGH INHIBITION OF THE INTERFERON RESPONSE

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MYELOID NEOPLASIA

miR-155 promotes FLT3-ITD–induced myeloproliferative disease through inhibition of the interferon response

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Key Points

- miR-155 promotes myeloproliferation in the bone marrow, spleen, and blood of mice carrying the FLT3-ITD mutation.
- miR-155 suppresses the IFN response in FLT3-ITD$^+$ mouse hematopoietic stem and progenitor cells, as well as FLT3-ITD$^+$ human AML cells.

FLT3-ITD$^+$ acute myeloid leukemia (AML) accounts for ~25% of all AML cases and is a subtype that carries a poor prognosis. microRNA-155 (miR-155) is specifically overexpressed in FLT3-ITD$^+$ AML compared with FLT3 wild-type (FLT3-WT) AML and is critical for the growth of FLT3-ITD$^+$ AML cells in vitro. However, miR-155's role in regulating FLT3-ITD–mediated disease in vivo remains unclear. In this study, we used a genetic mouse model to determine whether miR-155 influences the development of FLT3-ITD–induced myeloproliferative disease. Results indicate that miR-155 promotes FLT3-ITD–induced myeloid expansion in the bone marrow, spleen, and peripheral blood. Mechanistically, miR-155 increases proliferation of the hematopoietic stem and progenitor cell compartments by reducing the growth-inhibitory effects of the interferon (IFN) response, and this involves targeting of Cebpb. Consistent with our observations in mice, primary FLT3-ITD$^+$ AML clinical samples have significantly higher miR-155 levels and a lower IFN response compared with FLT3-WT AML samples. Further, inhibition of miR-155 in FLT3-ITD$^+$ AML cell lines using CRISPR/Cas9, or primary FLT3-ITD$^+$ AML samples using locked nucleic acid antisense inhibitors, results in an elevated IFN response and reduces colony formation. Altogether, our data reveal that miR-155 collaborates with FLT3-ITD to promote myeloid cell expansion in vivo and that this involves a multitarget mechanism that includes repression of IFN signaling. (*Blood*. 2017;129(23):3074-3086)

Introduction

Acute myeloid leukemia (AML) is an aggressive hematological malignancy that carries a poor prognosis. AML is a heterogeneous disease with a variety of genetic aberrations, including translocations and mutations, that can drive leukemic phenotypes. The most common genetic aberration in AML is a gain-of-function mutation in the FMS-like tyrosine kinase 3 (FLT3) receptor. FLT3 internal tandem duplication (ITD) in the juxtamembrane domain of the receptor occurs in ~25% of AML diagnoses and confers a poor prognosis.[1] FLT3 is a cell surface protein that promotes the proliferation and survival of the hematopoietic stem and progenitor cell (HSPC) compartments in response to FLT3 ligand.[2] However, FLT3-ITD mutations lead to constitutive, ligand-independent activation of this receptor,[3] conferring a growth and survival advantage.

Although FLT3-ITD is a common mutation observed in human AML and carries a poor prognosis, the mutation itself has not been shown to independently drive leukemic transformation in vivo. Rather, FLT3-ITD must collaborate with additional oncogenic mutations to trigger hematopoietic malignancy.[4-6] Introduction of human FLT3-ITD mutations into mice triggers a myeloproliferative disease (MPD) that resembles chronic myelomonocytic leukemia,[7-9] but does not lead to overt leukemia. Regardless, FLT3-ITD mouse models have proven useful in studying FLT3-ITD biology in hematologic malignancies.

MicroRNAs (miRNAs) are small noncoding RNAs that repress their target genes by binding to cognate sites in the 3′ untranslated region of their respective messenger RNA targets, thereby preventing their translation and/or triggering messenger RNA degradation. miRNA expression has been shown to be highly dysregulated in AML, including FLT3-ITD$^+$ AML, where microRNA-155 (miR-155) represents the most significantly overexpressed miRNA.[10-16] Overexpression of miR-155 alone in the hematopoietic compartment is sufficient to cause a myeloproliferative phenotype[17] resembling that seen in mice harboring FLT3-ITD mutations. Although the association between FLT3-ITD and miR-155 overexpression has been observed in primary human samples, and miR-155 has been shown to promote FLT3-ITD$^+$ cell line growth in vitro,[18,19] the relationship between FLT3-ITD and miR-155 has not been directly examined in vivo, and the downstream effects of miR-155 overexpression are still being deciphered.

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The RNA sequencing data reported in this article have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (accession number GSE86526).

The online version of this article contains a data supplement.

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In this study, we used a FLT3-ITD genetic mouse model and FLT3-ITD$^+$ human AML cells to study the collaboration between the FLT3-ITD mutation and miR-155 in promoting hematologic malignancy. We found that miR-155 substantially contributes to FLT3-ITD–induced MPD, and that knockdown of miR-155 in primary FLT3-ITD$^+$ AML samples reduces colony formation. Mechanistically, we show that miR-155 inhibits the response to interferon (IFN) in these model systems, and this involves direct repression of Cebpb. Interferon has previously been shown to exhibit an antiproliferative effect on early hematopoietic cells,[20-23] including in our FLT3-ITD mouse model.[24] Altogether, our study identifies a specific role for miR-155 in promoting the expansion of myeloid cells in FLT3-ITD–mediated disease in vivo, indicating that inhibition of miR-155 may be a promising new therapeutic approach for treatment of FLT3-ITD$^+$ AML.

Methods

A more extensive description of the methods can be found in the supplemental Methods, available on the *Blood* Web site.

Animals

All mice (wild-type [WT], 155$^{-/-}$, FLT3-ITD, and FLT3-ITD 155$^{-/-}$) were on a C57BL/6 background. FLT3-ITD mice were obtained from The Jackson Laboratory (stock no. 011112) and were homozygous for the FLT3-ITD mutation. Experimental procedures were performed with the approval of the Institutional Animal Care and Use Committee of the University of Utah.

Flow cytometric analysis

Splenocytes and bone marrow (BM) cells were harvested from mice and depleted of red blood cells prior to staining with specific fluorophore-conjugated antibodies. Antibody-stained cells were analyzed with a BD LSR Fortessa flow cytometer (BD Biosciences), and data analysis was performed by using FlowJo software.

Proliferation assay

FLT3-ITD and FLT3-ITD 155$^{-/-}$ mice were injected intraperitoneally with 150 μL of 5-bromo-2′-deoxyuridine (BrdU [BD Pharmingen]) at a concentration of 10 mg/mL 20 hours prior to BM harvest. BM cells were fixed and stained with an anti-BrdU antibody (BD Pharmingen) following the manufacturer's instructions and analyzed via flow cytometry.

BM chimera reconstitutions

Total BM was harvested from FLT3-ITD and FLT3-ITD 155$^{-/-}$ mice (45.2$^+$) and mixed in equal ratios with BM from WT (45.1$^+$) mice purchased from The Jackson Laboratory (stock no. 002014). Myeloproliferative phenotypes were evaluated at 3 months.

Expression profiling

Lineage-negative (Lin$^-$), c-Kit$^+$, Sca1$^+$ (LKS) cells were sorted by using flow cytometry, and total RNA was isolated using the miRNeasy spin column kit (Qiagen). RiboZero treatment/library preparation was performed at the University of Utah DNA Sequencing Core Facility, followed by stranded RNA sequencing by using Illumina HiSequation 2000 sequencing. Aligned reads (miRBase) were used in DESeq2 (version 1.10.1), which normalizes the signal and determines differential expression. The RNA sequencing data has been deposited in the National Center for Biotechnology Information Gene Expression Omnibus under GSE86526. Genes with multiple testing corrected P values $< .05$ were used in Ingenuity Pathway Analysis and Gene Set Enrichment Analysis (GSEA). Western blotting and quantitative reverse transcription polymerase chain reaction (qRT-PCR) were carried out by using standard procedures.

Cell culture and lentiCRISPR infections

Molm14 and MV4-11 cell lines were cultured in RPMI 1640 with fetal bovine serum and antibiotics. LentiCRISPR infections (155-CR1 and empty vector [EV]) were performed as described previously,[19] and cells were passaged for at least 10 days prior to analysis.

TCGA analysis

Mirbase20 miRNA expression data from IlluminaGA_miRNASeq (n = 188) and the RSEM gene expression data from IlluminaGA_RNASeqV2 (n = 173) were downloaded from The Cancer Genome Atlas (TCGA) Web site for the available AML samples. Somatic variants for the 305 mutated genes identified in the 200 TCGA AML patients were downloaded from the cBioPortal Web site (http://www.cbioportal.org/). AML samples were split into FLT3-ITD–positive samples (FLT3-ITD) and FLT3 mutation–negative samples (FLT3-WT). DESeq2 (version 1.10.1) was used to normalize the count data and detect differently expressed genes or miRNAs.

Cebpb overexpression

Lin$^-$ c-Kit$^+$ (LK) cells were sorted by flow cytometry and cultured in RPMI 1640–based medium with 50 ng/mL SCF. Cells infected with either an EV control (pMIG II-EV) or Cebpb-overexpressing vector (pMIG II-Cebpb) were passaged for 2 days in individual wells prior to RNA isolation.

Patient samples

Mononuclear cells from the peripheral blood of FLT3-ITD$^+$ AML patients at the Huntsman Cancer Institute (University of Utah) were Ficoll-separated and used for automated isolation of the CD34$^+$ fraction by using an autoMACS Pro (Miltenyi Biotech). Cells were used for methylcellulose colony assays or expanded in liquid culture to assess Annexin V or gene expression by qRT-PCR. Cells were treated with 100 nM LNA-155 or LNA-CTRL (Exiqon). Donors gave informed consent and studies were approved by the University of Utah Institutional Review Board (no. 00045880).

Statistics

Significant P values were determined by using an unpaired Student t test unless otherwise noted. Quantitative data are displayed as mean ± the standard error of the mean (SEM). P values are shown as indicated: *$P \leq .05$; **$P \leq .01$; ***$P \leq .001$; ****$P \leq .0001$; and not significant (ns) $P > .05$. The false discovery rate for GSEA plots was calculated by using Limma. All other statistics were performed in either GraphPad Prism 6.0 or Microsoft Excel.

Results

miR-155 promotes expansion of myeloid cells in the spleen and the blood of FLT3-ITD$^+$ mice

We generated mice homozygous for the FLT3-ITD mutation (denoted as FLT3-ITD) that also lack miR-155 (155$^{-/-}$) to test the function of miR-155 during FLT3-ITD–mediated pathogenesis in vivo (Figure 1A-B). Of note, mice homozygous for the FLT3-ITD mutation develop a chronic MPD during adulthood.[7-9] At 4 to 6 months of age, FLT3-ITD miR-155$^{+/+}$ (FLT3-ITD), FLT3-ITD 155$^{-/-}$, WT, and 155$^{-/-}$ mice were analyzed. We observed that FLT3-ITD–induced splenomegaly was significantly reduced in the absence of miR-155 (Figure 1C), both in terms of overall spleen weight and cellularity (Figure 1D). Spleens from FLT3-ITD animals showed extensive infiltration of maturing myeloid cells disrupting and replacing the white pulp and had a significant reduction in the red pulp as well as normal erythropoiesis (Figure 1E). Although various aspects of this disease process were still observed in the FLT3-ITD 155$^{-/-}$ group, their severity was noticeably diminished compared with their FLT3-ITD

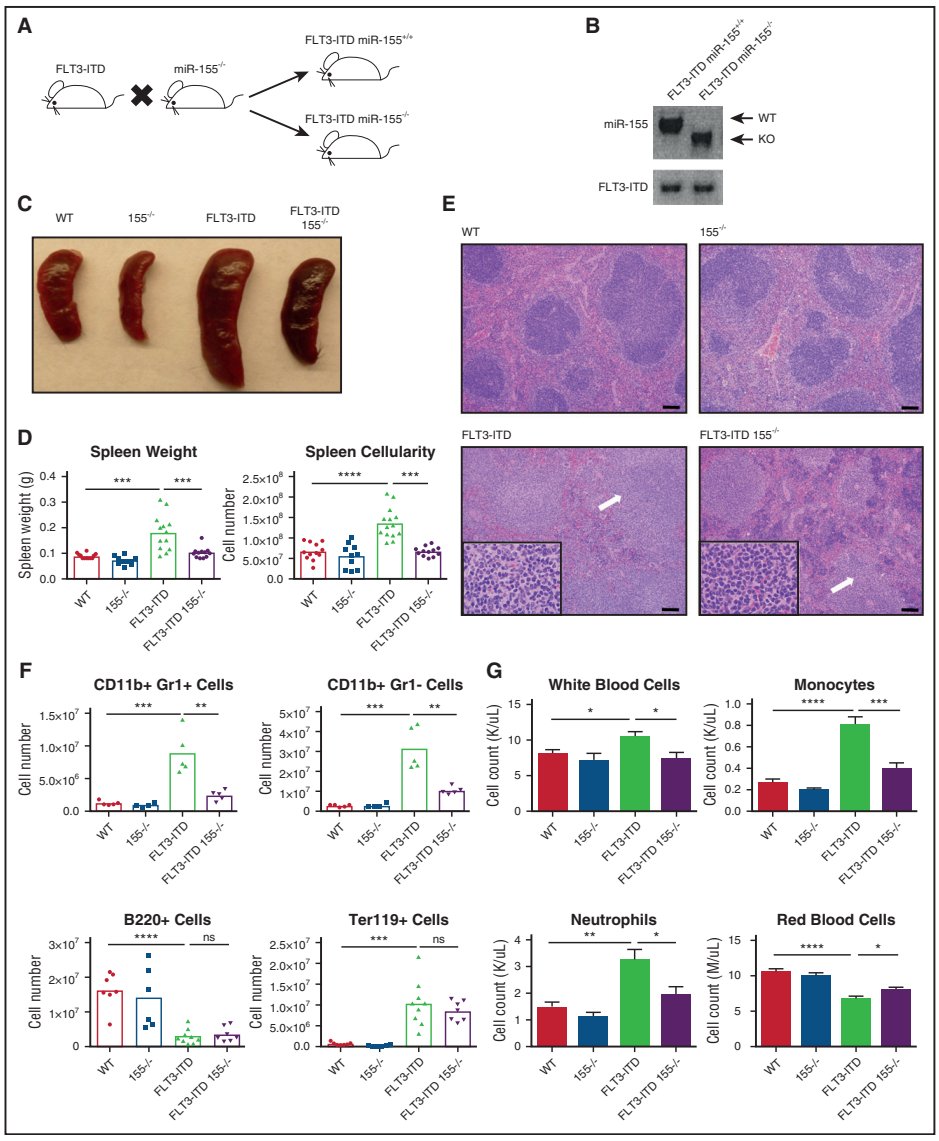

Figure 1. miR-155 promotes FLT3-ITD–mediated myeloid expansion in the spleen and blood. (A) Breeding strategy to create FLT3-ITD $155^{-/-}$ mice. (B) PCR confirming the presence of FLT3-ITD and loss of miR-155. (C) Representative spleens for the different mouse groups. (D) Spleen size quantified by both weight and overall cellularity. (E) Hematoxylin and eosin staining of spleens from different mouse groups. Representative images are shown. White arrows denote areas of myeloproliferation. Full-sized images, original magnification ×100; inset, original magnification ×1000. Scale bar, 100 μm. (F) Total numbers of $CD11b^+$ $Gr1^+$ cells, $CD11b^+$ $Gr1^-$ cells, $B220^+$ cells, and $Ter119^+$ cells in the spleen determined by flow cytometry. (G) Complete blood counts for various blood cells in different mouse groups. Data are representative of at least 3 independent experiments. Each point represents a sample from 1 mouse. Data represented as mean ± SEM. *$P \leq .05$; **$P \leq .01$; ***$P \leq .001$; ****$P \leq .0001$; ns, $P > .05$. KO, knockout.

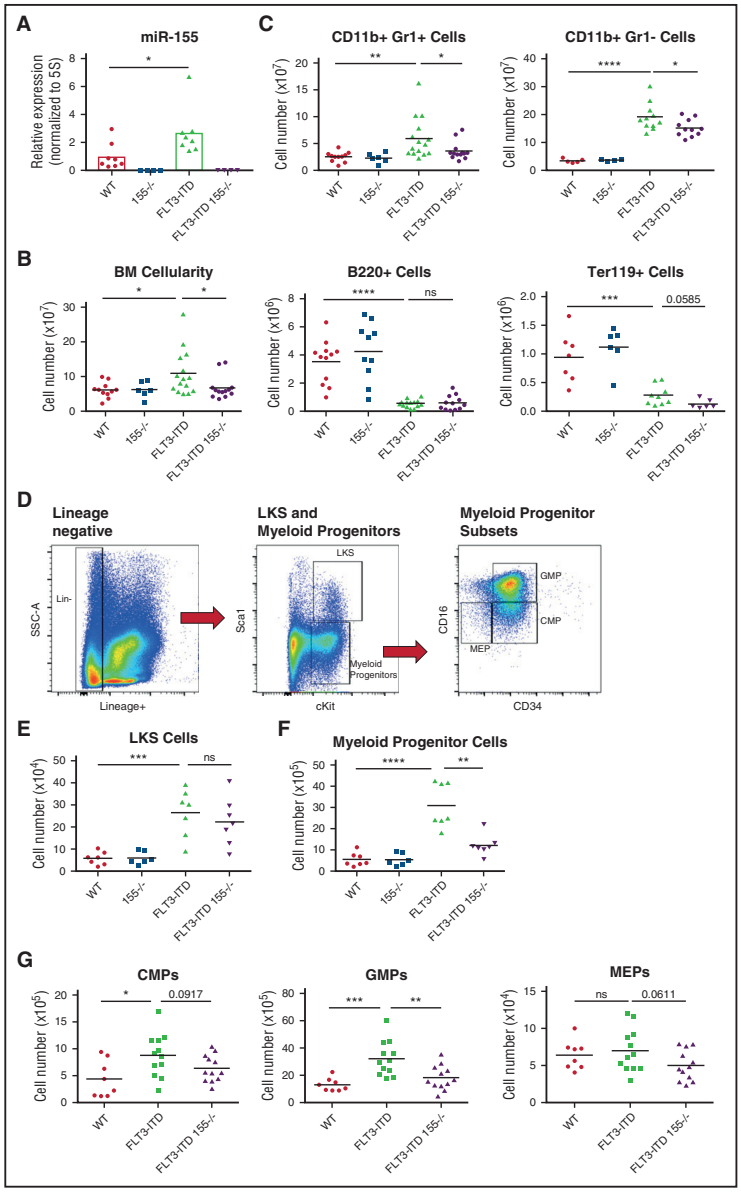

Figure 2. Expression of miR-155 in FLT3-ITD BM promotes expansion of myeloid progenitors. (A) Expression level of miR-155 in WT, $155^{-/-}$, FLT3-ITD, and FLT3-ITD $155^{-/-}$ mice in total BM determined by qRT-PCR. Expression normalized to 5S ribosomal RNA. (B) Total BM cellularity in different mouse groups. (C) Total numbers of $CD11b^+$ $Gr1^+$ cells, $CD11b^+$ $Gr1^-$ cells, $B220^+$ cells, and $Ter119^+$ cells in the BM of different mouse groups determined by flow cytometry. (D) Gating strategy for flow cytometric analysis of LKS and myeloid progenitor (Lin^-, c-Kit^+, $Sca1^-$) populations. (E) Total number of LKS cells in BM determined by flow cytometry. (F) Total number of myeloid progenitor cells (Lin^-, c-Kit^+, $Sca1^-$) in BM determined by flow cytometry. (G) Total common myeloid progenitor (CMP) (left), granulocyte-monocyte progenitor (middle), and megakaryocyte-erythrocyte progenitor (right) cells in BM determined by flow cytometry. Data are representative of at least 3 independent experiments. Each point represents a sample from 1 mouse. *$P \leq .05$; **$P \leq .01$; ***$P \leq .001$; ****$P \leq .0001$; ns, $P > .05$.

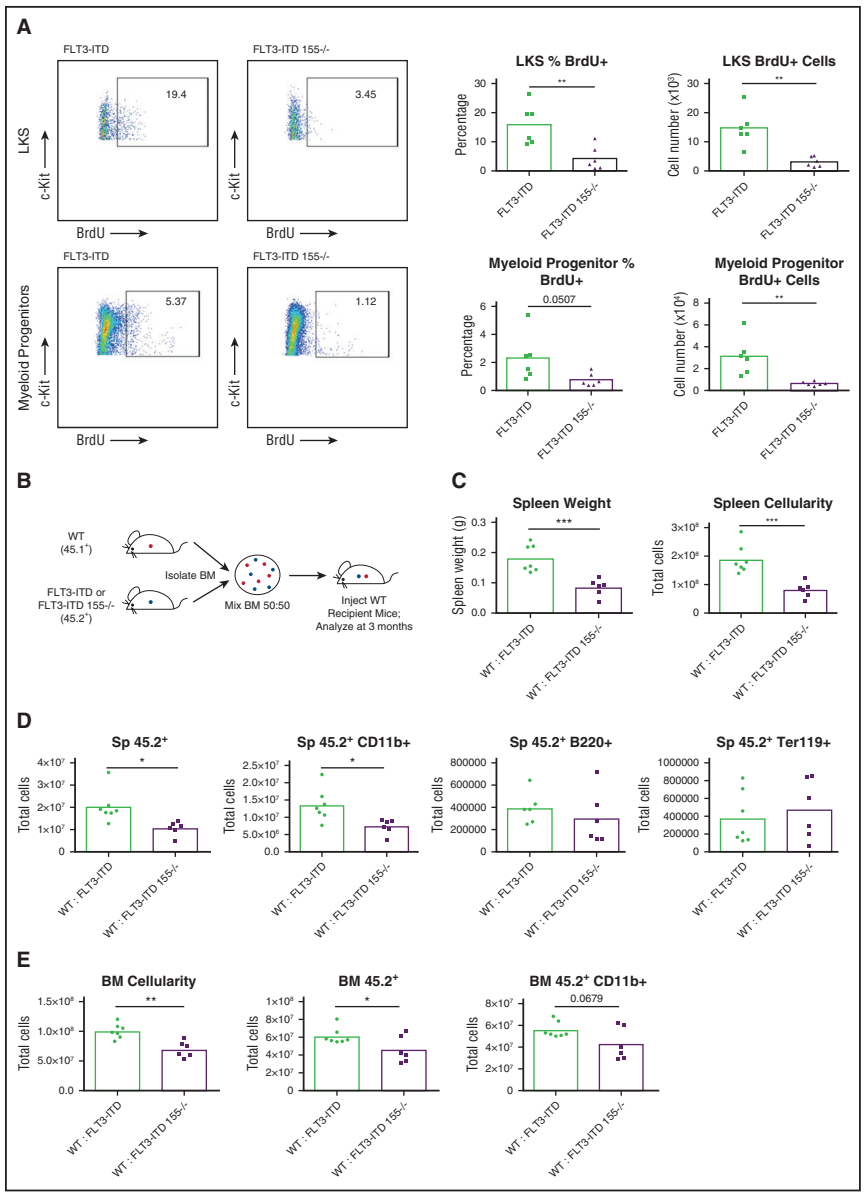

Figure 3. miR-155 promotes proliferation of the LKS and myeloid progenitor compartments and functions in a cell-intrinsic manner during FLT3-ITD–driven MPD. (A) Total BrdU$^+$ staining of LKS and myeloid progenitor cells (Lin$^-$, c-Kit$^+$, Sca1$^-$) in BM. Representative flow cytometric plots and percentages are shown. (B) Experimental strategy for producing WT:FLT3-ITD and WT:FLT3-ITD 155$^{-/-}$ BM chimeric mice. (C-D) Spleen analysis of WT:FLT3-ITD and WT:FLT3-ITD 155$^{-/-}$ BM chimeric mice at 3 months posttransplant. Total numbers of various cell populations determined by flow cytometry. (E) Analysis of BM from WT:FLT3-ITD and WT:FLT3-ITD 155$^{-/-}$ BM chimeric mice at 3 months posttransplant. Total numbers of various cell populations determined by flow cytometry. Data are representative of at least 2 independent experiments. Each point represents a sample from 1 mouse. *$P \leq .05$; **$P \leq .01$; ***$P \leq .001$.

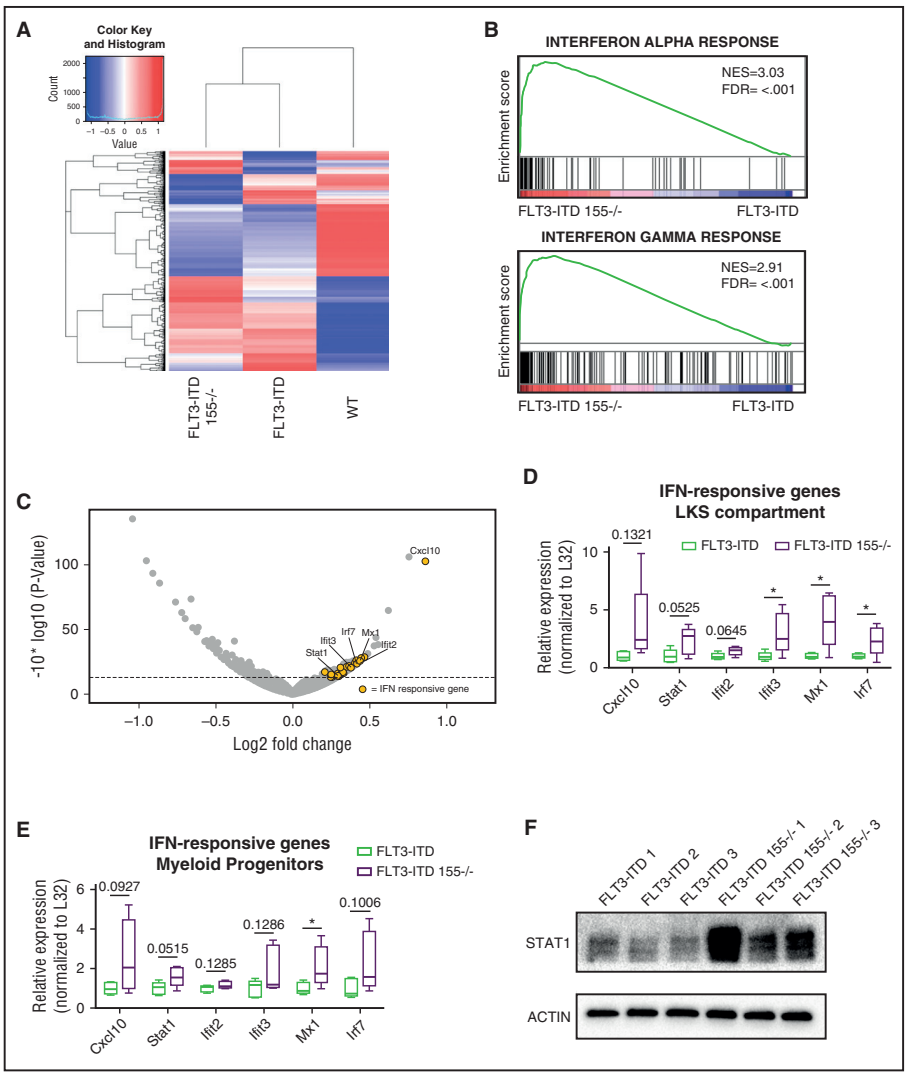

Figure 4. IFN signaling is increased in LKS and myeloid progenitor compartments of FLT3-ITD mice in the absence of miR-155. (A) Heat map showing gene expression differences in protein-coding genes identified by RNA sequencing of the LKS compartment of WT, FLT3-ITD, and FLT3-ITD $155^{-/-}$ mice. (B) GSEA of LKS compartment RNA sequencing data identifies the IFN-α response and IFN-γ response as hallmark differences between FLT3-ITD and FLT3-ITD $155^{-/-}$ mice. (C) Volcano plot showing LKS compartment gene expression changes in FLT3-ITD $155^{-/-}$ vs FLT3-ITD mice. Dashed line represents a *P* value = .05. Yellow dots indicate IFN-α/γ–responsive genes as annotated by GSEA. (D-E) qRT-PCR analysis of representative IFN-responsive genes in the LKS or myeloid progenitor compartment of FLT3-ITD or FLT3-ITD $155^{-/-}$ mice (n = 5 for each group). Expression normalized to L32, with the FLT3-ITD average set to a relative expression value of 1. (F) Western blot of STAT1 in LK-sorted BM of FLT3-ITD or FLT3-ITD $155^{-/-}$ mice; replicates represent individual mice. ACTIN serves as the loading control. *$P \leq .05$ FDR, false discovery rate; NES, normalized enrichment score.

counterparts. Flow cytometry on the spleen revealed a reduced total number of $CD11b^{+}$ $Gr1^{-}$ cells, primarily consisting of monocytes and dendritic cells, as well as a decrease in the neutrophilic $CD11b^{+}$ $Gr1^{+}$ population in the absence of miR-155 (Figure 1F; supplemental Figure 1A), indicating reduced splenic infiltration of mature myeloid cells.

In the peripheral blood, we observed a noticeable increase in white blood cells in the FLT3-ITD mice compared with WT mice, which is consistent with previous reports,[7] and this increase was significantly reduced in the FLT3-ITD $155^{-/-}$ group (Figure 1G; supplemental Figure 1B). The decrease in leukocytosis in FLT3-ITD $155^{-/-}$

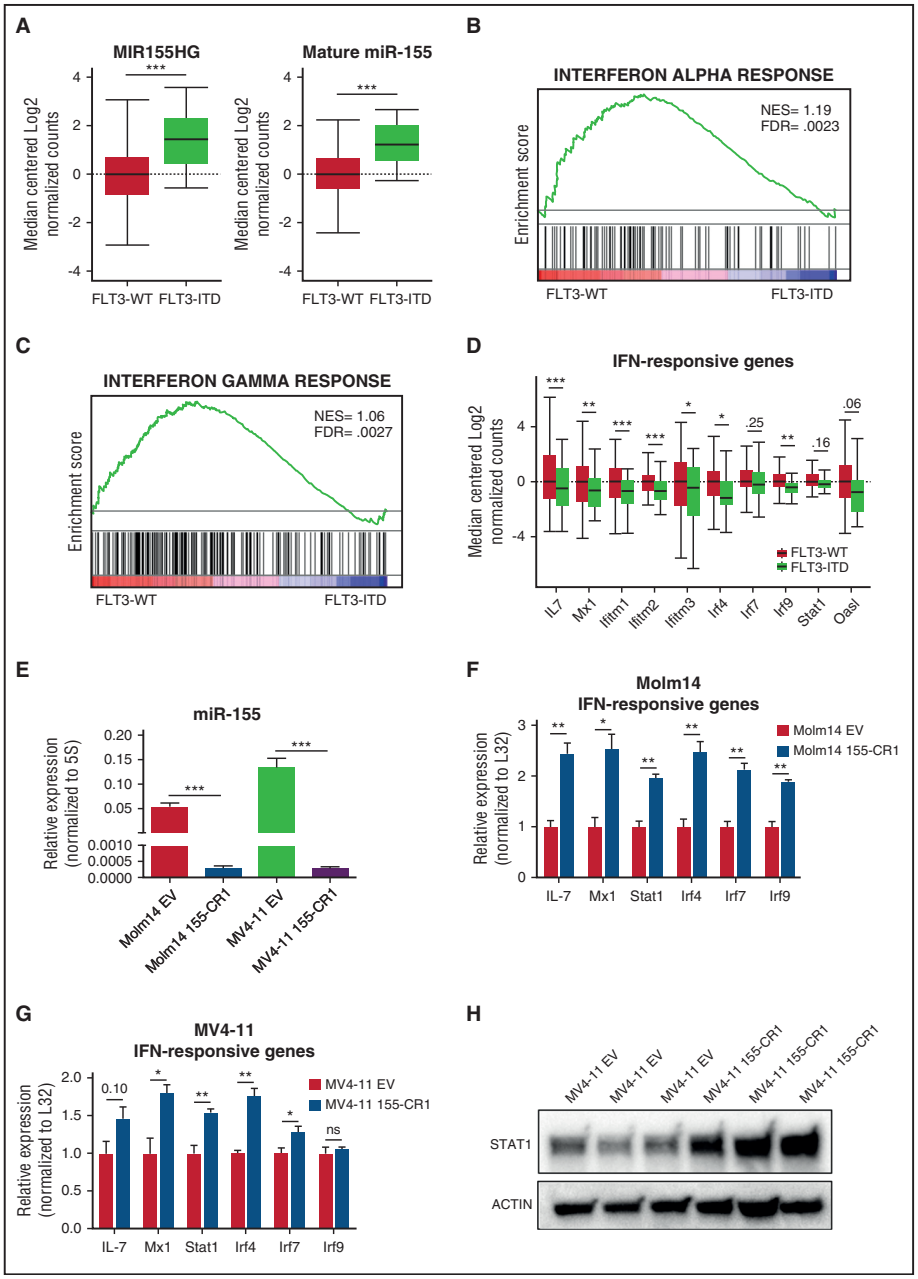

Figure 5.

compared with FLT3-ITD mice was attributed to decreased monocytosis and neutrophilia (Figure 1G). Although both FLT3-ITD and FLT3-ITD 155$^{-/-}$ mice were anemic, this phenotype was less severe when miR-155 was lacking. These findings demonstrate that deletion of miR-155 abrogates several aspects of the myeloproliferative phenotype observed in the spleen and blood of FLT3-ITD mice.

miR-155 drives myeloid cell production in the BM of FLT3-ITD mice by increasing granulocyte-monocyte progenitors

To determine the source of miR-155–dependent myeloproliferation in FLT3-ITD mice, we next analyzed the BM. FLT3-ITD BM cells displayed increased expression of miR-155 compared with BM from WT mice (Figure 2A), which is consistent with the clinical observation of elevated miR-155 levels in FLT3-ITD^{+} patient samples.[10,11] We also observed decreased BM cellularity in FLT3-ITD 155$^{-/-}$ compared with FLT3-ITD mice (Figure 2B). Flow cytometry revealed that this decrease in BM cellularity of FLT3-ITD 155$^{-/-}$ mice could largely be attributed to reduced CD11b^{+} Gr1^{+}, and CD11b^{+} Gr1^{-} cells (Figure 2C). In contrast, we did not observe a difference in the suppression of B220^{+} cells by FLT3-ITD when miR-155 was lacking. However, we did observe a slight decrease in Ter119^{+} cells in the BM of FLT3-ITD 155$^{-/-}$ mice compared with FLT3-ITD mice, suggesting that miR-155 may play some role in supporting erythroid development in the context of FLT3-ITD.

Next, we examined the impact of miR-155 on the HSPC population during FLT3-ITD–mediated disease. Total BM was isolated from the femurs and tibias of FLT3-ITD and FLT3-ITD 155$^{-/-}$ mice, and the depicted flow cytometry gating strategy was used to assess distinct HSPC populations (Figure 2D). There was no difference in the total number of LKS cells (Figure 2E), a compartment consisting of the earliest stem and progenitor cell populations.[25] However, there was a significant decrease in myeloid progenitor cells (Lin^{-}, c-Kit^{+}, Sca1^{-}) in FLT3-ITD 155$^{-/-}$ compared with FLT3-ITD mice (Figure 2F). This finding indicates that miR-155 is necessary for maintaining a robust myeloid progenitor pool in this disease context. On further analysis of myeloid progenitor subsets, we found that miR-155 is required for the increase in granulocyte-monocyte progenitor cells mediated by FLT3-ITD (Figure 2G; supplemental Figure 1C). We also observed a subtle decrease in megakaryocyte-erythrocyte progenitor cells in FLT3-ITD 155$^{-/-}$ mice, again suggesting that miR-155 may also be supporting erythroid development to some degree in FLT3-ITD animals. Altogether, these findings reveal a role for miR-155 in the myeloid progenitor compartment of the BM, where miR-155 is required for proper myeloid expansion in response to FLT3-ITD signaling.

miR-155 promotes the proliferation of the LKS and myeloid progenitor compartments in FLT3-ITD mice

After finding that miR-155 is critical for the myeloid-specific expansion seen in FLT3-ITD BM, we next sought to determine how miR-155 regulates myeloid cell numbers in the BM by assessing proliferation and cell survival. Using a BrdU incorporation assay, we found that miR-155 promotes proliferation of both the LKS and myeloid progenitor cell populations (Figure 3A). However, on analyzing Annexin V levels, we did not see a miR-155–dependent difference in LKS and myeloid progenitor cells undergoing apoptosis when comparing FLT3-ITD and FLT3-ITD 155$^{-/-}$ mice (supplemental Figure 2A), suggesting that miR-155 plays a more specific role in promoting proliferation of these cells in this premalignant context. These data indicate that miR-155 enhances FLT3-ITD–mediated MPD by increasing the proliferation of LKS and myeloid progenitor cells in the hematopoietic compartment.

A hematopoietic cell–intrinsic role for miR-155 during FLT3-ITD–mediated MPD

To determine if miR-155 functions in a cell-intrinsic manner to promote FLT3-ITD–mediated MPD, we generated mice with BM chimeras and assessed different disease parameters. Irradiated WT recipient mice were reconstituted with equal cell numbers of WT (45.1^{+}) BM and FLT3-ITD or FLT3-ITD 155$^{-/-}$ (45.2^{+}) BM and were analyzed at 3 months postreconstitution (Figure 3B). Markedly increased splenomegaly was observed in the WT:FLT3-ITD compared with WT:FLT3-ITD 155$^{-/-}$ mice (Figure 3C), a finding that correlated with the expansion of FLT3-ITD 45.2^{+} cells, namely 45.2^{+} CD11b^{+} myeloid cells, compared with FLT3-ITD 155$^{-/-}$ cells (Figure 3D). WT:FLT3-ITD animals also had increased BM cellularity compared with their WT:FLT3-ITD 155$^{-/-}$ counterparts, with an increased number of 45.2^{+} white blood cells that included 45.2^{+} CD11b^{+} myeloid cells (Figure 3E). Both the FLT3-ITD and FLT3-ITD 155$^{-/-}$ 45.2^{+} cells dominated the BM engraftment at 3 months postreconstitution (supplemental Figure 2B-C), and we did not observe significant differences in the small number of remaining WT 45.1^{+} BM cells between groups (supplemental Figure 2D). These findings indicate that miR-155 plays a hematopoietic cell–intrinsic role as it promotes the expansion of FLT3-ITD^{+} myeloid cells.

miR-155 inhibits endogenous IFN signaling in LKS cells and myeloid progenitors from FLT3-ITD mice

To decipher the mechanism by which miR-155 promotes proliferation of the LKS and myeloid progenitor pool, we performed RNA sequencing of sorted cells from the LKS compartment of WT, FLT3-ITD, and FLT3-ITD 155$^{-/-}$ mice. Interestingly, although the gene expression profiles of FLT3-ITD and FLT3-ITD 155$^{-/-}$ samples clustered away from the WT group, they also exhibited distinct gene expression patterns (Figure 4A). We then performed Ingenuity Pathway Analysis and GSEA to determine which pathways were impacted according to the observed gene expression differences. Overwhelmingly, the results pointed to the IFN response as being highly upregulated in the absence of miR-155 (Figure 4B; supplemental Figure 3A-B). IFN-α– and IFN-γ–responsive genes, as determined by GSEA, made up a large proportion of genes that were significantly elevated in FLT3-ITD cells lacking miR-155

Figure 5. The TCGA data set of human AML samples and miR-155 mutant AML cell lines identify an inverse correlation between miR-155 levels and the IFN response in FLT3-ITD^{+} AML. (A) Box plot showing MIR155HG and mature miR-155 expression levels in FLT3-WT and FLT3-ITD AML samples from the TCGA data set. (B-C) GSEA of the TCGA data set identifies the IFN-α response and IFN-γ response as hallmark differences between FLT3-ITD AML and FLT3-WT AML. (D) Expression level of representative IFN-responsive genes from the TCGA data set in FLT3-WT and FLT3-ITD AML. (E) Relative expression of miR-155 in Molm14 and MV4-11 cells infected with EV or 155-CR1 vectors determined by qRT-PCR. Expression normalized to 5S. n = 3 biological replicates for each condition. (F-G) qRT-PCR analysis of representative IFN-responsive genes in Molm14 (F) or MV4-11 (G) cells infected with either EV or 155-CR1 vectors. Expression normalized to L32, with the EV-infected cell average set to a relative expression value of 1. n = 3 biological replicates for each condition. (H) Western blot of STAT1 in MV4-11 cells infected with either EV or 155-CR1 constructs. Replicates represent cells grown in independent wells for 72 hours. ACTIN serves as the loading control. LentiCRISPR data represent at least 2 independent experiments. Data represented as mean ± SEM. *$P \leq .05$; **$P \leq .01$; ***$P \leq .001$; ns, $P > .05$. FDR, false discovery rate; NES, normalized enrichment score.

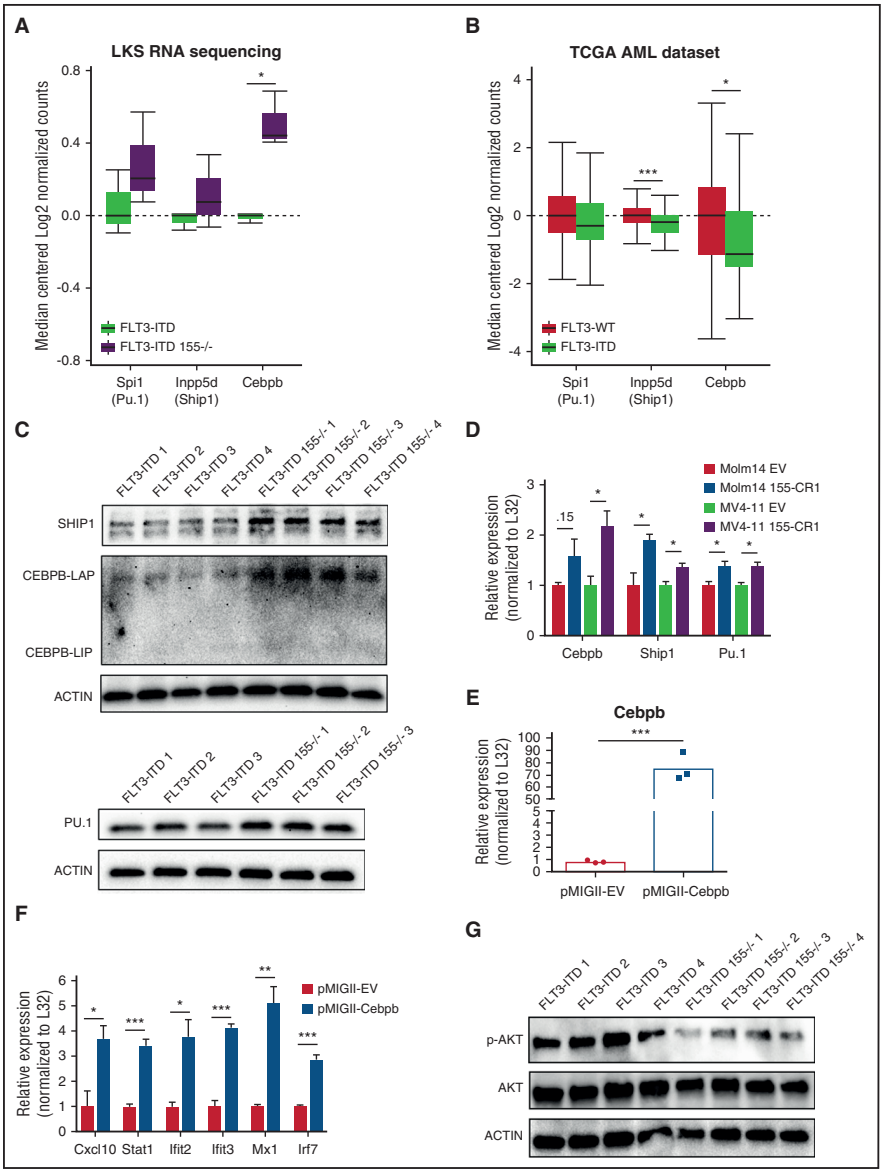

Figure 6. miR-155 represses multiple targets in mouse FLT3-ITD LK and human FLT3-ITD$^+$ AML cells, including the IFN gene regulator Cebpb. (A) Expression level of miR-155 targets in the LKS compartment of FLT3-ITD or FLT3-ITD 155$^{-/-}$ mice by RNA sequencing. (B) Expression level of miR-155 targets in the TCGA human AML data set. (C) Western blot of SHIP1, PU.1, and CEBPB in LK-sorted BM of FLT3-ITD or FLT3-ITD 155$^{-/-}$ mice; replicates represent individual mice. (D) qRT-PCR analysis of miR-155 targets Cebpb, Ship1, and Pu.1 in Molm14 and MV4-11 cells infected with either EV or 155-CR1 constructs. Expression normalized to L32, with the EV-infected cell average set to a relative expression value of 1. n = 3 biological replicates for each condition. (E-F) qRT-PCR analysis of FLT3-ITD LK cells infected with either the pMIGII-EV or pMIGII-Cebpb vector. Expression normalized to L32 with pMIGII-EV–infected cell average set to a relative expression value of 1. n = 3 biological replicates for each condition. (G) Western blot of phospho-AKT (p-AKT) and total AKT of LK cells from FLT3-ITD and FLT3-ITD 155$^{-/-}$ mice; replicates represent individual mice. Data represented as mean ± SEM. *$P \leq .05$; **$P \leq .01$; ***$P \leq .001$.

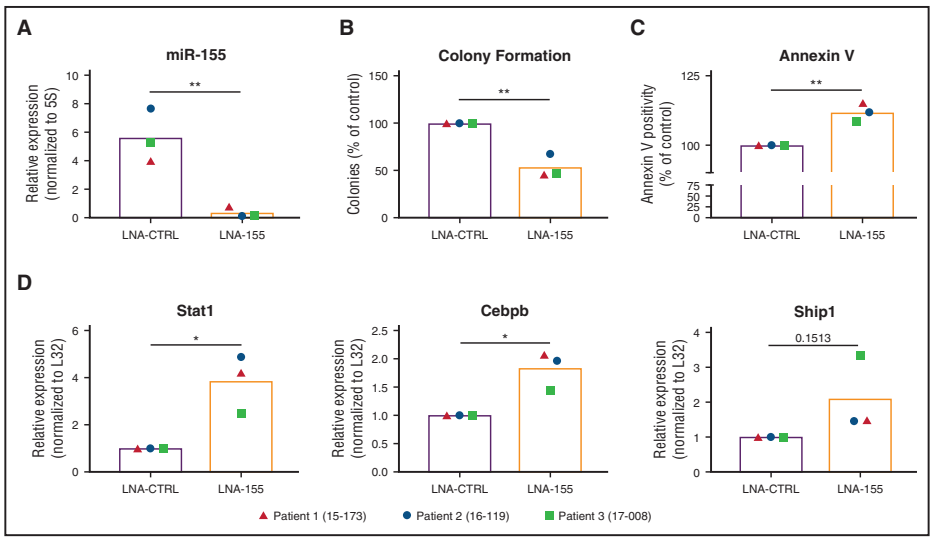

Figure 7. miR-155 promotes colony-forming potential and represses Cebpb and Stat1 expression in primary FLT3-ITD$^+$ AML cells. (A) miR-155 expression in primary FLT3-ITD$^+$ AML samples treated with a CTRL LNA or miR-155 LNA determined by qRT-PCR. Expression normalized to 5S ribosomal RNA. (B) Colony-forming potential of primary FLT3-ITD$^+$ AML samples grown in methylcellulose agar supplemented with 100 nM CTRL LNA or miR-155 LNA. (C) Annexin V staining of primary FLT3-ITD$^+$ AML samples treated with CTRL LNA or miR-155 LNA for 3 days in liquid culture. (D) qRT-PCR analysis of primary FLT3-ITD$^+$ AML samples treated with CTRL LNA or miR-155 LNA. Expression normalized to L32, with LNA-CTRL treated cell average set to a relative expression of 1. Each point represents an individual patient sample. *$P \leq .05$; **$P \leq .01$.

(Figure 4C; supplemental Figure 3C-D). Interestingly, although we found that the IFN response was moderately elevated in FLT3-ITD compared with WT LKS cells (supplemental Figure 4A), it was further increased in the FLT3-ITD 155$^{-/-}$ group, suggesting that the absence of miR-155 impairs the ability of FLT3-ITD to suppress the antiproliferative effect of BM regulatory cytokines. We further validated these RNA sequencing data using qRT-PCR to assay the expression levels of IFN-responsive genes in the LKS (Figure 4D) and myeloid progenitor compartments (Figure 4E), where we found the same overall trend in increased expression of IFN-responsive genes in the absence of miR-155, but only in the context of FLT3-ITD (supplemental Figure 4B-C). Lastly, we evaluated protein levels of STAT1, a master regulator of IFN responses, by western blotting and observed a large increase in STAT1 expression in FLT3-ITD LK cells deficient in miR-155 (Figure 4F). Altogether, these data support a model whereby miR-155 promotes proliferation of the LKS and myeloid progenitor pool in FLT3-ITD–mediated neoplasms by reducing the antiproliferative effects of IFN signaling in these cells.

Analysis of the TCGA human AML data set identifies increased miR-155 and decreased IFN signaling in FLT3-ITD$^+$ AML

To extend our findings from an animal model of FLT3-ITD–mediated disease into the clinical arena, we next analyzed sequencing results from human AML samples deposited in TCGA.[26] We sorted these 173 samples according to FLT3 status, placing patients with duplications in the FLT3 juxtamembrane domain in the FLT3-ITD group and patients without mutations in FLT3 in the FLT3-WT group. We found that MIR155HG, the miR-155 host gene, and mature miR-155 were significantly increased in FLT3-ITD$^+$ AML samples compared with FLT3-WT AML samples (Figure 5A), which is consistent with previous reports.[10-13] Next, we further analyzed these gene expression data from the categorized FLT3-WT and FLT3-ITD groups using GSEA and demonstrated that both the IFN-α and IFN-γ responses were significantly downregulated in FLT3-ITD$^+$ AML compared with FLT3-WT AML (Figure 5B-C), revealing an inverse correlation with miR-155 levels in FLT3-ITD$^+$ AML. This reduction can also be appreciated by examining the expression of representative IFN-responsive genes between the 2 groups (Figure 5D).

To further examine the connection between miR-155 and the IFN response, we deleted miR-155 in FLT3-ITD$^+$ human AML cell lines (MV4-11 and Molm14). Cells were transduced by using a previously developed miR-155 targeting lentiviral CRISPR/Cas9 construct (155-CR1),[19] and we subsequently observed significant miR-155 knockdown in both Molm14 and MV4-11 cell lines (Figure 5E). Reduced levels of miR-155 in Molm14 and MV4-11 cells resulted in significantly increased IFN signaling compared with cells infected with EV control (Figure 5F-G). In addition, we observed increased STAT1 protein levels in MV4-11 155-CR1 cells (Figure 5H). Together, these findings strongly correlate with data in our FLT3-ITD mouse model.

Multiple direct miR-155 targets are increased in FLT3-ITD$^+$ myeloid cells in the absence of miR-155, including the IFN regulator Cebpb

To determine which of the direct targets of miR-155 are involved in its regulation of FLT3-ITD–mediated disease, we first analyzed the expression of established miR-155 targets in our LKS sequencing data

set. Ship1, Pu.1, and Cebpb have all previously been shown by us and others to be directly targeted by miR-155[17,27] and may play important miR-155–dependent roles in FLT3-ITD pathogenesis.[18,28,29] We found increased expression of these targets in FLT3-ITD 155$^{-/-}$ compared with FLT3-ITD mice, with a statistically significant increase in Cebpb (Figure 6A). In the human AML TCGA data set, we found that expression of Spi1 (Pu.1), Inpp5d (Ship1), and Cebpb was lower in FLT3-ITD^{+} AML, with Ship1 and Cebpb reaching statistical significance (Figure 6B). Expression of these genes also inversely correlates with the higher expression of miR-155 (supplemental Figure 5), suggesting that they could be functional miR-155 targets in this disease setting. Next, we sorted LK cells from FLT3-ITD and FLT3-ITD 155$^{-/-}$ mice and evaluated protein levels of these miR-155 targets via western blot. Elevated expression of all 3 of these proteins was observed in FLT3-ITD 155$^{-/-}$ vs FLT3-ITD mice (Figure 6C), which is consistent with each of these being directly repressed by miR-155 in vivo. We also observed higher transcript levels of these targets in our 155-CR1–infected human FLT3-ITD^{+} cell lines (Figure 6D).

The miR-155 target Cebpb has previously been implicated in the regulation of IFN signaling.[30-32] To determine if elevated Cebpb levels could increase the IFN response, we overexpressed Cebpb in sorted LK cells (Figure 6E) and observed increased expression of IFN-responsive genes (Figure 6F). This was also true in Cebpb-overexpressing RAW264.7 cells, a murine myeloid cell line, treated with recombinant type I and type II IFN (supplemental Figure 6). This finding demonstrates that Cebpb is sufficient to induce the IFN response in myeloid cells and supports a model whereby miR-155 repression of Cebpb is involved in its ability to repress the IFN response in myeloid cells.

We also examined the downstream effect of miR-155's repression of Ship1, a known inhibitor of AKT activation.[33] Consistent with elevated Ship1 levels in FLT3-ITD 155$^{-/-}$ cells, we observed decreased AKT phosphorylation in the LK cells of FLT3-ITD 155$^{-/-}$ mice compared with FLT3-ITD mice (Figure 6G). Taken together, this finding indicates that miR-155 works through a multitarget mechanism that enables regulation of multiple relevant signaling pathways and responses that define FLT3-ITD myeloid cell biology.

Inhibition of miR-155 in primary FLT3-ITD^{+} AML samples leads to decreased colony formation and increased Stat1 expression

To assess the functional role of miR-155–mediated regulation of the IFN response in primary FLT3-ITD^{+} AML, we treated primary AML samples (n = 3, supplemental Table 1) with an LNA antisense oligonucleotide targeting miR-155 (LNA-155) or a scrambled control LNA oligonucleotide (LNA-CTRL) and assessed these cells for changes in the expression of Stat1, as well as colony formation in methylcellulose medium and Annexin V positivity. qRT-PCR confirmed miR-155 inhibition by LNA-155 at 100 nM (Figure 7A), and this correlated with a reduction of colony formation (Figure 7B) and an increase in apoptosis (Figure 7C). Consistent with data in our mouse model, miR-155 inhibition in primary FLT3-ITD^{+} AML samples correlated with increased expression of Stat1, Cebpb, and Ship1 (Figure 7D). These results provide further evidence that our findings in mice extend to primary human FLT3-ITD^{+} AML cells.

Discussion

miRNA expression is highly dysregulated in FLT3-ITD^{+} AML, a subtype of AML that confers a poor prognosis. Microarray and qRT-PCR–based methods have shown that miR-155 is among the most highly overexpressed miRNAs in FLT3-ITD^{+} AML.[10-16] We further substantiated these findings by analyzing the TCGA human AML data set, which contains sequencing data from 173 patients representing all major subtypes of AML. Our retrospective analysis confirmed that miR-155 was the most significantly upregulated miRNA in FLT3-ITD^{+} AML compared with FLT3-WT AML.

From a functional perspective, miR-155 has been shown by our group and others to be important for the growth of FLT3-ITD^{+} cells in vitro.[18,19] We recently identified miR-155 as a top growth-promoting miRNA in a FLT3-ITD^{+} leukemic cell line via a genome-wide CRISPR/Cas9 screen.[19] Importantly, both FLT3-ITD and miR-155 overexpression are each sufficient to cause MPDs in mice.[7-9,17] However, the impact of endogenous miR-155 on FLT3-ITD–mediated disease in vivo had not been previously explored. In the current study, we crossed FLT3-ITD and miR-155$^{-/-}$ mice to determine if these 2 molecules collaborate to induce MPD. Indeed, we found that miR-155 is critical for several aspects of disease development in FLT3-ITD mice, including myeloid expansion in the BM, spleen, and peripheral blood. Further analysis of the hematopoietic compartment revealed that miR-155 promotes disease in FLT3-ITD mice by increasing proliferation of the LKS and myeloid progenitor compartments.

Through RNA sequencing of the LKS compartment, we identified the IFN response as being substantially increased in FLT3-ITD 155$^{-/-}$ compared with FLT3-ITD mice. We also demonstrated that human FLT3-ITD^{+} AML samples had increased miR-155 and a decreased IFN gene expression signature when compared with FLT3-WT AML, which is consistent with our mouse data. It is well established that IFN signaling has growth-inhibitory effects on the hematopoietic compartment,[20-23] and inhibition of IFN signaling was recently discovered as a novel mechanism by which FLT3-ITD^{+} cells can avoid the antiproliferative effects of IFN-α and IFN-γ.[24] Our results indicate that miR-155 is clearly involved in this mechanism that subverts the antiproliferative effects of IFN in this setting. Our findings are also in line with a recent study demonstrating that miR-155 promotes proliferation of CD8^{+} T cells through inhibition of IFN signaling,[34] indicating that this mechanism is used by multiple cell types in vivo. Taken together, this work provides novel insights into the role of miR-155 in FLT3-ITD–mediated disease, where it helps HSPCs to escape the growth-inhibitory effects that are typically conferred by regulatory BM cytokines.

A number of studies have found that miR-155 can repress the expression of selected relevant targets, such as Ship1, Cebpb, and Pu.1, within FLT3-ITD^{+} AML cell lines.[18,28,29] Furthermore, we found that both Ship1 and Cebpb were decreased at the RNA level in FLT3-ITD vs FLT3-WT AML patient samples. To better understand our in vivo phenotype, we examined the regulation of these targets by miR-155 in sorted LKS and myeloid progenitors and found that these targets are indeed derepressed in early stem cell compartments genetically deficient for miR-155. Of note, Pu.1 and Cebpb are well-known myeloid commitment genes,[35] and reduction in either Ship1 or Pu.1 levels can give rise to myeloproliferation,[27,36] which further supports the functional relevance of their regulation by miR-155 in this context. In the case of Cebpb, it has been previously shown to promote the IFN response,[30-32] providing a connection between miR-155 and the repression of IFN-responsive genes. Indeed, we found that Cebpb could enhance the IFN response in murine myeloid cells, including LK cells. We also showed that AKT was less activated in the LK cells of FLT3-ITD 155$^{-/-}$ mice, likely due to increased levels of the miR-155 target and known AKT inhibitor, Ship1. Taken together, our data indicate that miR-155 acts on multiple targets, suggesting a complex mechanism of action that could involve both IFN signaling–dependent and –independent mechanisms. Sorting out the individual contributions of these targets to

the IFN response and/or additional mechanisms at play in this context will be a fascinating future area of study.

Our current results, coupled with other clinical observations that miR-155 upregulation correlates with the FLT3-ITD$^+$ subtype of AML that confers a negative prognosis, provide a physiologically relevant context where miR-155 plays a deleterious role in the myeloid compartment in vivo. We also demonstrate a functional role for miR-155 in FLT3-ITD$^+$ AML, because inhibition of miR-155 reduced survival and increased apoptosis in primary FLT3-ITD$^+$ AML samples, and this correlated with increased expression of Stat1, Cebpb, and Ship1. Taken as a whole, the current study provides strong evidence that miR-155 promotes FLT3-ITD–mediated MPD, at least in part, through its regulation of IFN signaling in the early hematopoietic compartment and argues that therapeutic targeting of miR-155 in FLT3-ITD$^+$ AML may warrant serious consideration.

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Authorship

Contribution: J.A.W. and R.M.O. designed the study; J.A.W., D.A.K., A.M.E., C.N.K., R.H., M.C.R., M.A., T.B.H., S.-H.L., and W.P.V. performed the experiments; T.L.M. performed the bioinformatics analysis; D.S.R. and R.R.M. performed the histopathology; J.A.W., D.A.K., J.L.R., and R.M.O. performed the data analysis; M.W.D. and A.B.P. provided patient samples; and J.A.W. and R.M.O. wrote the manuscript.

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CHAPTER 5

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Since the discovery of miRNAs as a novel class of short noncoding RNAs nearly two decades ago (1), innumerable studies by researchers across the globe have established miRNAs as playing an integral role in hematopoietic development and function. Perhaps it comes as no surprise that miRNA dysregulation in various hematopoietic lineages can have deleterious effects, including contributing to the development and progression of hematologic malignancies (2,3). It is now recognized that miRNAs can act as either oncogenes or tumor suppressors in a number of blood cancers, including AML (4).

AML is a deadly hematologic malignancy that leads to over 10,000 deaths each year in the United States alone. One subtype of AML with a particularly poor prognosis is AML containing gain-of-function mutations in the FLT3 receptor, known as FLT3-ITD mutations (5). A number of groups have previously shown that the miRNA expression profile is highly dysregulated in FLT3-ITD+ AML compared to FLT3-WT AML (6,7). However, which of these dysregulated miRNAs was functionally impacting disease progression remained unclear.

CRISPR-Cas9 screening to identify functionally relevant miRNAs in FLT3-ITD+ AML

To address this question, we utilized a genome-wide CRISPR-Cas9 screen to functionally assess which miRNAs were regulating cell growth of FLT3-ITD+ AML cells in vitro. First described by Shalem et al. in 2014 (8), the CRISPR-Cas9 library used in this study contained four unique guide sequences for every human miRNA gene, and three unique guide sequences to each protein-coding gene. The design of this library

allowed us to functionally screen both miRNAs and their putative mRNA targets simultaneously. Previous screening techniques did not afford researchers the ability to identify functional miRNA-mRNA target pairs in one high-throughput process, making our study one of the first of its kind.

Through the use of this innovative screening technology, we were able to identify miRNAs that both promoted and prevented the growth of a FLT3-ITD+ AML cell line in vitro (9), explained at length in Chapter 3. miR-150 was identified as our top candidate promoting the growth of these cells. We also analyzed the impact of miR-150's predicted mRNA targets on cellular growth, which led to the discovery of the tumor suppressor p53 as a novel target of miR-150 in this context. This miRNA-mRNA interaction has been identified in other types of cancer (10,11), but never before in AML. The relevance of miR-150 in clinical FLT3-ITD+ AML remains to be determined, but our study suggests that inhibition of miR-150 could have potent effects on the growth of cells containing this deadly mutation.

Beyond the individual miRNA candidates identified in this CRISPR-Cas9 screen, we made other interesting observations that could have broad impacts on future miRNA research. One of the most surprising results from this dataset was that levels of miRNA expression did not necessarily correlate with the functional significance elicited by the CRISPR-mediated knockdown of any given miRNA. For instance, we found that miR-155 was expressed at almost 10x greater level than miR-150 in our cell line of choice. However, knockdown of miR-150 had a slightly greater impact on the growth of these cells compared to miR-155. This result is most likely explained by the expression level and functional significance of the miRNA's target mRNAs. This finding suggests that

only studying miRNAs based on level of expression may not always be appropriate, and that other factors, such as predicted targets, should also be considered.

Our screen also supports a growing body of literature suggesting that miRNAs exert their effects through repression of multiple targets (12). While examining the effect of a particular miRNA on cell growth in this system, we found that knocking down many predicted targets had the opposite effect on growth, thus suggesting that a given miRNA has a number of putative targets that could potentiate its observed effect on growth. Although we did not individually validate these observations, we believe that this CRISPR-Cas9 screening system would allow for quick identification of functionally relevant miRNA targets, providing a more efficient system to study the downstream effects of miRNA dysregulation.

While this study provided evidence for the effectiveness of genome-wide CRISPR-cas9 screening to identify functionally relevant miRNAs in a given system, further optimization and more robust screens are needed to demonstrate the power of this approach. Future studies could incorporate multiple cell lines, or potentially even primary samples, to identify miRNAs regulating any number of AML cellular phenotypes, including proliferation, cell death, response to treatment, or drug resistance. Indeed, these efforts are already underway. Since the publication of our work in 2015, other groups have utilized similar methods to screen for genes that could affect the development of drug resistance in AML, including FLT3-ITD+ AML (13,14). While these studies did not specifically analyze noncoding RNAs, the same techniques could easily be applied to miRNA research.

miR-155 promotes myeloid expansion in a mouse model of FLT3-ITD-induced MPD

Our CRISPR-Cas9 screen also identified miR-155 as a top candidate promoting FLT3-ITD+ AML cell growth (9). We verified this result via knockdown of miR-155 with two independent CRISPR-Cas9 lentiviral constructs, confirming that inhibiting miR-155 expression could decrease the expansion of FLT3-ITD+ AML cells in vitro. Other groups have produced similar findings through alternate means of miR-155 knockdown (15,16). These findings correlate nicely with clinical data showing that miR-155 is highly overexpressed specifically in FLT3-ITD AML (6,15-17). However, whether miR-155 played a role in FLT3-ITD-driven myeloid malignancy in vivo and the downstream effects of miR-155 overexpression in the context of FLT3-ITD mutations was still undetermined.

We crossed mice homozygous for the FLT3-ITD mutation with miR-155 knockout mice to determine the specific role of miR-155 in the context of FLT3-ITD. FLT3-ITD miR-155-/- mice exhibited decreased myeloid expansion in the bone marrow, reduced splenomegaly, and decreased peripheral blood monocytosis and neutrophilia compared to their FLT3-ITD miR-155+/+ counterparts, thus indicating that miR-155 was crucial in promoting FLT3-ITD-mediated myeloproliferation (18). When examining the stem cell compartment, we found that miR-155 deficient animals had fewer myeloid progenitors than FLT3-ITD miR-155+/+ mice. This phenotype was attributed to miR-155's role in promoting proliferation of the HSPC and myeloid progenitor cell compartments in the bone marrow. These findings confirmed that miR-155 was promoting FLT3-ITD-mediated disease in vivo.

To better understand the downstream effects of miR-155 overexpression in the context of FLT3-ITD, we performed RNA sequencing on the HSPC population in FLT3-ITD 155+/+ mice and FLT3-ITD 155-/- mice. These data revealed that mice lacking miR-155 had an increased response to interferon, known to have a growth-suppressive effect on hematopoietic cells (19,20). These findings corresponded with human AML data from the The Cancer Genome Atlas database, where we found that FLT3-ITD+ AML samples, which have increased miR-155 expression, had a decreased interferon signature compared to FLT3-WT AML samples. We also found that Stat1, the master activator of interferon responses, increased when knocking down miR-155 levels in both FLT3-ITD+ AML cell lines and primary patient samples, further confirming this result.

This finding that miR-155 represses the interferon response in the context of FLT3-ITD was initially surprising. Inflammatory signals initiate miR-155 signaling under a normal physiologic conditions (21). miR-155 then represses anti-inflammatory targets that inhibit cellular proliferation and survival (22,23). FLT3-ITD aberrantly activates a number of the same inflammatory pathways that drive miR-155 expression. This suggests that FLT3-ITD could be coopting these inflammatory pathways in a cell-intrinsic manner to drive increased miR-155 expression, thus leading to increased proliferation and survival of these leukemic cells. This proinflammatory role for miR-155 in most immunological contexts is at odds with our finding that miR-155 inhibits a well-known inflammatory pathway, interferon signaling. However, other groups have also found miR-155 repressing interferon signaling in cells of hematopoietic origin (24,25), further confirming our observation. We attributed miR-155's repression of mRNA target Cebpb, a transcription factor known to activate interferon signaling (26,27), as being responsible

for this decreased interferon signature in FLT3-ITD+ AML cells. However, there is also the possibility the miR-155 could be repressing Stat1 directly through noncanonical targeting, as the Stat1 3'UTR was found to be loaded into the RISC complex in a miR-155-depending manner (28). Nonetheless, this is an interesting result that may provide a link between dysregulated inflammatory pathways and cancer.

A number of putative miR-155 targets in the HSPCs of FLT3-ITD mice were upregulated in the absence of miR-155, including Pu.1, Ship1, and Cebpb. Pu.1 and Cebpb are known regulators of myeloid development (29), and as mentioned above, we linked Cebpb to miR-155's repression of interferon responses. Ship1 is a known phosphatase that downregulates AKT activation and whose loss leads to a MPD in mice (30). Indeed, we found that Ship1 inhibition by miR-155 led to increased AKT activation, and thus increased proliferation and survival of myeloid progenitor cells. These findings suggest a multitarget effect of increased miR-155 expression in the context of FLT3-ITD.

miR-155 as a novel therapeutic target in FLT3-ITD+ AML

These findings suggest that miR-155 inhibition in FLT3-ITD+ AML could warrant consideration as a novel therapeutic approach. To this end, we treated primary FLT3-ITD+ AML samples with miR-155 inhibitors and observed increased apoptosis and decreased colony forming potential. This corresponded with our findings that CRISPR-mediated knockdown of miR-155 in FLT3-ITD+ AML cell lines decreased cellular growth, which was confirmed independently by Gerloff et al. (15). This begs the question if miR-155 inhibition could be used as combination therapy for FLT3-ITD+ AML. The most current targeted therapy for treatment of this deadly disease uses FLT3-

ITD-specific tyrosine kinase inhibitors (TKIs). One such inhibitor, Midostaurin, passed clinical trials within the last 12 months (31), and another, Quizartinib, may be shortly behind (32).

One consideration when proposing miR-155 therapy in combination with a FLT3-specific TKI is that these two drugs may be targeting the same pathway. Two independent groups have found that inhibition of FLT3 or downstream activators Stat5 and NFkB will decrease miR-155 expression (15,16); however, others claim that increased miR-155 expression occurs independently of FLT3-ITD (33). The clinical expression data and data from our FLT3-ITD mouse model suggest that FLT3-ITD must be driving increased miR-155 expression. This would suggest that FLT3-ITD inhibition alone would lower miR-155 expression, potentially decreasing the efficacy of miR-155 inhibitors as therapy. However, this may also mean that a combination therapy may decrease the risk of developing drug resistance during FLT3-ITD+ AML treatment, which is a serious concern in managing this disease (34). Adding miR-155 inhibition in combination with FLT3-specific TKIs will be of great interest and is certainly a future direction of this project.

The role of miR-155 in FLT3-ITD-driven leukemogenesis

Our studies demonstrate an interesting relationship between miR-155 and FLT3-ITD mutations, where FLT3-ITD drives miR-155 expression to increase myeloproliferation. Although FLT3-ITD has been associated with human AML and is considered a poor prognostic factor, the mutation itself has not been shown to independently drive leukemic transformation, but rather leads to an MPD similar to a

chronic myelomonocytic leukemia (35,36). Instead, FLT3-ITD is thought to be one of "multiple hits" and must collaborate with other oncogenic mutations in order to cause hematopoietic malignancy. However, these additional hits are not well understood and are just now being identified. An interesting question to explore will be if a mutation that increases miR-155 expression could collaborate with FLT3-ITD to cause leukemic transformation. Experiments investigating this hypothesis are currently ongoing.

There is some evidence of other mutations common in AML causing leukemic transformation when combined with FLT3-ITD in mouse models, including mutations in Tet2, Dnmt3a, and Npm1 (37-39). Interestingly, when analyzing miR-155 expression across all the common mutations found in AML using the TCGA database, we found that miR-155 expression was significantly increased in FLT3-ITD and NPM1 mutations (NPMc). A future direction in our lab is to generate a mouse model of FLT3-ITD+ NPMc+ AML and determine whether miR-155 is necessary for the development of AML. Other groups have examined the necessity of miR-155 for leukemic transformation in other mutational models of disease, including Hoxa9/Meis1-driven and MLL-rearranged AML (40,41). These groups found that miR-155 was not required for leukemogenesis, but did find that the leukemia was less severe in the absence of miR-155. This suggests that miR-155 was not a driver of AML in their model, but was certainly acting as a disease modifier. However, miR-155 expression in these models was not as dramatically increased as in FLT3-ITD+ NPMc+ AML, thus suggesting it could play a more significant role and perhaps act as a driver in this scenario. As highlighted in Chapter 2, varying levels of miR-155 expression in a number of AML models appears to have drastically different effects on the disease. Increased miR-155 expression in our

proposed model may be indispensable for AML transformation, or at the very least, the absence of miR-155 may delay leukemogenesis.

Concluding remarks

In conclusion, the findings described above and the work performed independently by other groups have now shown a clear role for miR-155 in promoting disease severity in FLT3-ITD+ AML. Future work by our lab will aim to further understand whether increased miR-155 expression is necessary for FLT3-ITD-driven leukemic transformation, or whether miR-155 is more of a disease modifier in FLT3-ITD+ AML. Regardless of the answer to this question, it is clear that further studies are clearly needed to investigate the therapeutic efficacy of miR-155 inhibition in this disease. Indeed, efforts by our group and others are being made in this area (42).

Beyond studying miR-155 in FLT3-ITD+ AML, the results of our CRISPR-Cas9 screen suggests that there could be a number of other miRNAs that regulate AML cell growth. This result is supported by over a decade of research showing that a variety of miRNAs clearly regulate nearly every aspect of the AML disease process, as highlighted in Chapter 2. Any one of these miRNAs could potentially be targeted for therapeutic benefit. Broadening this screening approach to include a number of different AML cell lines or applying it to primary patient samples may help identify miRNAs that regulate AML growth across a landscape of driver mutations, which could hold tremendous promise therapeutically. CRISPR-Cas9 screening could also be used to examine other aspects relevant to AML treatment, including identification of miRNAs that could be therapeutically targetable in combination with known AML chemotherapeutic agents, or

that could decrease the ability of AML cells to acquire drug resistance. Whether miRNA inhibition becomes a legitimate option for the treatment of AML may ultimately come down to improved delivery methods for miRNA-based therapeutics. Nonetheless, the findings described in this dissertation provide hope for a novel therapeutic approach targeting miRNAs in AML, a disease whose treatment often feels hopeless in the clinic.

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