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miR-590-5p, *miR-219-5p*, *miR-15b and miR-628-5p* are commonly regulated by IL-3, GM-CSF and G-CSF in acute myeloid leukemia

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Abstract

Aberrations in IL-3, GM-CSF and G-CSF induced signaling are frequently reported in acute myeloid leukemia (AML). Herein, we utilized a unique human myeloid leukemic cell line, AML-193, which responds to all three cytokines to analyze the regulation at microRNA level. Using real-time PCR-based miRNA expression profiling, we investigated miRNA signatures regulated by IL-3, GM-CSF and G-CSF for n=704 miRNAs. We discovered that in addition to regulating specific miRNAs, these cytokines also regulate common set of miRNAs, which includes *miR-590-5p*, *miR-219-5p*, *miR-15b* and *miR-628-5p*. Taken together, we have identified novel candidate miRNAs that may be instructive during leukemic and normal hematopoiesis.

Keywords

AML-193; AML; miRNA; IL-3; GM-CSF; G-CSF

Introduction

Hematopoiesis is mainly regulated by signaling cascades that are activated by a variety of cytokines [1]. IL-3, GM-CSF and G-CSF are predominant regulators for growth and differentiation of myeloid progenitors [2–4]. Interestingly, they all signal via a common JAK2-STAT5 pathway in myeloid progenitor compartments [5–8]. However, the mechanism through which STAT5 responds differentially to early-acting and lineage restricted cytokines, particularly in leukemic and stem/progenitor cells, is unknown. Previous studies investigated STAT5 response/activity to a mixture of factors, precluding analysis of pathways initiated by specific cytokines.

AML-193 is one of eight cell lines established from 50 patients with childhood acute leukemia [9,10]. Of those 8 lines, AML-193, which was derived from a 13-year-old female

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Authorship Contributions

All authors contributed to bench investigations and paper construction; A.F. performed miRNA profiling and analyses; P.S designed and directed all investigations.

Conflict of Interest

The Authors declare no conflict of interest related to this work.

with AML, was the only myeloid leukemic cell line that required conditioned media (from 5637 cell line) to grow and proliferate. Subsequently the active factors were identified as GM-CSF, G-CSF and Hematopoietin-I, which later came to be known as IL-1[9]. IL-3, a cytokine that supports the growth of hematopoietic stem cells, is also known to support the growth of AML-193 cells[11]. In addition, response to IL-3 confirms the expression of functional CD123 (alpha subunit of IL-3 receptor), which is known to be strongly expressed on leukemic stem cells (LSCs) and leukemic blasts [12]. Furthermore, AML-193 cells co-express both surface CD135 (FLT3 receptor) and FLT3 ligand [13], a well-established marker for leukemic and hematopoietic stem cells [14–16].

MicroRNAs (miRNAs), a class of naturally occurring noncoding RNAs of 17 to 30 nucleotides, are known to regulate gene expression by inducing translational inhibition and cleavage of their target mRNAs [17]. miRNAs play several crucial roles during hematopoiesis that include lineage decisions, stem cell progenitor transitions, niche control and other cell functions [18–20].

Recent investigations have linked miRNA expression with leukemia. The first miR that was discovered to have a role in myelopoiesis was miR-223, which was regulated by CEBPa transcription factor and was shown to target MEF2c transcription factor, a known promoter of myeloid progenitor differentiation [21]. Recently, miR-223 has been reported to target cell cycle regulator E2F1 and regulate cell cycle progression and myeloid proliferation [22]. Interestingly, trancriptional repressor GFI1, required for normal granulopoiesis, has been shown to negatively regulate the expression of miR-21 and miR-196b. Retinoic acid (RA/ATRA) induces the expression of *miR-15a/−16-1* and several *let-7* family members which target oncogenes BCL2 and RAS; and regulate RA induced differentiation in acute promyelocytic leukemia (APL) cells [23,24]. Baltimore and his colleagues showed that overexpression of human *miR-125b* caused a dose dependent myeloproliferative disorder that progressed to lethal myeloid leukemia in mice and also enhanced hematopoietic engraftment [18], which again reveals their potential to regulate hematopoietic output. A cluster of two miRNAs, *miR-15a* and *miR-16-1*, located at 13q14 was discovered to be deleted or down-regulated in approximately 60% of chronic lymphocytic leukemia (CLL) samples [25]. Furthermore, a recent study evaluated miRNA expression in 122 untreated adult acute myeloid leukemia (AML) cases using a microarray platform. AML patients with high expression of *miR-191* and *miR-199a* had significantly worse overall and event-free survival than AML patients with low expression [26]. Taken together, miRNAs can either act as tumor suppressors or as oncogenes and their altered levels can have functional relevance in leukemogenesis. Either single miRNA or signatures have demonstrated prognostic utility and complements the diagnostic information derived from cytogenetics, gene mutations and altered gene expressions.

Despite increasing evidence demonstrating a critical role for JAK2-STAT5 in leukemic and normal hematopoiesis, the distinct nature of signaling response regulated by early-acting (IL-3) and lineage-restricted cytokines (GM-CSF and G-CSF) is largely unresolved. We hypothesized that a unique response of leukemic myeloid progenitors to IL-3, GM-CSF, and G-CSF are possibly mediated in part by distinct regulation at the miRNA level. Therefore by utilizing a unique leukemic myeloid cell line, AML-193, that responds to both early and late acting cytokines, we attempted to profile IL-3, GM-CSF and G-CSF regulated miRNA signatures in leukemic myeloid progenitors and identify novel candidate miRNAs that may be instructive during leukemic and normal hematopoiesis.

Materials and methods

Cell culture

AML-193 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with -CSF, 5ng/mL IL-3, or 5ng/mL G-CSF.

RT-qPCR for STAT5 response factors

In analyses of bona fide STAT5 response factors, AML-193 cells were cultured for 16 hours in IMDM starvation media and were then exposed to either IL-3 (5ng/mL), GM-CSF (10ng/mL), or G-CSF (5ng/mL) for 90 minutes. RNA was extracted using an RNeasy Mini Kit (Qiagen) and then reverse transcription (QScript cDNA Synthesis Kit, cat # 95047-100, Quanta) and real-time quantitative polymerase chain reactions (iQ SYBR Green, i-Cycler, Bio-Rad) were performed. qPCR primer pairs were obtained for *Pim1* (NM_002648), *Cish* (NM_145071) and *Gapdh* (NM_002046) from SA Biosciences.

microRNA profiling and analyses

Post IL-3/GM-CSF/G-CSF treatment of AML-193 cells, miRNA was isolated using miRNeasy Mini Kit and RNeasy MinElute Cleanup Kit (cat # 217004 and 74204, Qiagen) to obtain high quality qPCR grade total RNA and microRNA. 400 ng of enriched miRNA per sample was used to perform reverse transcription to generate cDNAs (RT miRNA First Strand Kit, cat # 331401, SA Biosciences). The complete Human V2.0 miRNA Genome Array representing a total of n=704 miRNAs in 96-well format (MAH-100 and -200, SA Biosciences) was used to perform profiling. Four housekeeping small nuclear RNA (SNORD 48, 47, 44 and RNU6) were used for normalization. qPCR was performed using 2X SYBR Green master mix (SYBR Green/Fluorescein, cat # 330513, SA Biosciences) on an IQ5 cycler (BioRad). The raw Ct values obtained were loaded onto an online miRNA PCR Array Data Analysis web portal system, http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php, developed by SA Biosciences to retrieve fold change analysis using delta delta Ct method. For filtering criteria, greater than (>) 35 cycle threshold (Ct) scores were considered to be nonspecific and miRNAs that showed a raw Ct score > 35 were excluded from the final data analysis. Fold change filters were applied to select the miRNAs that were regulated more than 2-fold.

Results and Discussion

The process of hematopoiesis that involves HSC self-renewal and lineage commitment decisions is tightly regulated by extrinsic (bone marrow microenvironment, cell adhesion molecules, growth factors and cytokines) and intrinsic factors (signaling networks and transcription factors) [1]. miRNAs bring in another regulatory dimension to hematopoesis by selectively targeting signaling molecules, receptors, ligands and transcription factors and thus influence major outcomes related to HSC self renewal, differentiation, apoptosis and balance of myeloid/lymphoid compartments within the hematopoietic progenitor pool [27]. Importantly, miRNAs also contribute to oncogenesis, where an miRNA that targets oncogenes acts as a tumor suppressor and therefore decreased expression of such miRNAs contribute towards promoting tumorogenesis and comparably, up regulation of miRNAs that target tumor suppressors will also lead to oncogenesis [28,29]. Recently, dysregulation of miRNAs has been shown in leukemias where its genomic locations have been linked to breakpoint regions associated with chromosome aberrations and regions of amplification and loss of heterozygosity [30]. Importantly, aberrations in IL-3, GM-CSF, and G-CSF induced signaling are frequently reported in acute myeloid leukemia (AML) [31,32]. Here, we present outcomes from an miRNA profiling study that utilized a unique leukemic myeloid cell line, AML-193, which responds to both early and late acting cytokines, to investigate

distinct miRNA signatures regulated by IL-3, GM-CSF and G-CSF in leukemic myeloid progenitors.

In order to assess the Jak2-STAT5 responsiveness of various cytokines, AML-193 cells were cultured in the absence of cytokines for 16 hours before being treated with either IL-3 (5ng/mL), GM-CSF (50ng/mL) or G-CSF (50ng/mL) for 90 minutes. RNA was isolated and qPCR was performed on two bona fide STAT5 response genes *Pim1* and *Cish* [33–35]. The data was normalized using *GAPDH*. All three cytokines induced significant increases in *Pim1* and *Cish* transcripts (Figure 1B). Though the oncogenic role of JAK2-STAT5 is mediated through direct involvement in the up-regulation of genes such as *cyclin D*, *bcl-2*, *mcl-1*, *bcl-X_L*, and *myc* [36], the specific mechanisms by which JAK2-STAT5 critically controls preleukemic expansion in the myeloid lineage have not been defined. Identifying cytokine specific Jak2-STAT5-regulated miRNAs that govern this transition will provide candidate regulators for functional testing. Further, study of the distinct IL-3/GM-CSF/G-CSF induced miRNA signatures accompanying progenitor and lineage restricted stages will provide important mechanistic cues regarding the complexity of the regulatory network.

For miRNA profiling, AML-193 cells were initially exposed to IL-3 for 3 days followed by GM-CSF for 3 more days and subsequently to G-CSF for 3 days. We then profiled miRNA expression induced by IL-3, GM-CSF and G-CSF in AML-193 cells by treating the respective cohorts post growth factor deprivation with corresponding cytokines (Figure 1A). Using the complete Human V2.0 miRNA Genome Array platform representing a total of n=704 miRNAs, we analyzed the cytokine specific expression of miRNAs in AML-193 early myeloid leukemic cells. To further specify our search for miRNAs differentially induced by cytokines in AML-193, we added an additional filter. We only considered miRNAs that were at least 2-fold up- or down-regulated in their expression compared to untreated control. In addition, miRNAs having a Ct value of >35, which are likely due to background noise, were excluded from the analysis. Frequencies of false positives were avoided using these stringent filters. Using these empirically determined thresholds, we identified 90 miRNAs were up regulated by IL-3 and 339 miRNAs were down regulated (Figure 1C and 2B). GM-CSF elevated the expression of 65 miRNAs and decreased expression of 358 miRNAs (Figure 1C and 2B). G-CSF up regulated 10 miRNAs and down regulated 435 miRNAs (Figure 1C and 2B). Interestingly, this list of highly down regulated miRNAs includes Let-7a and miR-16. Tumor suppressors like Let-7a and miR-16 have been shown to target multiple oncogenes such as RAS, HMGA2 and BCL2, MCL1, CCND1, WNT3A respectively [25,37,38]. Significantly, down regulation of Let-7a and miR-16 have been reported in chronic lymphocytic leukemia (CLL) [39] and in addition, decreased levels of miR-16 has been found in blasts isolated from high-risk MDS (myelodysplastic syndrome) patients [40]. Therefore, it is possible that in AML associated with skewed IL-3 or GM-CSF signaling may result in the down regulation of these miRs and promote a proliferative phenotype for the blasts.

A detailed view of the comprehensive miRNA profiling outcome by three different cytokines is presented as three-way Venn diagram in Figure 2A. Interestingly, 301 miRNAs were commonly regulated by IL-3, GM-CSF and G-CSF. IL-3 specifically regulated 54 miRNAs and those miRNAs that were highly regulated included miR-362-39, miR-590-3p, miR-340, miR-24-2, miR-1183 and miR-99a (Figure 3A). GM-CSF specifically regulated miRNAs included let-7f, let-7a*, miR-195, miR-122, miR-376-c and miR-33a (Figure 3B). G-CSF specifically regulated set included miR-21*, miR-192*, miR-32*, miR-7-1*, miR-545* and miR-37-4a* (Figure 3C). Among the commonly regulated miRNAs, the ones that were subjected to high levels of regulation included miR-590-5p, miR-219-5p, miR-362-3p, miR-92-a1*, miR-378*, miR-548-3p, miR-29a*, miR-590-3p, miR-203, miR-363, miR-454, miR-340, miR-196a, miR-152, miR-10b, miR-24-2*, miR-10a,

miR-182, miR-27a*, and miR-199a-3p (Figure 4A). Interestingly, the commonly regulated miRNAs demonstrated a directional regulation in the order of IL-3>GM-CF>G-CSF (Figure 4A-C). A complete list of all miRNAs distinctly regulated as well as commonly regulated by IL-3, GM-CSF and G-CSF in AML-193 cells is provided as Supplemental Table 1, 2, 3, and 4 respectively. miR-10b has been shown to be up regulated in cytogenetically normal AML (CN-AML) with FLT3-ITD and nucleophosmin (NPM) mutations [26,41]. Likewise, in a previous Cancer and Leukemia Group B (CALGB) 19808 study, in AML patients with FLT3-ITD with wild type NPM1 or both, increased expression of miR-219-5p was associated with increased risk of an event [42]. Similarly and significantly, increased expression of miR-199a has been identified in AML patients with isolated Trisomy 8 that is associated with poor outcome [26]. Furthermore, increased expression of miR-199a is also part of a miRNA signature that is significantly up regulated in six solid tumors [43]. The currently observed up regulation of these select miRNAs by IL-3, GM-CSF and G-CSF in AML-193 cells provides an insight into the possible deviant regulatory mechanism responsible for the high expressions of these miRNAs in AML. Further analyses of the potential targets of these significantly regulated miRNAs revealed important functional roles for these putative targets in myeloid cell development and differentiation. For example, a few of the highly conserved targets of miR-590-5p included BMPR2 and PCBP2. Interestingly, comparative analysis of gene expression on Agilent 22K oligonucleotide microarrays in purified CD34+ cells from the blood of myeloid metaplasia patients showed decreased expressions of BMPR2 [44]. BMPR2 is also a conserved target of miR-125b and significantly, Baltimore and his colleagues showed that overexpression of human miR-125b caused a dose dependent myeloproliferative disorder that progressed to lethal myeloid leukemia in mice and also enhanced hematopoietic engraftment [18]. Furthermore, BMPR2 has been recently shown to possess tumor suppressive roles in mammary epithelia, and disruption of BMPR2 expression results in accelerated metastases of mammary carcinoma [45]. Therefore, down regulation of BMPR2 by miR-590-5p may promote the IL-3 induced proliferation of early myeloid leukemic progenitors. Further, miR-590-5p is also predicted to target poly(C) binding protein 2 (PCBP2), which has been recently shown to be highly expressed in hematopoietic stem cells [46]. Importantly, deficiency of PCBP2 in K562 cells results in p53 independent induction of cyclin-dependent kinase inhibitor (CDKN1A) and G1 arrest [47]. Therefore, up regulation of miR-590-5p may attenuate onset of myeloid differentiation program, nonetheless, the direct effect of miR-590-5p on BMPR2 and PCBP2 in myelopoiesis will be explored in our future studies. Another directionally regulated miRNA that was discovered in the present studies was miR-219-5p. A highly conserved 8-mer target of miR-219-5p is a TGFβ/BMP induced signaling protein Smad4 [48]. Importantly, Smad4 has been showed to bind directly to Hoxa9 and inhibit Hoxa9-Nup98 induced AML [49]. Most significantly, a recent study employing Smad4 −/− hematopoietic stem and progenitor cells (HSPCs) revealed a major negative regulatory role for Smad4 in Hoxa9 or Hoxa9-NUP98 induced AML [50]. Disruptions of Smad4-Hoxa9 interaction in the cytoplasm leads to activation of the TGF-β pathway and apoptosis; deletion of Smad4 in HSPCs leads to the acceleration of or Hoxa9-NUP98 induced AML [50]. One of miR-219-5p's highly conserved target genes is Growth arrest and DNA damage 45 beta (Gadd45beta), one of the primary response genes induced during myeloid differentiation along with MyD88, Gadd45a, Gadd45g and Gadd34 [51]. Interestingly, Gadd45beta and Gadd45a knockout mice exhibit deficiencies in coping with hematological stresses like acute cytokine stimulation, myeloablation and inflammation [52,53]. Significantly, a recent study discovered that miR-219 is one of the eight up regulated miRNAs in AML patients with 11q23 balanced translocation [26]. The third most highly induced miRNA in our studies was miR-362-3p. miR-362-3p is predicted to target Sox17, an inhibitor of Wnt signaling pathway. The promoter of Sox17 has been shown to be hypermethylated in AML patients [54]. Interestingly, the Wnt/beta-catenin pathway has recently been shown to be required for the development of leukemic stem cells (LSCs) in

AML [55]. Therefore, silencing of Sox17 by elevated expression of *miR-362-3p* may result in a similar up regulation of Wnt signaling pathway and promote growth of LSCs in AML.

One of the most down regulated miRNA in our data set was a known STAT5 target miR-15b* [56]. miR-15 has been shown to target Bcl-2 and inhibit apoptosis and promote cell survival [56]. Importantly, *miR-15a* and *miR-16-1*, located at 13q14 were discovered to be deleted or down-regulated in approximately 60% of chronic lymphocytic leukemia (CLL) samples [25]. Further, a recent study demonstrated that miR-15 and -16 target Cyclin E and conversely, inhibition of miR-15 and -16 enhanced E2F1-induced G(1) S transition [57]. More importantly, a new study found that levels of miR-15 and -16 were significantly down regulated in a majority of the patients with prostate tumor [58]. Therefore, it is tempting to speculate that GM-CSF mediated down regulation of miR-15 in leukemic cells may promote survival of blasts or even earlier leukemic myeloid progenitor pool or LSCs. Another highly down-regulated miR was miR-628-5p. One of the highly conserved predicted targets of miR-628-5p is Foxo3/Foxo3a [59]. Recent clinical studies have implicated an important prognostic role for Foxo3 expression in AML. Elevated Foxo3 expression was shown to be associated with a poorer prognosis in AML with normal cytogenetics [60]. Further, highly phosphorylated Foxo3 has been predicted to be an independent adverse prognostic factor in AML [61]. In the light of these reports that reveal high expressions of Foxo3 in AML, it is possible that decreased levels of miR-628-5p caused by IL-3 signaling in leukemic progenitors may be playing a major role in promoting increased Foxo3a expressions in AML.

In summary, we have discovered for the first time novel miRNA profiles regulated by IL-3, GM-CSF and G-CSF in an acute myeloid leukemia progenitor cell model. Importantly, we have established a cytokine regulated miRNome for leukemic myeloid progenitors and set the stage for future investigations in leukemic stem cells to delineate the pathological roles of dysregulated miRNAs in AML. It will be interesting to examine how many of these miRNAs are directly regulated by STAT5 as the outcomes from such investigation may provide important leads to develop combinatorial therapeutic strategy for AML subtypes with STAT5 dysregulation via use of STAT5 specific inhibitors [62]. Furthermore, these novel miRNA signatures may have therapeutic implications for targeting dysregulated miRNAs by antagomir strategy or miRNA replacement therapy, paving the way for the development of novel miRNA-based therapeutic interventions in AML.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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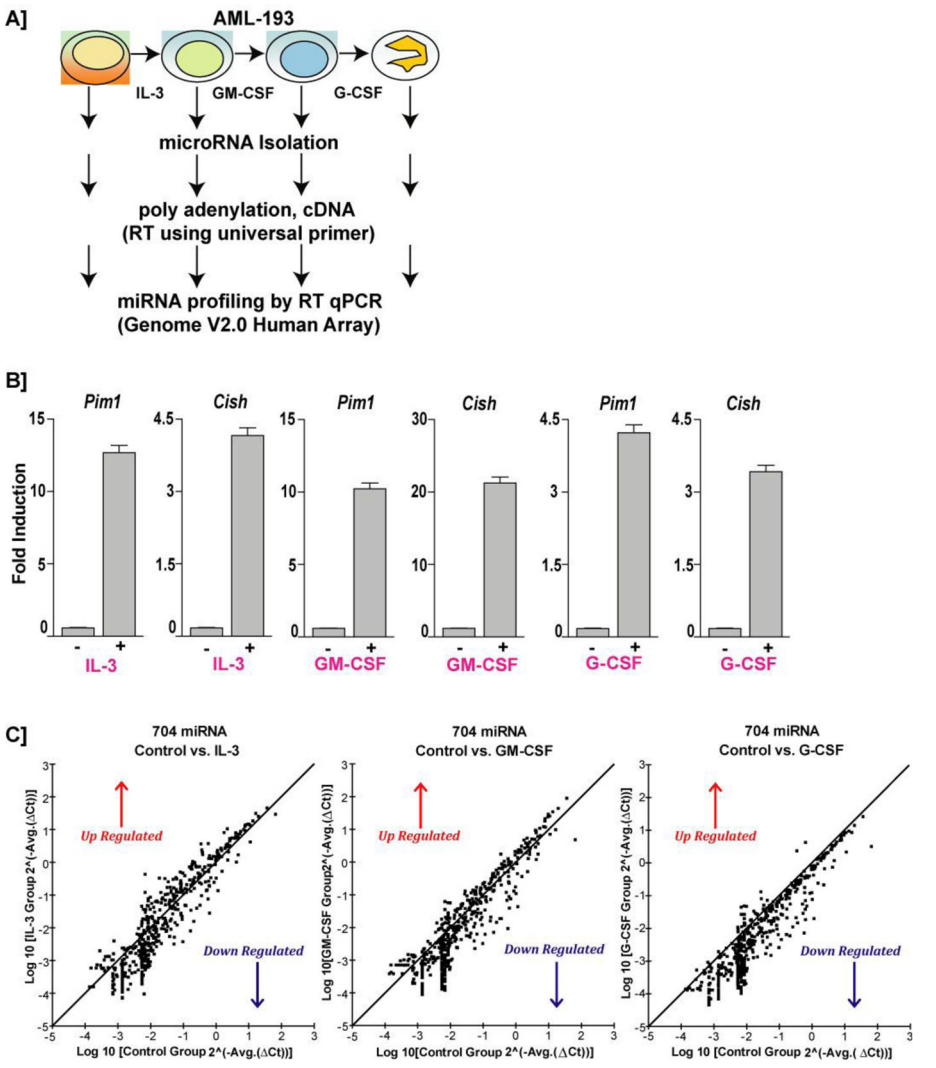

Figure 1. AML-193 as a model system for testing IL-3, GM-CSF, and G-CSF induced miRNA profiling via potential JAK2-STAT5 pathway

A. Schematic diagram of overall experimental strategies for IL-3, GM-CSF and G-CSF induced miRNA profiling in AML-193 cells. **B.** In order to confirm the JAK2-STAT5 responsiveness to cytokines, AML-193 cells were cultured under growth factor deprivation conditions for 16 hours and were treated with either IL-3 (5ng/mL) or GM-CSF (50ng/mL) or G-CSF (50ng/mL) for 90 minutes. RNA was isolated and RTqPCR was performed on two bona fide STAT5 response genes *Pim1* and *Cish*. The data was normalized using *GAPDH*. Data are representative of two independent experiments performed in triplicate (mean +/− SEM). **C.** Scatter plot showing the comparison of IL-3, GM-CSF and G-CSF induced miRNA expression profiling in AML-193 cells. Fold change filters were applied to select the miRNAs that were regulated more than 2-fold.

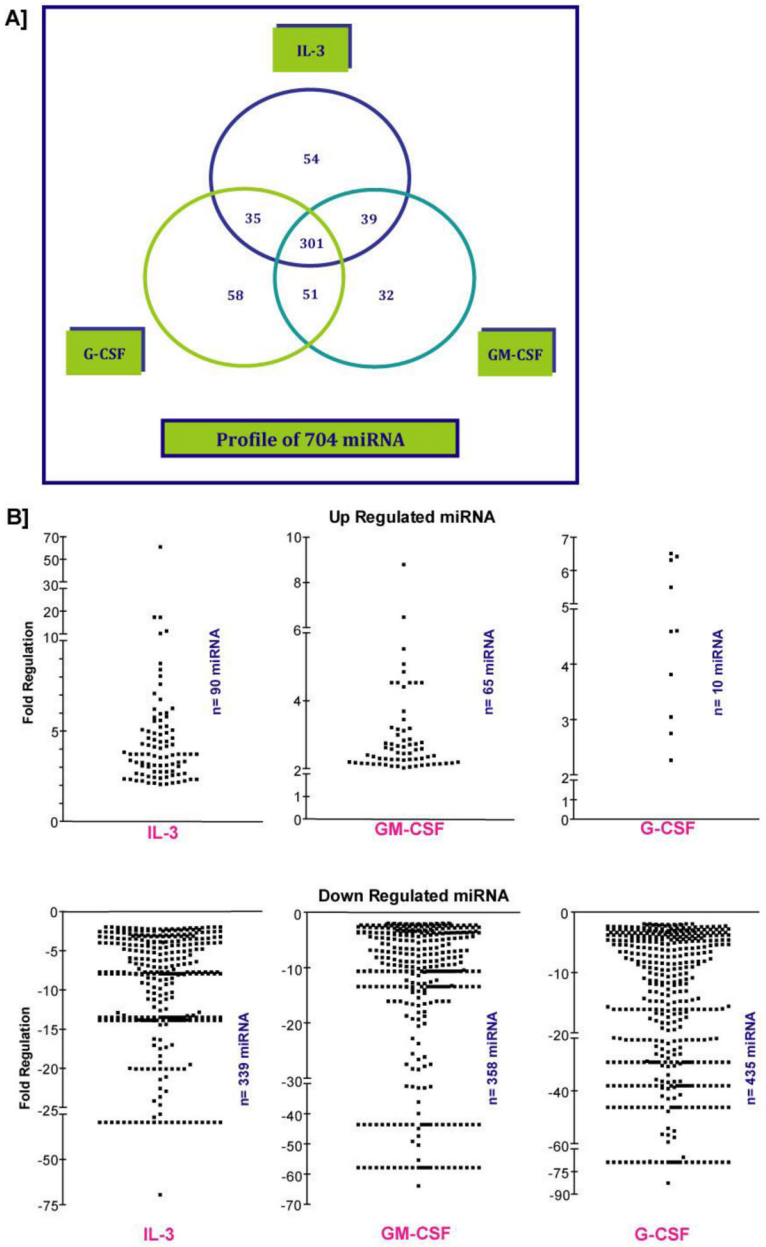

Figure 2. Profiling outcomes of IL-3, GM-CSF, and G-CSF regulated miRNA in AML-193 cells

A. A three-way Venn diagram illustrating the comprehensive miRNA profiling outcome by three different cytokines. 301 miRNAs were commonly regulated by IL-3, GM-CSF and G-CSF. **B**. Individual scatter plots for up- and down- regulated miRNAs. 90 miRNAs were upregulated by IL-3 and 339 miRNAs were down regulated. GM-CSF elevated the expression of 65 miRNAs and decreased expressions of 358 miRNAs. G-CSF upregulated 10 miRNAs and downregulated 453 miRNAs. As previously mentioned, fold change filters were applied to select the miRNAs that were regulated more than 2-fold.

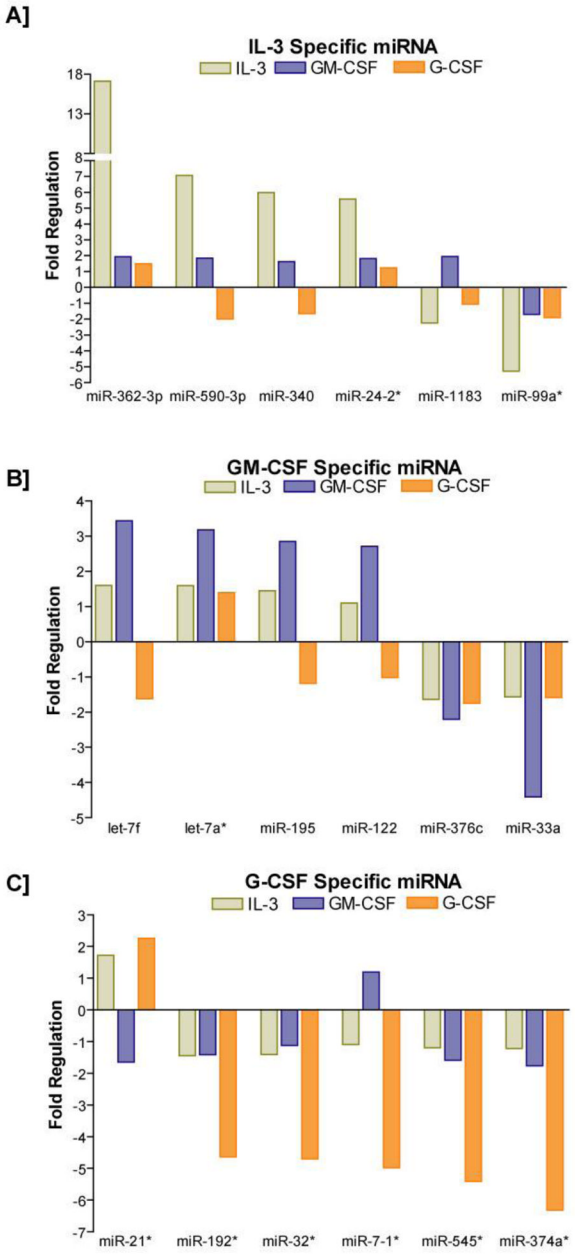

Figure 3. Specific miRNAs independently regulated by IL-3, GM-CSF, and G-CSF in AML-193 cells

A. IL-3 specifically regulated 54 miRNAs and highly regulated miRs included miR-362-39, miR-590-3p, miR-340, miR-24-2, miR-1183 and miR-99a. **B.** miRNAs specifically regulated by GM-CSF included let-7f, let-7a*, miR-195, miR-122, miR-376-c and miR-33a. **C.** miRNAs specifically regulated by G-CSF included miR-21*, miR-192*, miR-32*, miR-7-1*, miR-545* and miR-37-4a*.

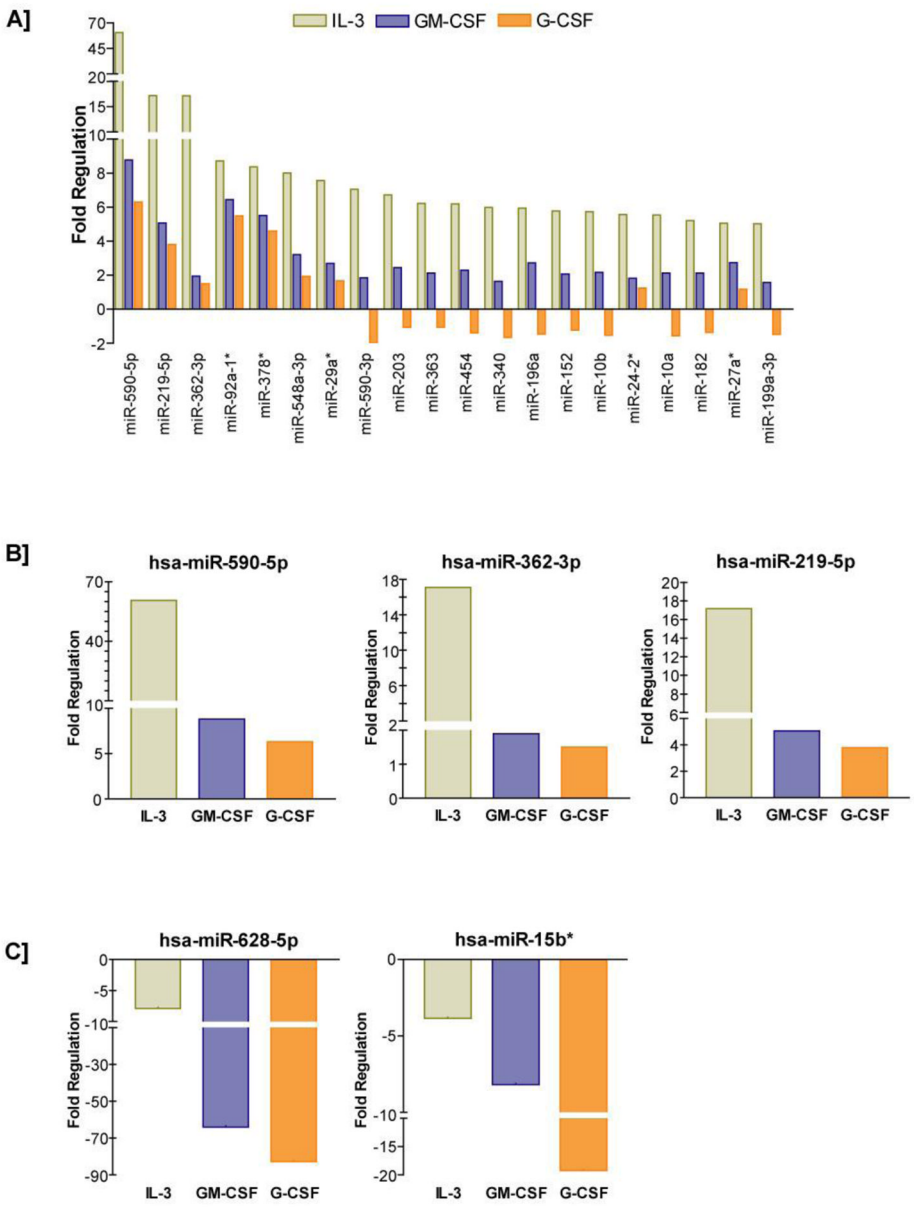

Figure 4. IL-3, GM-CSF, and G-CSF directionally regulate specific miRNAs in AML-193 cells

A. 301 miRNAs were commonly regulated by IL-3, GM-CSF and G-CSF and miRNAs that were subjected to high levels of regulation included miR-590-5p, miR-219-5p, miR-362-3p, miR-92-a1*, miR-378*, miR-548-3p, miR-29a*, miR-590-3p, miR-203, miR-363, miR-454, miR-340, miR-196a, miR-152, miR-10b, miR-24-2*, miR-10a, miR-182, miR-27a*, miR-199a-3p. **B.** Top three miRNAs (miR-590-5p, miR-362-3p and miR-219-5p) that were predominantly upregulated byIL-3, GM-CSF and G-CSFare individually graphed. **C.** Top two miRNAs (miR-628-5p and miR-15b*) that were predominantly down-regulated byIL-3, GM-CSF and G-CSF are individually graphed.