TUMORIGENESIS AND NEOPLASTIC PROGRESSION

miR-378-3p Knockdown Recapitulates Many of the Features of Myelodysplastic Syndromes

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Myelodysplastic syndromes (MDS) are clonal neoplasms of the hematopoietic stem cell that result in aberrant differentiation of hematopoietic lineages caused by a wide range of underlying genetic, epigenetic, and other causes. Despite the myriad origins, a recognizable MDS phenotype has been associated with miRNA aberrant expression. A model of aberrant myeloid maturation that mimics MDS was generated using a stable knockdown of miR-378-3p. This model exhibited a transcriptional profile indicating aberrant maturation and function, immunophenotypic and morphologic dysplasia, and aberrant growth that characterizes MDS. Moreover, aberrant signal transduction in response to stimulation specific to the stage of myeloid maturation as indicated by CyTOF mass cytometry was similar to that found in samples from patients with MDS. The aberrant signaling, immunophenotypic changes, cellular growth, and colony formation ability seen in this myeloid model could be reversed with azacitidine, albeit without significant improvement of neutrophil function. (*Am J Pathol* 2021, 191: 2009–2022; <https://doi.org/10.1016/j.ajpath.2021.07.006>)

Myelodysplastic syndrome (MDS) is a group of clonal hematopoietic stem cell (HSC) disorders, predominantly of the elderly population, that carry an increased risk of transformation to an acute myeloid leukemia.¹ MDS is characterized by cytopenias with dysplasia in one or more lineages of the bone marrow.^{1,2} MDS has been associated with a myriad of molecular genetic, epigenetic, and inflammatory lesions.^{2–9} However, a recognizable phenotype of MDS used by pathologists and clinicians in the diagnosis of MDS includes granulocytic dysplasia (including hypogranulation and hypolobation), myeloid maturational dys-synchrony by flow cytometry, neutropenia, and aberrant function of neutrophils that manifest as increased risk of infections. miRNAs are critically important in the regulation of the cellular phenotype and are differentially expressed at different stages in hematopoietic maturation.^{10,11} Thus, the numerous causal lesions may funnel through a common pathway of dysregulated miRNAs in the pathogenesis of MDS.

miRNAs are small, noncoding RNAs of approximately 22 nucleotides that bind imperfectly to the 3' untranslated region and other regions of target mRNAs to repress their translation and/or stability.^{10,11} Recent studies have confirmed disordered miRNA expression in patients with

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MDS, including a study from this research group that identified eight miRNAs that are highly discriminatory between MDS and normal control samples.^{12–17}

miR-378-3p was underexpressed in 80% of cases across MDS subtypes but only 10% of control normal bone marrows.¹² miR-378-3p underexpression is associated with hypermethylation of its promoter, and targets of miR-378-3p are accordingly overexpressed.^{7,12} An antisense to miR-378-3p was introduced by lentiviral vector in HSCs and acute myeloid leukemia cell lines that can be induced to differentiate, creating a myeloid maturational model of MDS. This model demonstrated a transcriptional profile that indicated aberrant maturation and function and showed immunophenotypic, morphologic, growth, and functional defects that characterize MDS. Moreover, aberrant signal transduction in myeloid response to stimulation mimicked that seen in samples from patients with MDS. With the use of this model, azacytidine treatment reversed some of phenotypic alterations, albeit with no significant improvement of neutrophil function.

Materials and Methods

Cell Lines and Cell Cultures

HL60 cells were purchased from ATCC (Manassas, VA) and maintained in Iscove's modified Dulbecco's medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Fisher Scientific, Grand Island, NY). Human bone marrow cells were obtained from patients diagnosed with MDS or normal controls under appropriate institutional review board approval [Brigham and Women's Hospital (BWH), Boston, MA]. Cells were cultured in RPMI 1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum. CD34-positive primary human HSCs were isolated from patient bone marrow and mobilized peripheral blood samples under investigational review board approval (Vanderbilt University Medical Center) using EasySep Human CD34 Positive Selection Kit (catalog number 18056, StemCell Technologies Inc., Cambridge, MA).

ZIP Lentivirus Infection

miRZip-378a-3p (ZIP) and scramble (SCR) pGreenPur lentivectors (catalog number CS970A-1, System Biosciences, Palo Alto, CA) encode either an miRZip hairpin interfering RNA designed to generate the full-length antisense miR-378a-3p or an empty vector, respectively. HL60 or HSCs were seeded in 24-well plates and infected with a multiplicity of infection of 20 with ZIP or SCR using polybrene transduction reagent (8 µg/mL) (catalog number TR-1003-G, Millipore, Burlington, MA) by spin transduction (1500 × *g*, 2 hours). Initial transduction efficiency was measured by flow cytometry on day 3. After puromycin (catalog number P8833; Sigma-Aldrich, Burlington, MA) (2 µg/mL) treatment on day 3 through day 8, selection

efficiency was measured by flow cytometry. Expression of the ZIP construct normalized to U6 was confirmed by real-time quantitative polymerase chain reaction using QuantiMir (catalog number RA420A-hU6, System Biosciences), using the ABI 7300 Real Time PCR System (Applied Biosystem, Foster City, CA). Expression of miR-378-3p normalized to U6 was assessed by real-time quantitative polymerase chain reaction using TaqMan miRNA assays (catalog number 4427975, Applied Biosystems). The presence of green fluorescent protein (GFP) in both ZIP and SCR vectors was confirmed by polymerase chain reaction and fluorescent microscopy (Eclipse TE2000-S Inverted Microscope, Nikon, Melville, NY).

Cell Maturation and Proliferation

HL60 cells stably transduced with ZIP, SCR, or untransduced (UNT) were plated at a density of 2.5×10^5 cells/mL in triplicate. The cells were treated with all-*trans* retinoic acid (ATRA, 1 µmol/L) (catalog number R2625; Sigma-Aldrich), azacytidine (0.5 µmol/L) (catalog number 2385; Sigma-Aldrich), or combined ATRA and azacytidine for 9 days. Cell number and viability were quantified by hemocytometer using Trypan blue staining (catalog number 25900CI, Corning, Manassas, VA). CD34-positive HSCs were induced to mature with granulocyte colony-stimulating factor (G-CSF; 100 ng/mL) (catalog number 300-23, Peprotech, Rocky Hill, NJ) and stem cell factor (20 ng/mL) (catalog number 300-07, Peprotech).

Morphologic Assessment

Cells were centrifuged at $91 \times g$ (Shandon-Cytospin4, Thermo Electron Corporation, Waltham, MA) for 3 minutes, fixed in 100% methanol, and stained with Wright-Giemsa (catalog number 9380-16, Rica Chemical Company, Arlington, TX) for 15 minutes. Cells were morphologically binned as immature (myeloblasts and promyelocytes), intermediate (myelocytes and metamyelocytes), or mature myeloid cells (bands and segmented neutrophils) and scored for dysplasia on a per cell basis and represented as a percentage of total analyzed cells. Dysplastic features included hypoblobation (bilobed or monolobed forms), hypogranulation, megaloblastoid change (nuclear/cytoplasmic dyssynchrony), and apoptotic bodies. Myeloid cells without these features were considered without significant atypia. Scoring was performed by two pathologists, one blinded to the conditions (Y.D.) and one not blinded to the conditions (A.S.K.). The blinded results are included in the table, but results were concordant between the pathologists.

Transcriptional Profiling by mRNA Sequencing

A library was constructed from total RNA using the TruSeq Small RNA sample preparation kit (Illumina, San Diego, CA) and sequenced on an Illumina HiSeq 2500. The raw

sequencing reads in BCL format were processed through CASAVA-1.8.2 for FASTQ conversion and demultiplexing. The RTA chastity filter was used, and only the pass filter reads were retained for further analysis.

mRNA Sequencing Data Analysis

RNA-sequencing data went through multiperspective quality control procedures following established guidelines.¹⁸ Alignment was performed by TopHat 2 (Center for Computation Biology, Johns Hopkins University, Baltimore, MD).¹⁹ Gene quantification using both Cufflinks version 2.2.0 (University of Washington, Seattle, WA)²⁰ and HTSeq version 0.13.5 (Python Software Foundation, Fredericksburg, VA)²¹ was converted to differential expression analysis using Cuffdiff from the Cufflinks package and MultiRankSeq version 1.2.0 (Center for Quantitative Sciences, Vanderbilt University, Nashville, TN),²² respectively, which is a combination of DESeq,²³ edgeR,²⁴ and baySeq.²⁵ A list of the most differentially expressed genes was generated from all four methods based on a weighted flexible compound covariate method.²⁶ Data (heatmaps and functional pathways) were visualized with permission using Ingenuity Pathway Analysis (Qiagen Inc., Germantown, MD).²⁷

Colony-Forming Units Assay

HL60 cells stably expressing ZIP or SCR were seeded in 35-mm culture dishes in quadruplicate with a density of 2.2×10^5 cells/mL in methylcellulose (MethoCult H4535; StemCell Technologies). Cells were cultured in the presence or absence of azacytidine (0.5 $\mu\text{mol/L}$), ATRA (1 $\mu\text{mol/L}$), or combined azacytidine and ATRA for 7 days. Colonies were counted and imaged using an inverted microscope and gridded scoring dishes.

Cell Migration Assay

ZIP, SCR, or UNT HL60 cells were treated with ATRA (1 $\mu\text{mol/L}$) for 7 days. Viable cells were enriched by Ficoll-Paque purification (catalog number GE17-1440-02, Sigma-Aldrich). Cells (1×10^5) seeded in the upper wells of 96-well Transwell plates with 5- μm pores (catalog number 3388, Corning, Lowell, MA) were allowed to migrate into the lower wells that contained the mixture of chemotactic factors, *N*-formylmethionine-leucyl-phenylalanine (10 nmol/L; catalog number F3506, Sigma-Aldrich), complement component 5a (C5a, 1 nmol/L; catalog number 2037, R&D System, Minneapolis, MN), and lipopolysaccharides (10 $\mu\text{g/mL}$; catalog number L-8274, Sigma-Aldrich) for 120 minutes at 37°C. Cells from the lower wells were stained with alamarBlue HS (catalog number A50100, Invitrogen, Eugene, OR) for 120 minutes and fluorescence read at 570/610 nm.

Flow Cytometry

Cell surface markers were measured by flow cytometry in two laboratories. Cells were harvested by centrifugation, washed with phosphate-buffered saline, and incubated with antibodies for 20 to 30 minutes in the dark. In the Flow Cytometry Core of the Tennessee Valley Healthcare System, Veterans Affairs, two panels were used. For analysis of HL60 cells, the following antibodies were obtained from BD Biosciences (San Jose, CA): 7-aminoactinomycin D (catalog number 559925), CD11b-phycoerythrin (PE) (catalog number 340712), CD13-allophycocyanin (APC) (catalog number 557454), CD16-V450 (catalog number 560474), and CD45-V500 (catalog number 560777). CD33—proprotein convertase 7 (PC7) was obtained from Beckman-Coulter (Indianapolis, IN) (catalog number A54824). For the analysis of CD34-positive HSCs, the following antibodies were obtained from BD Biosciences: CD11b-APC (catalog number 550019), CD13—Brilliant Violet (BV42) 1 (catalog number 562596), CD16-APC-cyanine 7 (catalog number 560195), and CD34—peridinin-chlorophyll-protein (catalog number 340666). CD33-PC7 was obtained from Beckman-Coulter (catalog number A54824). Samples were run on a BD FACSCanto and data analyzed on FACSDiva software version 6.1.3 (BD Biosciences). Co-expression of GFP with the myeloid cell surface marker confirmed analysis of transduced cells. The flow panel used in subsequent studies at BWH included the following antibodies: CD45-APC-H7 (catalog number 560178, BD Biosciences), CD13—fluorescein isothiocyanate (FITC) (catalog number 11-0138-42, eBioscience Inc., San Diego, CA), CD16-BV421 (catalog number 302038, Biolegend, San Diego, CA), and CD11b-PE (catalog number 301306, Biolegend). At the Dana Farber Cancer Institute Flow Cytometry Core, specimens were analyzed on a BD LSR Fortessa, and data were analyzed by FlowJo software version 10.2 through version 10.6.1 (BD Biosciences).

Cytometry Time of Flight (CyTOF) Mass Cytometry

Patient Sample Acquisition, Treatment, and Preparation
Bone marrow aspirates from patients with active myelodysplasia ($n = 32$) and controls ($n = 32$) were obtained under institutional review board approval from the BWH (Table 1 and Supplemental Table S1). After treatment with azacytidine (5 $\mu\text{mol/L}$) (catalog number A2385, Sigma-Aldrich) or dimethyl sulfoxide overnight at 37°C, erythrocytes were removed with ACK lysing buffer (catalog number A10492-01, Gibco). Cells were adjusted to 1×10^6 per sample and incubated with 1:1000 cisplatin (catalog number 201064, Fluidigm, San Francisco, CA) for 2 minutes.

Cell Surface Staining and Cytokine Stimulation

Cells (patient samples or cell lines) were incubated for 20 minutes at room temperature with a 100- μL cocktail of 16

Table 1 Summary of Patient Characteristics

Characteristic	Patients with MDS (<i>n</i> = 32)	Controls (<i>n</i> = 32)	<i>P</i>
Sex, <i>n</i>			
Male	26	20	0.098
Female	6	12	
Age, mean (range), y	65 (39–85)	59 (28–76)	0.055
BM diagnoses, <i>n</i>			
MDS-SLD	1		
MDS-MLD	14		
MDS-EB1	6		
MDS-EB2	8		
CMML-0*	2		
MDS/MPN, NOS*	1		
Negative for neoplasm		26	
PCN ($\leq 10\%$ involved)		5	
LPL ($\leq 10\%$ involved)		1	
CBC counts, mean			
WBCs, / μ L (in thousands)	3.49	6.49	0.0003
ANC, μ L (in thousands)	1.56	4.43	<0.0001
HGB, g/dL	10.00	12.93	<0.0001
MCV, fL	92.13	93.02	0.6194
Platelets, / μ L (in thousands)	110.78	211.34	0.0001
PB blasts, %	0.31	0.01	0.1098
BM blasts, %	6.34	0.91	<0.0001

*The two cases of CMML and one case of MDS/MPN, NOS, are dysplastic-type MDS/MPNs and are accordingly included in this study.

ANC, absolute neutrophil count; BM, bone marrow; CBC, complete blood cell; CMML, chronic myelomonocytic leukemia; EB, excess blasts; HGB, hemoglobin; LPL, lymphoplasmacytic lymphoma; MCV, mean cell volume; MDS, myelodysplastic syndrome; MDS/MPN, NOS, myelodysplastic/myeloproliferative neoplasms not otherwise specified; MLD, multilineage dysplasia; PB, peripheral blood; PCN, plasma cell neoplasm; SLD, single-lineage dysplasia; WBC, white blood cell.

metal conjugated cell surface marker antibodies obtained from the Lederer Laboratory of BWH (Table 2), followed by stimulation with granulocyte-monocyte colony stimulating factor (100 ng/mL) (catalog number 215-GM-010, R&D Systems, Minneapolis, MN), interferon- γ (100 ng/mL) (catalog number 285-IF-100, R&D Systems), and phorbol 12-myristate 13-acetate (50 nmol/L) (catalog number P8139, Sigma-Aldrich, St. Louis, MO) for 10 minutes at 37°C. Cells were fixed with 4% paraformaldehyde and permeabilized with methanol.

Cells were barcoded using Cell-ID 20-Plex Pd Barcoding Kit (catalog number S00114, Fluidigm Corporation, South San Francisco, CA). Samples were incubated in BarCode Reagent for 15 minutes at room temperature and washed with cell staining buffer. Barcoded samples were then pooled and rinsed.

Cells were incubated at room temperature for 60 minutes with a cocktail of 16 metal conjugated intracellular antibodies (1:100 dilution of each antibody) (Table 2). Cells were fixed with paraformaldehyde, resuspended in cell staining buffer, and stored at 4°C overnight.

Data Acquisition by Mass Cytometry

On day 2, cells were incubated with intercalator Ir (191 and 193) (1:4000 with phosphate-buffered saline) for 20 minutes at room temperature. Cells were resuspended with EQ beads and analyzed on the mass cytometry (Helios, Fluidigm). Events were gated using Cytobank (Cytobank Inc., Santa

Clara, CA)²⁸ and binned as immature, intermediate, or mature myeloid cells based on expression of CD33 and the variable expression of CD45, CD34, CD117, CD123, CD16, CD11b, and CD13. For patient samples, mature monocytes were also gated by expression of CD14. T, B, and natural killer lymphoid cells were excluded using CD3, CD4, CD8, CD20, CD16, and CD56. Erythroid cells were excluded using CD235a. Gating assignments were confirmed by SPADE analysis in Cytobank software version 7.2.1 (Cytobank Inc., Santa Clara, CA).²⁸ Expression of all markers was normalized to the unstimulated state.

Statistical Analysis

For cell line-based experiments, data are expressed as means $\pm$ SD or SE of triplicates. Experiments were performed at least three times. Statistical analyses were performed by analysis of variance followed by Tukey's multiple comparison test or by *t*-test as appropriate. *P* < 0.05 was considered to be significant. Replicates of mass cytometry CyTOF data were averaged across experiments per experimental condition for each intracellular marker expression level and analyzed by the *t*-test. Expression levels were all normalized to the unstimulated paired samples, and Δ expression levels were calculated (condition 1 – condition 2). For patient CyTOF mass cytometry data, mean expression levels for the control and myeloid samples were obtained and analyzed by the *t*-test. After

Table 2 List of CyTOF Mass Cytometry Antibodies, Clone, and Isotope Label

Target	Clone catalog number	Isotope
Cell surface markers		
CD3	UCHT1	115In
CD45	HI30	141Pr
CD11b	M1/70	142Nd
CD34	581	143Nd
CD235a	HI264	144Nd
CD16	3G8	145Nd
CD8a	RPA T8	146Nd
CD13	WM15	147Sm
CD20	2H7	148Nd
CD123	6H6	151Eu
CD14	M5E2	152Sm
CD33	WM53	154Sm
CD4	RPA T4	155Gd
CD56	HCB6	162Dy
CD117 cKit	10422	158Gd
CD274/PD-L1	29E.2A3	170Er
Intracellular markers		
Akt	Poly6034	150Nd
c-PARP	F21-852	164Dy
Cleaved caspase 3 (Asp175)	D3E9	156Gd
Ki-67	8D5	161Dy
Phospho-JAK2 (Y1008)	D4D8	153Eu
Phospho-NF-κB p65 (Ser536)	93H1	159Tb
Phospho-STAT1 (Y701)	58D6	160Gd
Phospho-PLCγ1 (Tyr783)	D6M9S	165Ho
Phospho-p38 MAPK (Thr180/Tyr182)	D3F9	166Er
Phospho-p44/42 MAPK (T202/Y204)/Erk1/2	D13.14.4E	167Er
Phospho-cleaved CBL (Tyr700)	D1D67	169Tm
Phospho-STAT4 (Y693)	D2E4	171Yb
Phospho-4E-BP1 (T37/46)	236B4	172Yb
Phospho-STAT5 (Y694)	D47E7 XP (R)	174Yb
Phospho-S6 (p70RSK) S235/236	D57.2.2E XP R	175Lu
Phospho-CREB (S133)	87G3	176Yb

c-PARP, cleaved poly (ADP-ribose) polymerase; CREB, cAMP response element-binding protein; MAPK, mitogen-activated protein kinase; PLCγ1, phospholipase C γ1; STAT, signal transducer and activator of transcription.

normalization, delta expression levels were calculated (control-myeloid).

Results

Transduction by ZIP Results in Reduced Expression of miR-378-3p

HL60 cells and CD34 HSCs were transduced with ZIP or SCR lentiviral vectors. After puromycin selection, ZIP and SCR cells were confirmed to express GFP by fluorescent microscopy, flow cytometry, and PCR, whereas only ZIP cells expressed the miRZIP-378-3p by real-time quantitative polymerase chain reaction. Compared with UNT and SCR, ZIP cells demonstrated reduced expression of miR-378a-3p ([Supplemental Figure S1](#)). Initially, several other miRNAs, including combinations, were targeted (data not shown); however, eventually miR-378-3p was selected for full

analysis detailed in this article because it had the most robust phenotype, as detailed below.

ZIP Induces a Transcriptional Profile of Aberrant Cellular Function and Growth

mRNA sequencing of duplicate HL60 transductions was followed by comparisons using a combination ranking derived from DEGseq, edgeR, and baySeq methods. The top 2000 discriminatory genes for the comparisons were input into Ingenuity Pathway Analysis with DEGseq, edgeR, and baySeq data. *P* values and fold change data were provided separately. All three algorithms identified the same most impacted cellular functions in ZIP compared with SCR cells with significant deactivation of cell movement, signaling, and phagocytosis ([Figure 1](#), [Supplemental Table S2](#), and [Supplemental Figure S2](#)). DEGseq and baySeq provided the identical six pathways with a score ≥ 35 , whereas edgeR

Figure 1 Snapshot from Ingenuity Pathway Analysis, demonstrating key diseases and functions affected by knockdown of miR-378-3p by mirZIP-378-3p. Most highly affected pathways include those that involve cell movement and function. Key pathways are predominantly underexpressed as indicated by the blue end of the color scale of z scores that represent decrease of implicated biologic function (blue) (versus increased function in orange). **A:** Hematologic system development and function; **B:** organismal injury and abnormalities; **C:** cell-to-cell signaling and interaction; **D:** cancer; **E:** tissue development; **F:** cellular development; **G:** cell growth and function; **H:** lymphoid tissue structure; **I:** lipid metabolism; **J:** small molecule biochemistry; **K:** gastrointestinal disease; **L:** cell function and maintenance; **M:** hematopoiesis; **N:** cellular compromise; **O:** organismal death; **P:** cardiovascular disease; **Q:** infectious disease; **R:** free radical scavenging; **S:** cellular movement; **T:** inflammatory response; **U:** immune cell trafficking; **V:** cell death and survival; **W:** immunologic disease; **X:** reproductive system disease; **Y:** skeletal and muscular disorders; **Z:** endocrine system disorders; **AA:** embryonic development; **BB:** organismal development; **CC:** tissue morphology; **DD:** respiratory disease; **EE:** connective tissue development; **FF:** metabolic disease; **GG:** antimicrobial response; **HH:** inflammatory disease; **II:** renal and urologic disease; **JJ:** cell signaling; **KK:** cellular growth and proliferation; **LL:** molecular transport; **MM:** neurologic disease; **NN:** connective tissue development; **OO:** hepatic system disease; **PP:** dermatologic disease; **QQ:** hepatic and digestive system disease; **RR:** hematologic disease; **SS:** gene expression; **TT:** organ development; **UU:** cell migration; **VV:** posttranslational modification; **WW:** vitamin and mineral metabolism; **XX:** humoral immune response and hypersensitivity response; **YY:** carbohydrate metabolism; **ZZ:** protein synthesis and cellular function and maintenance.

provided seven pathways that met the criteria. These top-scoring pathways encompassed a total of 328 key proteins involved in cellular maturation, growth and proliferation, cellular movement, DNA replication, and cell death and survival (Supplemental Table S3).

ZIP Causes Dysplasia and Apoptosis

UNT, SCR, and ZIP HSCs were induced to mature by G-CSF treatment over 9 days. Wright-Giemsa–stained cytopins of the cultured cells were scored for cell maturation and dysplasia (Table 3 and Figure 2, A–C). Although there was some effect of the transduction on both ZIP and SCR cells, of mature neutrophils, 100% of ZIP cells demonstrated abnormal bilobed (8% of neutrophils) and monolobated neutrophils (92% of neutrophils) (bilobed: $P = 0.050$ versus SCR and $P = 0.027$ versus UNT; monolobated: $P = 0.015$ versus SCR and $P = 0.0034$ versus UNT). By contrast, 71% of SCR cells were abnormally lobated, including 49% monolobated and 22% bilobed neutrophils, and only a few UNT cells were abnormally lobated. ZIP cells also demonstrated significantly increased apoptosis ($P = 0.0021$ versus SCR and $P = 1.15 \times 10^{-6}$ versus UNT). Similarly, the HL60 cells were studied under the analogous

conditions (Table 3) after treatment with ATRA for 7 days. Despite a higher background of cellular atypia (as defined in Materials and Methods) with the HL60 UNT, SCR, and ZIP cells compared with the primary HSCs, the ZIP cells were significantly left-shifted compared with both UNT and SCR cells with increased immature cells ($P = 0.0148$ versus UNT), increased intermediate myeloid cells ($P = 0.045$ versus UNT, $P = 0.0255$ versus SCR), and decreased mature forms ($P = 0.003$ versus UNT, $P = 0.029$ versus SCR). Similar results were identified in NB4 cells (data not shown). Morphologic atypia was not statistically different between the ZIP cells and either set of control cells.

ZIP Induces Immunophenotypic Dyssynchronous Maturation

Both HL60 and CD34 HSCs were induced to mature by treatment with ATRA for 7 days (HL60) or G-CSF for 9 days (CD34 HSCs), respectively. Flow cytometry was then performed using a panel of myeloid maturation markers (Figure 2, D–G). ZIP cells exhibited decreased CD11b (HL60 cells: $P = 0.0035$; CD34 HSCs: $P = 0.0019$) but increased CD13 expression over SCR (HL60 cells: $P = 0.0090$; CD34 HSCs: $P = 0.0055$). Because CD11b

Table 3 Manual Differential of Morphologic Stages of Myeloid Maturation and Dysplasia in UNT, SCR, and ZIP Cells in Primary HSCs (Mean of 4 Replicate Experiments, with Goal of 100 Cell Counts per Experiment) and the HL60 Cell Line (Mean of 4 Replicate Experiments, with Goal of 200 Cell Counts per Experiment)

Morphologic feature (mean lobes)	UNT, %	SCR, %	ZIP, %	<i>P</i> value		
				SCR versus ZIP	UNT versus ZIP	UNT versus SCR
HSCs						
Neutrophils (3–5 lobes)	30.50	7.00	0.00	0.0500	0.0001	0.0009
Neutrophils (2 lobes)	6.50	5.25	0.50	0.0500	0.0267	0.4918
Neutrophils (1 lobe)	0.25	12.00	5.75	0.0154	0.0034	0.0026
Intermediate myeloid	34.25	23.75	9.25	0.0344	0.0099	0.2209
Immature myeloid	25.25	13.75	3.00	0.0481	0.0036	0.0904
Apoptotic body	3.25	38.25	81.50	0.0021	<0.0001	0.0033
HL60s						
Mature myeloid without significant atypia	32.12	26.25	4.41	0.0290	0.0027	0.5951
Mature myeloid and dysplastic	8.68	4.50	1.13	0.2319	0.0796	0.2599
Intermediate myeloid without significant atypia	32.07	28.75	53.92	0.0255	0.0450	0.7353
Intermediate myeloid and dysplastic	2.67	1.00	1.68	0.5664	0.7692	0.3198
Immature myeloid without significant atypia	12.22	29.25	27.36	0.8691	0.0148	0.3353
Immature myeloid and dysplastic	0.67	0.50	2.59	0.2129	0.3133	0.9929
Apoptotic body	11.56	9.75	8.92	0.7429	0.4120	0.1961
HL60s, azacytidine treated						
Mature myeloid without significant atypia	36.79	34.92	26.59	0.7848	0.0628	0.2641
Mature myeloid and dysplastic	6.68	8.54	1.80	0.2722	0.1013	0.5838
Intermediate myeloid without significant atypia	39.25	21.83	35.58	0.4003	0.6572	0.0100
Intermediate myeloid and dysplastic	3.71	0.13	1.14	0.0622	0.1305	0.0263
Immature myeloid without significant atypia	5.84	19.27	22.01	0.8391	0.0619	0.2413
Immature myeloid and dysplastic	0.39	1.02	0.88	0.8811	0.5935	0.9601
Apoptotic body	7.33	14.29	12.01	0.7937	0.2318	0.4792

HSCs, human hematopoietic stem cells; SCR, scramble; UNT, untransduced; ZIP, miRZIP-378-3p.

typically increases with maturation and CD13 typically increases in the later stages of maturation (after decreasing early in maturation), these findings indicate true dyssynchrony. Similar dyssynchrony was seen with other markers of maturation (CD16, CD33, CD34, and CD45) ([Supplemental Table S4](#)).

ZIP Inhibits Colony Formation and Cell Proliferation

UNT, SCR, and ZIP HL60 cells were assessed for the ability to form methylcellulose colonies. ZIP cells exhibited diminished colony formation ability compared with SCR cells ($P = 0.005$) ([Figure 3](#), A and B). Cell proliferation studies were performed by viable cell enumeration on days 0, 3, 5, and 7. No significant difference was found between the growth of UNT and SCR cells ($P = 0.49$), whereas ZIP cells were significantly less proliferative ($P = 0.017$ versus UNT and $P = 0.026$ versus SCR) ([Figure 3](#), C and D).

ZIP Inhibits Cell Migration

Because of the need to sufficiently expand cells for maturational studies, all subsequent studies were performed on HL60 cells alone. The HL60 cell line was chosen because of its ease of expansion compared with HSCs,²⁹ its superior transduction efficiency compared with NB4 cells, and its well-known ability to mature into the full spectrum of myeloid maturation

morphologically and immunophenotypically.³⁰ To investigate the effects of ZIP on cell migration, UNT, SCR, and ZIP HL60 cells were treated with ATRA (1 $\mu\text{mol/L}$) for 7 days. In response to a cocktail of neutrophil chemotactic factors (*N*-formylmethionine-leucyl-phenylalanine, C5a, and lipopolysaccharides), ZIP cells exhibited significantly reduced migration compared with UNT and SCR cells ($P = 0.0044$ and 0.0063 , respectively) ([Figure 3D](#)).

ZIP Induces Immunophenotypic Evidence of Aberrant Signaling

Single-cell phenotyping by CyTOF mass cytometry was performed on UNT, SCR, and ZIP HL60 cells in response to stimulation by a cocktail of known neutrophilic stimulants (granulocyte-monocyte colony stimulating factor, interferon- γ , and phorbol 12-myristate 13-acetate). Cells were induced to mature for 7 days with ATRA before stimulation and CyTOF mass cytometry assessment. The myeloid cells were binned into three stages of maturation by cell surface markers followed by assessment of intracellular signaling proteins ([Supplemental Figure S3](#)). As shown in the heatmap in [Figure 4A](#) and in [Supplemental Table S5](#), differences were found in pathway use by ZIP cells compared with both SCR and UNT cells, including underutilization of phospho (p)–ERK, pS6, and p-STAT1 across all stages of maturation. This underutilization of p-STAT1 was especially prominent in the most

Figure 2 Morphologic and immunophenotypic dysplasia of miRZip-378a-3p (ZIP). **A–C:** Human CD34-positive hematopoietic stem cells (HSCs) were transfected with ZIP or scramble (SCR) vector and induced to mature by treatment with granulocyte colony-stimulating factor (G-CSF) for 9 days. **Arrows** indicate hypolobated neutrophils. **A:** untransduced human HSCs. **B:** HSCs transduced with SCR. **C:** HSCs transduced with ZIP. **D–G:** Myeloid markers of maturation on ZIP and SCR cell on days 0 (D0), Day 7 (D7) of all-*trans* retinoic acid treatment for HL60 cells and day 2 (D2) and day 9 (D9) of G-CSF treatment for HSCs. **D and E:** CD11b expression on HL60 cells (**D**) and HSCs (**E**). **F and G:** CD13 expression on HL60 cells (**F**) and HSCs (**G**). * $P < 0.05$. Original magnification, $\times 40$ (Wright-Giemsa stain) (**A–C**).

mature ZIP cells ($P = 0.014$ versus UNT). Interestingly, ZIP cells had decreased Ki-67 in the most immature cells ($P = 0.020$ versus SCR cells).

ZIP Signaling Aberrations Mimic Those Seen in Patient Samples

The optimal validation for any human model system is the concordance with primary patient samples. To ascertain whether the signaling aberrations seen in the ZIP myeloid model were representative of patients with MDS, bone marrow aspirate samples were obtained from 32 patients with myelodysplasia and 32 controls ([Table 1](#) and [Supplemental Table S1](#)) and were analyzed by CyTOF mass cytometry. As shown in [Figure 4C](#), the MAPK pathway and p-STAT1 were significantly underresponsive to granulocytic stimulation in patients with MDS compared with controls at all stages of myeloid maturation, mirroring that seen in the ZIP cells ([Supplemental Table S6](#)). By contrast, p-CBL and JAK2 were more highly responsive in MDS myeloid cells, similar to that seen in the ZIP cells. Mature MDS myeloid cells had significant activation of caspase-3 and c-PARP ($P = 0.033$ and 0.012 , respectively), whereas Ki-67 was underexpressed in immature MDS cells ($P = 0.002$), as was seen in the ZIP cells.

Azacytidine Reverses Differentiation Skewing of ZIP Cells

The DNA methyltransferase inhibitor azacytidine is used clinically in the treatment of MDS.³¹ HL60 cells having

undergone ATRA-induced maturation with and without azacytidine were examined morphologically. Azacytidine markedly improved the differentiation of the ZIP cells, eliminating all significant differences in maturation from the UNT or SCR cells seen with ATRA alone ([Table 3](#) and [Figure 5](#)).

Azacytidine Mitigates Cell Signaling Abnormalities of ZIP Cells

UNT, SCR, and ZIP HL60 cells were induced to mature with ATRA in the presence of azacytidine before stimulation and CyTOF mass cytometry analysis. As shown in the heatmap of signaling molecules ([Figure 4B](#)), azacytidine mitigated the expression of p-CBL in the mature ZIP cells and expression of caspase-3 and cPARP in immature cells compared with untreated cells ([Figure 4A](#)). Interestingly, there was marked activation of caspase-3 and c-PARP in mature cells with azacytidine treatment, potentially consistent with azacytidine toxicity in the mature cells. In addition, there was increased AKT expression relative to UNT in the mature myeloid cells treated with azacytidine in marked reversal of that seen in the untreated cells.

Effect of Azacytidine on Aberrant Signaling in Patient Samples

Patient samples were incubated overnight with azacytidine before stimulation and CyTOF mass cytometry analysis. As shown in [Figure 4D](#), some reversal of the signaling transduction pathway use occurred with azacytidine compared with untreated cells ([Figure 4C](#)). Azacytidine appears to

Figure 3 Colony formation and proliferation of untransduced (UNT), scramble (SCR), and miRZip-378a-3p (ZIP) cells. **A** and **B**: Colony formation in methylcellulose of SCR and ZIP cells. **C**: Time course of cell proliferation of UNT, SCR, and ZIP cells in triplicate. **D**: Neutrophil migration experiments were performed using the Transwell system assay and UNT, SCR, or ZIP cells treated with all-*trans* retinoic acid (1 μ mol/L) for 7 days. Data are expressed as the fold change compared with basal in each group. * $P < 0.05$. Original magnification (inverted microscope), $\times 10$ (**A**).

mitigate the abnormal use of p-CBL and JAK2 in intermediate and mature myeloid cells and of c-PARP and caspase-3 in mature myeloid cells, findings similar to those seen in the ZIP cell line.

Azacytidine Restores the Growth of ZIP Transduced Cells on Cell Proliferation and Colony Formation

SCR and ZIP HL60 cells were treated with azacytidine to test the effects of azacytidine on ZIP-induced cell growth inhibition. As previously shown (Figure 3C), ZIP markedly decreased cell proliferation compared with control (Figure 6, A and B). However, low-dose azacytidine (100 nmol/L) but not higher-dose (500 nmol/L) azacytidine reversed the cell growth defect such that the difference between the SCR and ZIP cells treated with low-dose azacytidine was not significant. ZIP cells also responded differently to azacytidine than SCR cells in the colony formation assay; although the SCR cells treated with azacytidine demonstrated further

attenuation of colony formation, ZIP cells did not (Figure 6C and Supplemental Figure S4).

Azacytidine Has no Effect on Neutrophil Migration in ZIP Cells

HL60 cell maturation was induced by ATRA treatment for 7 days. Cells were then incubated with either azacytidine or vehicle control (dimethyl sulfoxide) overnight. The Transwell system assay (Fisher Scientific, Waltham, MA) was used to detect cell migration. As previously shown (Figure 3D), ZIP HL60 cells demonstrated reduced cell migration compared with UNT and SCR groups, but this effect was not modified by azacytidine treatment (Figure 6D).

Discussion

The lack of adequate model systems hampers the study of MDS. Primary MDS cells are challenging to culture because of their poor viability *ex vivo*.³² MDS models, historically

Figure 4 Heatmaps of relative use of intracellular signaling pathways by CyTOF mass cytometry. Expression levels are normalized to the unstimulated paired condition, and the difference between two test arms after normalization represented on the heat map scale. The myeloid cells are binned into three stages of maturation (immature, intermediate, and mature). **A** and **B**: miRZip-378a-3p (ZIP) expression levels shown relative to either untransduced (UNT) or scramble (SCR) cells with all-*trans* retinoic acid (ATRA) (**A**) or ATRA plus azacytidine (**B**). Green indicates lower expression in the ZIP cells, whereas red indicates higher expression in the ZIP cells. **C** and **D**: Expression levels from samples of patients with myelodysplasia compared with control samples treated with dimethyl sulfoxide vehicle control (**C**) or azacytidine (**D**). Green indicates lower expression in the patient cells, whereas red indicates higher expression in the patient cells compared with controls. * $P < 0.05$. Casp-3, caspase-3; CREB, cAMP response element-binding protein; c-PARP, cleaved poly (ADP-ribose) polymerase; MAPK, mitogen-activated protein kinase; MDS, myelodysplastic syndromes; p-, phospho; PLCγ1, phospholipase C γ1.

created from a single genetic lesion, have been plagued by the assumption that the biology of this one lesion recapitulates the whole etiologic diversity of MDS. Recent advances in multiplex gene editing and patient-derived xenograft models have greatly increased the avenues for exploration of molecular diversity and clonal evolution but do not provide a single model of MDS but rather many genetically distinct models.^{33,34}

Rather than targeting this molecular diversity, this study focused downstream on the miRNA alterations that regulate the broadly recognizable MDS phenotype. In particular, one miRNA, miR-378-3p, has been implicated in the pathogenesis of MDS^{12,35,36} and oncogenesis in general.^{37–41} Although other miRNAs that were significantly differentially expressed in MDS compared with the controls in the discovery and validation sets from the initial study in this area¹² were initially examined with the an eye toward potential for combinatorial effects, miR-378-3p alone appeared to recapitulate much of the MDS phenotype and was the focus of this study.

The ZIP cells developed in this study demonstrated significant aberrations in cellular maturation, growth and proliferation, and cellular movement across several different methods. These features mimic the cytopenias and dysplasia seen in patients with MDS. Increased apoptosis of MDS

cells occurs *ex vivo* and in this ZIP model.⁴² Criteria for the use of flow cytometry in the diagnosis of dysplasia associated with MDS include the asynchronous expression of CD13/CD11b or CD13/CD16, all markers that had aberrant dyssynchrony in this ZIP model.⁴³ The diminished growth and viability of the ZIP cells under standard culture conditions and decreased colony formation ability are also seen in primary MDS cells *ex vivo*,^{32,44} whereas overexpression of miR-378-3p has been associated with increased tumor sphere formation.⁴⁵ Finally, neutrophils from patients with MDS demonstrate decrease migration, as seen in the ZIP cells.^{46–48}

A previous study from this group detailed some of the predicted targets of miR-378-3p and demonstrated the expression of differentiation of one of these targets, suppressor of fused, in the bone marrow biopsies of patients with MDS compared with controls. It discussed the possible function of suppressor of fused in MDS, particularly in the erythroid findings of the disease.¹² A second potential target, transducer of ERBB2 (TOB2), was also implicated in this earlier report and could explain many of the cell growth/proliferation defects seen in this study. TOB2 is an anti-proliferative tumor suppressor that inhibits cell cycle progression, reportedly through repression of cyclin D1.⁴⁹ TOB2 is directly regulated by miR-378-3p through a

Figure 5 Azacytidine reverses the effects of miRZip-378a-3p (ZIP) on HL60 maturational morphologic features. ZIP and scramble (SCR) cells were assessed in presence of all-*trans* retinoic acid (ATRA) with dimethyl sulfoxide vehicle control or azacytidine. **A–F:** Morphologic assessment was performed on cytopins on SCR (**A**, **C**, and **E**) and ZIP cells (**B**, **D**, and **F**) at day 0 (D0) (**A** and **B**) and day 7 (D7) of ATRA (**C** and **D**) or ATRA and azacytidine (**E** and **F**). **C** and **D:** The untreated SCR cells demonstrate increased mature forms (**C**), whereas the ZIP cells show more intermediate forms (**D**). **E** and **F:** With ATRA treatment, there is an increase in the number of mature myeloid forms in the ZIP cells (**F**), such that there is no significant difference between SCR and ZIP (**E** versus **F**). Original magnification, $\times 40$ (Wright-Giemsa stain).

luciferase reporter assay that uses the 3' untranslated region of *TOB2*, demonstrating that overexpression of miR-378-3p results in decreased *TOB2* expression.⁴⁹ Derepression of *TOB2* expression by knockdown of miR-378-3p might therefore result in the observed decreased cell proliferation. What role *TOB2* or other targets of miR-378-3p might play in the function of the myeloid cells remains to be seen.

To explore the signal transduction alterations that might underlie these functional defects, signaling patterns of ZIP cells were compared with those of patients with myelodysplasia. The use of primary patient samples was selected as the optimal comparator rather than use of these cells in a secondary model system, such as a mouse model. The MAPK and STAT1 pathways were found to be significantly underresponsive to granulocyte stimulation in both patients with MDS and ZIP cells at all stages of maturation. Similar parallels were seen between the patient samples and the cell model with decreased Ki-67 expression of immature myeloid cells and increased caspase-3 activation in mature myeloid cells. Although preliminary associations between signaling pathway use and the specific genetic findings in these samples from patients with MDS were examined, the

number of samples with any specific genetic lesion was too small for any robust associations to be made and will need to await larger studies.

Finally, the utility of this model was demonstrated by studying the effects of azacytidine on the various defects in MDS. Azacytidine was the first compound approved by the US Food and Drug Administration for the treatment of all types of MDS. Although cytotoxic at high doses, azacytidine at low doses has been previously reported to improve differentiation.³¹ In these studies, azacytidine increased maturation of ZIP cells and mitigated the expression changes in the signaling pathways seen in the ZIP cells. In addition, azacytidine was able to partially reverse the growth and colony-forming defects in the ZIP cells, although no effect on neutrophil function was observed. The effect of azacytidine is intriguing in these studies. It has been previously demonstrated that the promoter of miR-378-3p is hypermethylated in MDS.⁷ However, the decreased expression of miR-378-3p in this experimental model was not caused by hypermethylation but by the presence of an antisense oligonucleotide. Although the most cited effect of azacytidine is on DNA methylation,

Figure 6 Azacytidine reverses the effects of miRZip-378a-3p (ZIP) on HL60 cell proliferation, colony formation, and migration. **A:** Time course of cell proliferation with two different azacytidine doses. **B:** Comparison of the different groups at the end of day of treatment (10 days). **C:** Quantification of cell colony formation in each group [dimethyl sulfoxide (DMSO) or azacytidine] after culture for 7 days. **D:** Effects of azacytidine on cell migration. * $P < 0.05$.

azacytidine may also be incorporated into RNA and affect tRNA methyltransferase levels, resulting in defective mRNAs and tRNAs.^{50,51} This alteration in the mRNA and tRNA complements in the cells caused by azacytidine administration may negate the loss of the translational repression of miR-378-3p targets by the antisense. It is well known that the effects of azacytidine are multifactorial. This study suggests that, at least at low doses, azacytidine may exert a beneficial normalizing effect on the myeloid cells in addition to the known cytotoxic effects at higher dosages.^{52,53}

Admittedly, this study examined the effect of knocking down only a single miRNA, although several dysregulated miRNAs (both up- and down-regulated) have been reported in MDS.^{12–17} It is possible that the combinatorial mix of these aberrancies is required to recapitulate fully the findings in patients with MDS. However, it is remarkable that a single knockdown of miR-378-3p is effective in mimicking

so many of the myeloid findings in MDS. The extension of this model to the other hematopoietic lineages involved in the MDS and to interactions of those hematopoietic cells with their microenvironment should be examined in the future.

In conclusion, this study uses a miRNA implicated in MDS to create an miRNA-based cell line model of MDS. This myeloid model recapitulates many of the phenotype aberrations in MDS and can be used in the study of MDS biology and potentially in the identification of novel therapies for MDS.

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Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2021.07.006>.

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