Epigenetically Aberrant Stroma in MDS Propagates Disease via Wnt/β-Catenin Activation

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Abstract

The bone marrow microenvironment influences malignant hematopoiesis, but how it promotes leukemogenesis has not been elucidated. In addition, the role of the bone marrow stroma in regulating clinical responses to DNA methyltransferase inhibitors (DNMTi) is also poorly understood. In this study, we conducted a DNA methylome analysis of bone marrow–derived stromal cells from myelodysplastic syndrome (MDS) patients and observed widespread aberrant cytosine hypermethylation occurring preferentially outside CpG islands. Stroma derived from 5-azacytidine–treated patients lacked aberrant methylation and DNMTi treatment of primary MDS stroma enhanced its ability to support erythroid differentiation. An integrative expression analysis revealed that the WNT pathway antagonist FRZB was aberrantly hypermethylated and underexpressed in MDS stroma. This result was confirmed in an independent set of sorted, primary MDS-derived mesenchymal cells. We documented a WNT/β-catenin activation signature in CD34⁺ cells from advanced cases of MDS, where it associated with adverse prognosis. Constitutive activation of β-catenin in hematopoietic cells yielded lethal myeloid disease in a NUP98–HOXD13 mouse model of MDS, confirming its role in disease progression. Our results define novel epigenetic changes in the bone marrow microenvironment, which lead to β-catenin activation and disease progression of MDS.

Introduction

Myelodysplastic syndromes (MDS) comprise a heterogeneous group of acquired clonal bone marrow disorders characterized by varying degrees of cytopenias, morphologic and functional abnormalities of hematopoietic cells, and the risk of transformation into acute myeloid leukemia (AML; ref. 1). Studies have traditionally focused on hematopoietic cells in an effort to understand hematologic disease development with the goal of pursuing therapeutic solutions. The hematopoietic cells in MDS have been shown to contain numerous genetic and epigenetic aberrations (2) and these studies have helped elucidate the pathobiology of MDS. However, there is growing evidence that microenvironmental defects also contribute to ineffective hematopoiesis and, hence, progression of the disease (3, 4).

The bone marrow microenvironment consists of a mixture of different cell types—mostly represented by stromal or megakaryocytic cells, with the bone marrow stromal cells (BMSC) playing a key role in MDS leukemogenesis. The bone marrow microenvironment influences malignant hematopoiesis, but how it promotes leukemogenesis has not been elucidated. In addition, the role of the bone marrow stroma in regulating clinical responses to DNA methyltransferase inhibitors (DNMTi) is also poorly understood. In this study, we conducted a DNA methylome analysis of bone marrow–derived stromal cells from myelodysplastic syndrome (MDS) patients and observed widespread aberrant cytosine hypermethylation occurring preferentially outside CpG islands. Stroma derived from 5-azacytidine–treated patients lacked aberrant methylation and DNMTi treatment of primary MDS stroma enhanced its ability to support erythroid differentiation. An integrative expression analysis revealed that the WNT pathway antagonist FRZB was aberrantly hypermethylated and underexpressed in MDS stroma. This result was confirmed in an independent set of sorted, primary MDS-derived mesenchymal cells. We documented a WNT/β-catenin activation signature in CD34⁺ cells from advanced cases of MDS, where it associated with adverse prognosis. Constitutive activation of β-catenin in hematopoietic cells yielded lethal myeloid disease in a NUP98–HOXD13 mouse model of MDS, confirming its role in disease progression. Our results define novel epigenetic changes in the bone marrow microenvironment, which lead to β-catenin activation and disease progression of MDS.

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mesenchymal cells, macrophages, fibroblasts, adipocytes, endothelial cells, osteoblasts, and glial cells (5–7). The microenvironment is critically important in supporting the growth of hematopoietic stem and progenitor cells and is a source of growth factors that drive the self-renewal and differentiation of the hematopoietic cells. Alterations in the marrow niche can, therefore, result in hematopoietic disorders such as MDS. In fact, a recent study showed that genetic deletion of Dicer in marrow mesenchymal progenitor cells led to myelodysplasia and development of leukemia in vivo (8). Another study demonstrated that activation of β-catenin in the murine osteoblastic niche led to MDS/AML, further supporting the role of stromal dysfunction in the genesis of these diseases (9). Yet, studies of human MDS marrows have generally not revealed gene mutations or cytogenetic alterations as seen in hematopoietic cells (10, 11), and the molecular basis of stromal dysfunction in human MDS is currently not known. Because epigenetic alterations are important in regulating gene transcription and are present in the hematopoietic cells in MDS, it is conceivable that such alterations also occur in the stromal cells. Furthermore, even though the DNA methyltransferase inhibitor, 5-azacytidine (5-Aza), is approved for therapy of MDS, its hypomethylating actions, presumed to affect the hematopoietic cells, fail to show a strong correlation with therapeutic responses, leading us to hypothesize that methylation patterns in stroma may be relevant.

To evaluate the epigenome of the MDS marrow microenvironment, we used the HpaII tiny fragment Enrichment by Ligaction-mediated PCR (HELP) assay to study cytosine methylation patterns in primary stromal cells from patients with MDS. The HELP assay relies on differential digestion by a pair of isochizomer enzymes, HpaII and MspI, which differ on the basis of their methylation sensitivity. The HpaII and MspI genomic representations can be cohybridized to a custom microarray and their ratio can be used to indicate the methylation of particular CCGG sites at these loci. The HELP assay has been successfully used to reveal novel epigenomic alterations in leukemias, MDS, and other cancers (12–14). Here we used this assay to demonstrate that MDS stromal cells contain aberrant hypermethylation that affects the Wnt/β-catenin pathway. We further showed that β-catenin activation cooperates and results in a lethal MDS or myeloid leukemia in an MDS mouse model. We showed that treatment with 5-Aza leads to abrogation of hypermethylation in stromal cells and, in fact, enhanced the ability of MDS stromal cells to support hematopoiesis (of healthy donor cells) in vitro. Taken together, these results support the concept that aberrant epigenetic marks in MDS stroma contribute to the disease progression and can be targeted for therapeutic interventions with DNMT inhibitors.

**Materials and Methods**

Cell lines, MDS stromal samples, and nucleic acid extraction

The HS 27a human stroma cell line was derived from a healthy marrow donor and immortalized by transduction with a human papillomavirus E6/E7 construct (15). The KG1a cell line was obtained and authenticated from ATCC by STR profiling in 2015. Cell lines were low passage and were regularly checked for mycoplasma contamination monthly by Plasmotest (Invivogen). Bone marrow samples were obtained from patients diagnosed with MDS and from controls after obtaining informed consent, as approved by the Fred Hutchinson Cancer Research Center and Albert Einstein College of Medicine Institutional Review Boards (Supplementary Table S1). The patient sample collection was conducted in accordance with declaration of Helsinki. Bone marrow mononuclear cells (MNC) were isolated using Ficoll–Paque gradient separation. Bone marrow cells, 25–30 × 10⁶ cells per 75-mL cell flasks were cultured in nonhematopoietic expansion medium (Miltenyi Biotec Inc.) at 37°C in 5% CO₂, with weekly medium replacement until adherent cells reached 70% confluence. Adherent cells were analyzed by flow cytometry. CD45⁻ hematopoietic cells were discarded immunomagnetically (Miltenyi Biotec). The resulting CD45⁻, low passage, bone marrow-stromal cells were used for DNA and RNA extraction.

**DNA methylation analysis by HELP assay**

The HELP assay was carried out as described previously (14, 16) to determine methylene of 50,000 CpGs corresponding to 14,000 genes. Detailed description provided in Supplementary Methods [GEO (GSE60233)]. Pathway analysis was performed using the IPA software (17). The list of hypermethylated genes was examined for enrichment of conserved gene-associated regions using the Molecular Signatures Database (MsigDB; ref. 18). Transcription factor (TF) binding sites in the differentially methylated regions was determined by the HOMER algorithm (19).

**Quantitative DNA methylation analysis by mass array epityping**

Validation of HELP microarray findings was carried out by MALDI-TOF mass spectrometry using EpiTYPER by MassArray on bisulfite-converted DNA as described previously (20, 21).

**HELP-tagging analysis for HS27 stromal cells**

HS27a stromal cells were grown to 80% confluence in RPMI1640 and then cocultured with KG1a cells (CD45⁻) at a ratio of 1:3 for 48 hours. Adherent cells were washed, trypsinated, and depleted of residual KG1a cells by CD45 MicroBeads (Miltenyi Biotec Inc.). DNA was isolated from HS27a cells and used for high resolution HELP tagging assay as previously performed (22, 23) for methylation status of 1.8 million CpGs.

**Gene expression analysis**

Gene expression data were obtained using Affymetrix Human Genome U133A 2.0 or Plus2 GeneChips; mRNA isolation, labeling, hybridization, and quality control were carried out as described before (24).

**Hematopoietic progenitor cell assays and flow cytometry**

Hematopoietic progenitor colony formation was determined by clonogenic assays in methylcellulose, as in our previous studies (25, 26). Bone marrow-stroma cells from controls and MDS patients were expanded and treated with either 0.5 μmol/L 5-Aza (CD45⁻) containing 500 healthy CD34⁺ cells was layered on top. At day 10, colony formation was assessed, and FACS analysis conducted using CD34−, PE, CD45−, PE/Cy7, CD14−, PB, CD11b−, APC, glycoporphin A−, PE-Cy5, CD71−, FITC β-catenin antibodies.

**IHC for FRZB**

MDS patient stromal cells were cultured onto four-chambered slides with two chambers having received no 5-Aza treatment and...
two chambers receiving 5-Aza (0.5 μmol/L for 5 days). IHC for FRZB was performed with FPR-3 (H-170) rabbit mAb (catalog no. sc-13941; Santa Cruz Biotechnology) and matched isotype control diluted to 1:100, for 30 minutes.

**Mice**

The doxycycline-inducible constitutively active β-catenin mice (K/H2-Col1A1-tetO-CTNNI1S33Y/Rosa-rTa; S33Y) were generated by the Hochedlinger laboratory (details in Supplementary Methods; ref. 27). These mice were crossed to transgenic mice expressing a NUP98–HOXD13 fusion gene in hematopoietic tissues, resulting in a transgenic NUP98–HOXD13 mouse with doxycycline-inducible constitutively active β-catenin. All experimental mice were heterozygous in both Col1a1 (S33Y under tetOP) and Rosa26 (rTa) loci and for the NUP98–HOXD13 transgene. Transgenic primary NUP98–HOXD13 mice ages 14 to 18 months were used for analysis and were verified to display clinical hallmarks of MDS and cytopenias. Details on FACS analysis and bone marrow transplantation are provided in supplementary methods.

**Analysis of WNT signature in MDS cohort**

WNT target genes obtained from a comprehensive database (http://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes) that were expressed in MDS derived and healthy CD34+ cells were analyzed in a large cohort of gene expression profiles (28) and used to calculate a composite score.

**Primary MDS mesenchymal cell isolation and RNA-seq analysis**

Control bone marrow was obtained from donors for allo-genetic transplantation [median age: 45 (35–61)], after approval by the IRB of Erasmus Medical Center (29). Mesenchymal cells from human MDS patients were FACS sorted using the FACSAria III systems (BD Biosciences) with the following antibodies using optimized dilutions: CD45-PE-Cy7 (1:200), CD235a-BV421-A (1:100), CD271-PE (1:100), CD105-APC-Cy7 (1:50). Sorted cells were kept in TRIzol (Ambion). Smarter Ultra Low RNA Kit for Illumina Sequencing (Clontech) was used for cDNA synthesis according to the manufacturer’s protocol. Sample preparation, sequencing, demultiplexing, and alignment were performed as previously described (30) with modifications specific to the application of Smarter Kit. Details on analysis are provided in Supplementary Methods.

**Results**

**Primary stromal cells in MDS are characterized by aberrant hypermethylation**

Primary cultures of stromal cells were established from MDS bone marrow samples and controls (Supplementary Table S1 with clinical characteristics). The MDS samples included patients who had been treated (MDS Tx) and never been treated (MDS UnTx) with the DNMT inhibitor 5-Aza. Controls were age matched and had blood counts in the normal range. CD45–nonhematopoietic cells from the cultures were immunomagnetically sorted and used for DNA/RNA extraction after low passage numbers (up to three passages). Genome-wide cytose methylation was analyzed by the HELP assay, which uses differential methylation-specific digestion by HpaII and MspI followed by amplification, two color labeling and hybridization to quantitatively determine individual promoter methylation of 50,000 CpG loci covering 14,000 promoters (13, 31). Unsupervised clustering based on cytosine methylation profiles demonstrated that untreated MDS stromal cells were epigenetically distinct from healthy controls (Fig. 1A), whereas MDS stromal cells from 5-Aza–treated patients clustered closer to healthy controls. Next, to determine the qualitative epigenetic differences between these groups, we performed a supervised analysis of the respective DNA methylation profiles. A volcano plot comparing the differences between mean methylation of individual loci between MDS stromal cells and controls plotted against the significance [log (P value) based on t test] of the difference was used to represent these data shown in Fig. 1B. We observed that MDS stromal cells were characterized by aberrant hypermethylation when compared with controls (3626 hypermethylated vs. 306 hypomethylated loci in untreated MDS stromal cells). Comparison of 5-Aza–treated samples demonstrated a lesser degree of methylation and an epigenomic pattern similar to that in healthy controls (Fig. 1C and D).

Even though aberrant methylation in MDS stromal samples occurred genome wide (Fig. 1E), there was a significant enrichment at chromosomal regions chr13q15, chr7q32, chr4q21, chr7q31, chr3q13, chr2p12, and chr8q24 when compared with the genomic distribution of all HpaII fragments from the HELP array (P < 0.05; MDSig Program). Furthermore, to determine whether these hypermethylated loci shared any common DNA elements, we performed a search for TF-binding sites enriched in these regions. We observed a significant over-representation of binding sites for Sp1, NIF, MYB, and other TFs in differentially methylated regions in MDS stroma (P < 0.05; HOMER Program; Fig. 1F). Finally, we sought to determine if CpG islands were predominantly affected by differential methylation in MDS stromal cells. We observed that a large proportion of aberrantly methylated loci were located outside of CpG islands (test of proportions, P < 0.01; Fig. 1G), consistent with recent data demonstrating epigenetic changes at less CpG dense regions of the genome (32).

**Important functional pathways are epigenetically dysregulated in MDS stroma**

Gene expression analysis on MDS stromal cells also showed transcriptomic differences when compared to controls (Fig. 2A). Most transcriptional changes were seen in the untreated MDS stromal samples and consisted of aberrantly underexpressed genes (Fig. 2B). Because of cell limitations due to low passage numbers we did not get adequate RNA from samples MDS14 and MDS11. Samples MDS18 and MDS5 were thus used for gene expression analysis and clustered similarly to untreated and 5-Aza–treated status. There were very few differences between 5-Aza–treated stromal samples and healthy controls (Fig. 2C and D). Thus, these data demonstrated both methylocnic and transcriptomic changes in primary MDS stromal samples. Integrative analysis revealed that differentially expressed genes that were also accompanied by aberrant methylation belonged to important functional pathways, such as those controlling cell morphology, signaling, and transport (Supplementary Table S2). Determination of epigenetically regulated signaling pathways included those controlling WNT/β-catenin signaling, integrin signaling, and other
Figure 1.
Widespread epigenetic alterations are seen in MDS stroma. A, Unsupervised clustering of primary MDS stromal cells from untreated patients (MDS UnTx), MDS stromal cells from patients treated with 5-Aza (MDS Aza), and healthy controls shows that MDS UnTx stroma has a distinct DNA methylation profile (hierarchical clustering, wards). B, Volcano plot shows that most of differentially methylated genes in stroma from untreated patients are hypermethylated. C and D, Comparison of 5-Aza-treated stroma with Untx MDS stroma and healthy controls shows that 5-Aza-treated samples do not have increased numbers of hypermethylated loci. E, Circos plots show that aberrant hypermethylation (blue) occurs throughout the genome and is more frequent than aberrant hypomethylation (orange). F, TF-binding sites that are enriched at differentially methylated regions (DMR) are shown with motifs. G, DMRs in Untx MDS stroma were predominantly present in non-CpG island locations (87.9%) and were significantly different from the distribution of HpaII loci in the whole HELP array (61.8%; test of proportions, P < 0.001).
metabolic pathways and included WNT antagonists FRZB (SFRP3) and SFRP1 and cellular receptors belonging to the integrin and ephrin families (Fig. 2E; Supplementary Table S3).

FRZB is repressed and the Wnt/β-catenin pathway activated in highly purified mesenchymal cells from human MDS patients

Next, we sought to confirm the relevance of these epigenetic and transcriptional aberrancies in ex vivo expanded stromal cells. We interrogated a recently established transcriptome database of prospectively isolated, highly FACS purified CD45−/CD235−/CD31−/7AAD−/CD271+/CD105+ mesenchymal cells from a cohort of MDS patients (Fig. 3A; Supplementary Table S4; ref. 29). The mesenchymal nature of CD271+ cells was confirmed by their colony-forming unit-fibroblast (CFU-F) capacity and differential expression of mesenchymal, osteolineage, and HSPC-regulatory genes. Massive parallel RNA sequencing was performed on these purified mesenchymal cells in comparison to their normal counterparts obtained from allogeneic bone marrow donors (29).

FRZB (SFRP3) was significantly underexpressed in mesenchymal cells from MDS (n = 12) in comparison to normal controls (n = 10; Fig. 3B, t test, P = 0.02). This was further corroborated by significantly increased expression of the gene encoding β-catenin (CTNNB1; Fig. 3C; t test, P < 0.001). Together, the data confirm that reduced expression of SFRP3 in expanded stromal cells is of relevance to mesenchymal biology in human disease.

WNT pathway antagonists are epigenetically silenced in MDS stroma and can lead to activation of β-catenin in cocultured HSCs

Next, to further validate the hypermethylation and underexpression of FRZB, we evaluated whether the epigenetic changes observed in primary cells could be replicated in vitro. Human stromal HS-27 cells were cocultured with the leukemic cell line KG1a, and cytosine methylation changes were analyzed at a high resolution by next-generation sequencing-based HELP-Tagging assay. Coculture led to hypermethylation of various CpGs in the FRZB and SFRP1 promoters in stromal cells (Fig. 4A; Supplementary Fig. S1).
methylated CpGs were located in canonical CpG islands and 2kB flanking regions (CpG shores) in the promoter region. Other differentially methylated sites were also validated by sequencing and quantitative massarray epityper analysis (Supplementary Fig. S2). Underexpression of the WNT antagonist FRZB was also validated by qRT-PCR analysis in primary expanded MDS stromal samples (Fig. 4B). FRZB protein levels were shown to be epigenetically downregulated in an independent set of three MDS Stromal cultures (MDS 21–23) and increased after in vitro treatment with 5-Aza (Fig. 4C; Supplementary Fig. S3). Having shown underexpression of secreted WNT antagonist FRZB in MDS stroma, we next wanted to determine whether this can lead to activation of WNT/β-catenin in surrounding HSCs. We used specific siRNAs to knockdown FRZB in primary human MSCs (Fig. 4D) and cocultured them with primary marrow-derived CD34+ cells in methylcellulose. Intracellular flow cytometry demonstrated that coculture with FRZB knockdown stromal cells led to activation of nuclear β-catenin in CD34+, CD45+ hematopoietic cells (Fig. 4E and F).

Treatment with 5-Aza improves the ability of MDS stroma to support erythropoiesis

Having shown that MDS stroma contains hypermethylated loci that are not seen in 5-Aza treated samples, we wanted to test the efficacy of Aza treatment on the stroma. Primary MDS stromal samples (MDS19, 20) were pretreated with 5-Aza for 5 days and then cocultured with healthy human CD34+ cells. Coculture with MDS stromal cells from 2 patients (mock-treated, not exposed to 5-Aza) led to dysplastic colony formation (Fig. 4G and H). When primary MDS stroma was pretreated with 5-Aza the numbers of hematopoietic colonies increased, and colonies were of larger size than in mock-treated controls (Fig. 4G and H). Next, cocultured hematopoietic cells were collected and examined for differentiation markers by FACS. There was a significant increase in erythroid differentiation after coculture with Aza treated MDS stroma, as evident from increased percentages of proerythroblasts, basophilic, and mature erythrocytes (Fig. 4I). Furthermore, we grew low passage stromal cells from another MDS patient treated them with 5-Aza after FRZB knockdown. We observed that 5-Aza consistently led to increased erythroid differentiation in cocultured healthy CD34+ cells. The erythroid differentiation caused by 5-Aza was partially inhibited in the presence of FRZB knockdown (Supplementary Fig. S4), thus demonstrating a potential role of FRZB expression in 5-Aza-mediated effects on MDS stroma.

Activation of β-catenin leads to disease progression in vivo

Downregulation of the Wnt pathway antagonists FRZB and SFRP1 suggest that β-catenin activation may contribute to MDS pathogenesis. To test the role of activated β-catenin in the context of MDS, we utilized an established murine model of MDS, the NUP98–HOXD13 transgenic model (NHD13). This model recapitulates many of the salient features of MDS including pancytopenias accompanied by hypercellular or normocellular bone marrow at 4 to 7 months (33–36). Also, 12% to 17% of the marrow contains dysplastic erythroid, myeloid, and rare megakaryocytic cell types (33). Similar to patients with MDS, a significant cohort of the primary mice can progress and develop an aggressive AML. However, if the bone marrow of the NHD13 mice is transplanted, the recipient animals succumb to a fully penetrant form of MDS that rarely and only after 1 year progresses to AML (35). Although the NHD13 transplanted bone marrow cells engraft poorly, they still retain the clinical features of MDS (~10%–20% chimerism, data not shown) (35). To test if β-catenin can alter MDS disease, the NHD13 transgene was crossed into a tetracycline inducible and constitutively activated human β-catenin (KH2- Col1A1-tetO-CTNN1S33Y/Rosa-rtTA; S33Y) overexpression mouse model described previously (27).

We performed bone marrow transplants of C57BL6 (WT), S33Y, NHD13 or compound NHD13/S33Y cells into lethally irradiated CD45.1+ recipients (Fig. 5A). After equivalent engraftment was verified in the peripheral blood (Fig. 5B), mice were treated with doxycycline to constitutively activate β-catenin (S33YOE and NHD13/S33YOE) and were then followed for disease progression. At 4 months, flow cytometric analysis revealed that the NHD13/S33Y mice had an increase in mature

Figure 3.
Transcriptional analysis of SFRP5 and Wnt target genes in primary mesenchymal cells in human MDS. A, Highly purified CD45−CD235− 7AAD−CD34−CD45RO+ mesenchymal cells were FAC5-sorted and subjected to massive parallel RNA sequencing and quantitative mass-array epityper analysis (Supplementary Fig. S2). Other differentially methylated sites were also validated by sequencing. B, Reduced FRZB (SFRP5) expression was seen in MDS samples when compared with healthy controls (t test, P < 0.05). C, Increased expression of CTNNB1 in seen in MDS MSCs. *, P < 0.05; **, P < 0.001; MDS n = 12; normal controls (n = 10).
WNT antagonist FRZB is hypermethylated and underexpressed in MDS stroma and treatment of MDS stroma with 5-Aza increases hematopoietic activity. A, DNA methylation analysis by HELP-tagging assay shows hypermethylation of selected loci (marked by arrows) in the FRZB promoter in the HS27 stromal cells that are cocultured with KG1a cells. Dark green denotes CpG islands, whereas light green denotes CpG shores. B, qRT-PCR shows decreased expression of FRZB in untreated MDS samples (n = 4) when compared with control stroma (n = 4) or Aza-treated MDS (n = 2; t test, P < 0.05). C, IHC shows increased expression of FRZB in MDS stroma treated with 5-Aza (0.5 μmol/L for 5 days). D, siRNA-mediated knockdown of FRZB was achieved in primary MSCs. Coculture with FRZB knockdown MSCs led to increased nuclear β-catenin in CD45+ cells (representative image shown in E; t test, P < 0.01, N = 2, F). Healthy CD34+ cells were grown with MDS stromal cells (MDS19 and MDS20) in methylcellulose media. G, MDS stromal cells (MDS19 and MDS20) that were pretreated with 5-Aza for 5 days led to greater colony formation from healthy CD34 cells (t test, P < 0.001). H, Dysplastic colonies seen after coculture of healthy CD34 cells with MDS stroma (left). 5-Aza pretreatment leads to increased size of colonies (right). I, FACS analysis of cocultured cells shows increase in glycophorin A-positive cells in 5-Aza–treated MDS stromal cocultures (n = 2; t test, P < 0.5). J, Increased percentages of all stages of erythroid cells are seen in 5-Aza–pretreated stromal cocultures.
and immature myeloid cells among the donor cells (Fig. 5C and D). In addition, the S33Y/E, NHD13 and NHD13/S33Y/E animals all had reduced white blood cell counts compared with the control mice (Fig. 5E). Surprisingly, the NHD13/S33Y/E succumbed to a lethal myeloid disease with a median latency of 388 days whereas other mice were followed until 451 days and then sacrificed to assess their phenotype (Fig. 5F). The NHD13/S33Y/E mice mainly died of a myeloid leukemia (5 of 8) with enlarged spleens and increased white blood cell counts whereas the other mice died of an MDS-like phenotype (3 of 8; Fig. 5G and H). These data suggest that activation of β-catenin drives a lethal and aggressive myeloid disease with an increased likelihood of transformation.

WNT activation signature is present in MDS and is marker of adverse prognosis

Next, we wanted to determine whether WNT pathway activation was seen in independent cohort of MDS samples. The expression patterns of known WNT pathway targets (Supplementary Table S5) was determined in transcriptomic data from a cohort of 183 MDS marrow CD34+ cells and 17 healthy controls. Numerous WNT targets including MYC, JUN, FZD7, and others (Fig. 6A; Supplementary Fig. S5) were found to be overexpressed in MDS samples (18/39 overexpressed; Supplementary Fig. S5) when compared to healthy controls and demonstrated a trend towards elevated mean expression in higher risk preleukemic refractory anemia with excess of blasts.
Because WNT/β-catenin pathway controls numerous downstream genes, we next developed a composite signature based on degree of activation of all expressed downstream targets (Supplementary Table S5) and correlated it with clinical subtypes of MDS. We observed that the higher risk cases of MDS with higher blast counts (RAEB) and higher propensity of transformation to AML had the highest levels of WNT pathway activation when compared to lower risk refractory anemia (RA) and refractory anemia with ringed sideroblasts (RARS) subtypes (Fig. 6B; t test, \( P < 0.05 \)). Correlation with overall survival also revealed that a higher WNT activation signature correlated with shorter overall survival with a median of 2.95 years in patients with high levels of WNT activation vs 5.24 years in patients with low WNT expression (Fig. 6C; log-rank \( P = 0.037 \)). These data taken together (Fig. 7A and B) with in vivo murine data demonstrate that stroma-mediated WNT activation is a pathogenic and prognostic event in MDS progression.

**Discussion**

We show that marrow stroma in patients with MDS is aberrantly hypermethylated and that these marks are abrogated in stroma derived from 5-Aza–treated patients. These findings demonstrate that the marrow microenvironment is also affected by epigenetic alterations and contributes to the MDS pathophysiology. These data raise the possibility that the marrow niche may also be targeted by epigenetically active drugs (DNMT inhibitors).

Even though numerous studies have highlighted the importance of the marrow microenvironment in the pathogenesis of bone marrow failure and malignant disorders, it is not understood how the stroma is reprogrammed to perpetuate the diseased phenotypes. Previous studies have evaluated stromal cells in MDS for cytogenetic alterations and have generally failed to show a high incidence of these abnormalities (21, 37–39). A recent study showed cytogenetic abnormalities in 16% of mesenchymal stem cells in MDS, but none of them showed any mutations (10). Nevertheless, the stroma is presumed to participate in the pathogenesis of ineffective hematopoiesis in MDS and has been shown previously to have altered gene expression patterns (40). Our findings of epigenetic alterations that affect important regulatory pathways provide a molecular basis to explain microenvironmental reprogramming in MDS. A different recent study reported that deletion of Dicer in the bone marrow niche in mice induced MDS/AML with ineffective hematopoiesis and dysmorphic hematopoietic cells (8). Dicer is involved in microRNA processing, providing further evidence that epigenetic dysregulation in the microenvironment can lead to hematopoietic alterations.
Figure 7.
Proposed model of stroma-mediated activation of WNT/β-catenin signaling in MDS. A, Aberrant methylation and underexpression of WNT/β-catenin antagonists FRZB and SFRP1 is seen in MDS stroma. Activation of β-catenin leads to disease progression in vivo and a WNT/β-catenin activation signature correlates with advanced disease in human samples. B, 5-Aza-treated MDS stroma samples have higher FRZB levels and in vitro treatment can lead to increased erythroid differentiation. LSC, leukemia stem cells.

Another recent report showed that an activating mutation of β-catenin in the osteoblastic niche can lead to an MDS and AML-like phenotypes in vivo (9). Activation of β-catenin in the osteoblastic niche was present in a large proportion of MDS patients and led to upregulation of Jagged in osteoblasts, which resulted in altered hematopoiesis via activation of Notch signaling in hematopoietic stem cells (9). Building on these findings, our study provides a mechanism for β-catenin activation in the niche. We demonstrate reduced expression of FRZB (SFRP3) via epigenetic silencing and consequent activation of the Wnt/B-catenin pathway, not only in ex vivo expanded cells but also in primary mesenchymal cells directly isolated from patient marrows. These findings are, to our knowledge, the first describing molecular congruence between ex vivo expanded stromal cells and their in situ counterparts and provide novel insights into the lineage hierarchy of the stromal system in human bone marrow. The data support the view that a small population of mesenchymal colony-forming stem and progenitor cells (CFU-F, comprising <2% of CD271+ cells in MDS; Chen and colleagues, manuscript submitted) is epigenetically altered in human MDS and maintains the CD271+ reticulocyte network in the bone marrow.

In addition, we demonstrate that β-catenin activation within hematopoietic cells can result in an aggressive myeloid disease and transformation using a model of MDS, providing a complementary mechanism for disease progression (Fig. 7). Our study is in line with other reports that suggest that β-catenin can cooperate with HOXA9/MEIS1 in GMPs and with BCR-ABL in CML models (41, 42). Another study demonstrated that haploinsufficiency of del(5q) genes, Egr1 and Apc, cooperate with Tp53 loss to induce AML in mice (43). Our study shows that β-catenin activation in the NHD mouse model of MDS model can cause transformation of hematopoietic cells. It has also been demonstrated that hematopoietic cells are sensitive to differential levels of cell intrinsic WNT signaling, with varying effects of expression levels on HSCs, myeloid precursors, and T lineage precursors during hematopoiesis (44). Previous studies have also suggested that constitutive β-catenin activation in normal hematopoietic stem and progenitor cells resulted in a block in differentiation and rapid lethality of the mice (45, 46). In contrast to this study, our model failed to alter hematopoietic differentiation or cause lethality, unless we combined our model with NHD13. One potential explanation for these disparate findings is that our model utilizes a different promoter to activate β-catenin (Col1A1/Rosa vs. the endogenous promoter). Therefore, we suggest that this mouse model allows for the study of β-catenin activation without the toxicity that was previously observed. The NHD13 model used in our studies demonstrates dysplasia and cytopenias coupled with progression to accelerated disease, thus serving as a representative model of human MDS (36, 47, 48). Taken together, we provide a novel mechanism for epigenetic suppression of the WNT pathway antagonists FRZB and SFRP1 in the niche, which can be epigenetically reversed by 5-Aza treatment.

5-Aza and decitabine are inhibitors of DNMT, approved for treatment of MDS. These drugs lead to hematopoietic improvements and 5-Aza has been show to prolong survival. Even though the mechanism of action is presumed to be reversal of aberrant DNA methylation in hematopoietic cells, studies so far have not been able to correlate aberrant hypermethylation in pretreatment samples with response. All of these studies have examined hematopoietic cells - our data raise the possibility that stromal methylation may also have to be considered as a factor in the therapeutic efficacy of these drugs. In addition to altering transcription of various important genes in the stromal compartment, hypomethylation caused by 5-Aza may also affect TF binding dynamics. We observed that binding sites of many TFs with roles in hematopoiesis such as Myb and NF1 were enriched in differentially methylated regions in MDS stroma. Thus, taken together, our results demonstrate that MDS stromal cells have widespread epigenetic alterations that

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modify the disease pathophysiology and can be targeted by DNMT inhibitor treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.D. Bhagat, S. Chen, M. Bartenstein, D. Von Ahrens, G.S. Choudhary, N. Ramachandra, I. Mantzaris, V. Sukriehan, P. Bhagat, O. Giricz, A. Wickrema, C. Yeung, K. Gritsman, P. Aplan, J. Zhang, S. Mukherjee, A. Pellagatti, J. Boulwood, B. Will, M.H.G.P. Raaijmakers, H.I. Deeg
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References
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