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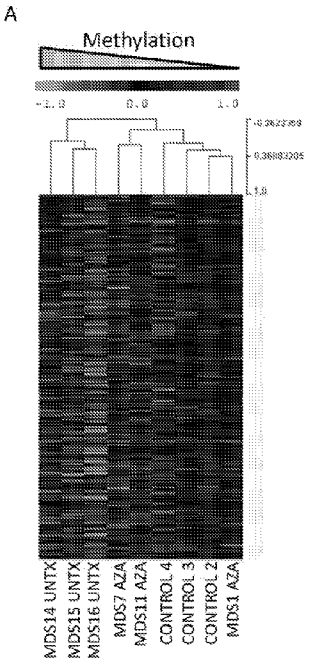
(54) Title: METHODS AND COMPOSITIONS FOR THE TREATMENT OF MYELODYSPLASTIC SYNDROME

(57) Abstract: The present invention provides methods and compositions for the treatment and prevention of myelodysplastic syndrome (MDS), and for the prevention of acute myeloid leukemia (AML) in MDS patients. In some embodiments such methods comprise administration of active agents that are Wnt/βcatenin pathway inhibitors. In some such methods delivery of such active agents is targeted to bone marrow stromal cells. Some such methods also involve first determining whether a subject is a candidate for treatment.



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METHODS AND COMPOSITIONS FOR THE TREATMENT OF MYELODYSPLASTIC SYNDROME

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of U.S. Provisional Patent Application No. 62/327,972 filed on April 26, 2016, the contents of which are hereby incorporated by reference in their entirety.

STATEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made with government support under grant numbers HL116336, DK103961, and DK101989 awarded by the National Institutes of Health. The government has certain rights in the invention.

INCORPORATION BY REFERENCE

[0003] For countries that permit incorporation by reference, all of the references cited in this disclosure are hereby incorporated by reference in their entireties. In addition, any manufacturers' instructions or catalogues for any products cited or mentioned herein are incorporated by reference. Documents incorporated by reference into this text, or any teachings therein, can be used in the practice of the present invention. Documents incorporated by reference into this text are not admitted to be prior art.

BACKGROUND

[0004] Myelodysplastic syndromes (MDS) comprise a heterogeneous group of acquired clonal bone marrow (BM) disorders characterized by varying degrees of cytopenias, morphological and functional abnormalities of hematopoietic cells and the risk of transformation into acute myeloid leukemia (AML) (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 micro-environmental defects also contribute to ineffective hematopoiesis and, hence, progression of the disease (3,4).

[0005] The bone marrow microenvironment consists of a mixture of different cell types – mostly represented by stromal or 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 beta-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. As such there remains a need in the art to elucidate the mechanisms underlying stromal dysfunction in human MDS. Furthermore, there remains a need in the art to provide methods and compositions for treating or preventing such stromal cell dysfunction in MDS. The present invention addresses these needs.

SUMMARY OF THE INVENTION

[0006] Some of the main aspects of the present invention are summarized below. Additional aspects are described in the Detailed Description of the Invention, Examples, Drawings, and Claims sections of this disclosure. The description in each section of this disclosure is intended to be read in conjunction with the other sections. Furthermore, the various embodiments described in each section of this disclosure can be combined in various different ways, and all such combinations are intended to fall within the scope of the present invention.

[0007] The present invention is based, in part, upon certain new discoveries that are described further in the "Examples" section of this patent disclosure. In particular, it has now been found that aberrant epigenetic DNA methylation in bone marrow stromal cells leads to activation of Wnt/βcatenin signaling pathways and contributes to the progression of MDS, and that inhibition of such Wnt/βcatenin signaling can reverse MDS progression. Building on these discoveries, the present invention provides certain new and improved methods and compositions for the treatment of MDS.

[0008] Accordingly, in one embodiment the present invention provides a method of treating or preventing MDS in a subject in need thereof, the method comprising contacting the subject's bone marrow stromal cells with an effective amount of an active agent that inhibits the Wnt/βcatenin signaling pathway.

[0009] In another embodiment the present invention provides a method of treating or preventing MDS, the method comprising: administering to a subject in need thereof an effective amount of an active agent that inhibits the Wnt/βcatenin signaling pathway in bone marrow stromal cells.

[00010] In a further embodiment the present invention provides a method of preventing AML in a subject that has, or that is suspected of having, MDS, the method comprising contacting the subject's bone marrow stromal cells with an effective amount of an active agent that inhibits the Wnt/βcatenin signaling pathway.

[00011] In yet another embodiment the present invention provides a method of preventing AML in a subject in need thereof, the method comprising: administering to a subject that has, or that is suspected of having, MDS, an effective amount of an active agent that inhibits the Wnt/βcatenin signaling pathway in bone marrow stromal cells.

[00012] In some of such embodiments the active agent is selected from the group consisting of Wnt-C59, LGK-974, X-AV-939, ICG-001, IWR-1-endo, IWR-1-exo. KY02111, IWP-2, FH535, WIK14, IWP-L6, PNU-74654, CCT036477, OMP-18R5, dimethoxy curcumin, Dickkopf(DKK), Axin, FRZB, SFRP1, and SFRP3. For example, in some embodiments the active agent is a Porcn inhibitor, such as Wnt-C59 or LGK-974.

[00013] In some of such embodiments the active agent is a DNA methyltransferase (DNMT) inhibitor, such as 5-azacytidine or decitabine.

[00014] In some embodiments the active agent is targeted to stromal cells in the bone marrow of the subject by localized delivery to the bone marrow niche (such as the bone marrow stromal cell niche). In some embodiments the active agent is targeted to stromal cells in the bone marrow of the subject using a targeting agent, such as an antibody or antibody-fragment that binds to an antigen on the surface of bone marrow stromal cells.

[00015] In some embodiments the subject is a mammal. In some embodiments the subject is a human. In some embodiments the subject has a severe form of MDS, and/or is determined to be at high risk of progression to AML. In some embodiments the subject has MDS that comprises refractory cytopenia with unilineage dysplasia (RCUD), refractory anemia with ringed sideroblasts (RARS), refractory cytopenia with multilineage dysplasia (RCMD), refractory anemia with excess blasts-1 (RAEB-1), refractory anemia with excess blasts-2 (RAEB-2), myelodysplastic syndrome associated with isolated del(5q), or unclassified myelodysplastic syndrome (MDS-U).

[00016] In some embodiments the active agent is administered to the subject at any suitable dose, for example as determined using a suitable dosing study, such as a dose-escalation study. For example, in some of those embodiments where the active agent is LGK974 or Wnt-C59, the active agent may be administered to the subject at a dose from about 0.1mg/kg/day to about 1000 mg/kg/day, or from about 0.5mg/kg/day to about 500 mg/kg/day, or more preferably from about 5mg/kg/day to about 50 mg/kg/day.

[00017] In some embodiments the active agents may be administered to a subject in conjunction with one or more additional agents useful in the treatment of MDS, such as, for example, cytarabine (ara-C), idarubicin, topotecan, fludarabine, 5-azacytidine (Vidaza), decitabine (Dacogen), thalidomide, lenalidomide (Revlimid), anti-thymocyte globulin (ATG), cyclosporine, granulocyte colony stimulating factor (G-CSF, Neupogen, or filgrastim), granulocyte macrophage-colony stimulating factor (GM-CSF, Leukine, or sargramostim), pegfilgrastim (Neulasta), erythropoietin (Epo or Procrit), darbepoetin alfa (Aranesp), or oprelvekin (Neumega, interleukin-11, or IL-11).

[00018] Similarly, in some embodiments the active agents may be administered to a subject in conjunction with treating the subject with one or more additional treatment regimens useful in the treatment of MDS, such as, for example, bone marrow transplantation, stem cell transplantation, and/or chemotherapy.

[00019] In some embodiments the present invention provides various compositions, such as pharmaceutical compositions that may be useful in the treatment or prevention of MDS, or in the prevention of AML. In some embodiments such compositions may comprise one or more of the active agents listed above. In some embodiments such compositions may comprise a

Wnt/βcatenin signaling pathway inhibitor (such as one or more of the active agents listed above), and a targeting agent for targeted delivery of the active agent to bone marrow stromal cells. For example, in some embodiments, such a targeting agent may comprise an antibody, or antibody fragment, that can bind to an antigen on the surface of bone marrow stromal cells.

[00020] In some embodiments the present invention provides methods for determining whether a subject is a candidate for treatment using any of the compositions or methods provided herein. In some of such embodiments the subject is also subsequently treated using the compositions and/or methods provided herein.

[00021] For example, in some embodiments the present invention provides methods for determining whether a subject is a candidate for treatment with a composition or method as described herein, wherein such methods involve performing an assay to detect and/or measure DNA methylation in bone marrow stromal cells, or activity of Wnt/βcatenin signaling pathways in bone marrow stromal cells, or one or more of the other indicators of MDS or MDS progression or MDS prognosis described in the Examples section of this patent application, and wherein if the subject exhibits higher levels of DNA methylation in his or her bone marrow stromal cells, or higher levels of activity of Wnt/βcatenin signaling pathways in his or her bone marrow stromal cells, or higher levels of FRZB (SFRP3) or SFRP1 expression in his or her bone marrow stromal cells, as compared to the levels found in control bone marrow stromal cells (such as control bone marrow stromal cells from a subject that does not have MDS, or from a subject that has been successfully treated for MDS, or from a subject or cells that have been treated with a DNMT inhibitor, such as 5-azacytidine or decitabine), then the subject is a candidate for treatment. Suitable assays for measuring DNA methylation in bone marrow stromal cells are provided in the Examples section of this patent disclosure. In those embodiments that involve performing an assay to detect and/or measure DNA methylation in the bone marrow stromal cells, methylation may be measured or assessed genome wide, or may be measured or assessed at one or more chromosomal regions selected from the group consisting of chr12q15, chr5q32, chr4q21, chr7q31, chr3q13, chr2p12 and chr8q24, or may be measured or assessed outside of CpG islands. Similarly, a subject may be determined to be a candidate for treatment if the candidate exhibits DNA hyper-methylation in bone marrow stromal cells genome-wide, or at one or more chromosomal regions selected from the group consisting of

chr12q15, chr5q32, chr4q21, chr7q31, chr3q13, chr2p12 and chr8q24, or outside of CpG islands.

[00022] Each of the embodiments described in the above paragraphs, or elsewhere herein, that involves performing an assay to determine whether a subject is a candidate for treatment, may, in some embodiments, also comprise a preliminary step of obtaining a sample of bone marrow stem cells from the subject, or obtaining a sample of genomic DNA from bone marrow stem cells from the subject.

[00023] Similarly, each of the embodiments described in the above paragraphs, or elsewhere herein, that involves performing an assay to determine whether a subject is a candidate for treatment, may, in some embodiments, also comprise a subsequent step of treating the subject using one of the methods or compositions provided herein. For example, in some embodiments the methods of the present invention involve first determining whether a subject has DNA hyper-methylation in his or her bone marrow stromal cells (for example by comparison to suitable control cells), and then if the subject does have DNA hyper-methylation in his or her bone marrow stromal cells, subsequently treating the subject with one of the active agents described herein, such as, for example, a DNA methyltransferase (DNMT) inhibitor (such as 5-azacytidine or decitabine).

[00024] These and other embodiments of the present application are described further in the following sections of this patent application.

BRIEF DESCRIPTION OF THE FIGURES

[00025] **Fig. 1A-G**. Widespread Epigenetic alterations are seen in MDS stroma. Unsupervised clustering of primary MDS stromal cells from untreated patients (MDS UnTx), MDS stromal cells from patients treated with 5-Azacytidine (MDS Aza) and healthy controls shows that MDS UnTx stroma has a distinct DNA methylation profile (Hierarchical clustering, Wards) (**Fig. 1A**). Volcano plot shows that most of differentially methylated genes in stroma from untreated patients are hyper-methylated (**Fig. 1B**). 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 (**Fig. 1C, D**). Circos plots show that aberrant hypermethylation occurs throughout the genome and is more frequent than aberrant hypomethylation. (**Fig. 1E**). Transcription factor binding sites that are enriched at differentially

methylated regions (DMRs) are shown with motifs (**Fig. 1F**). 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 Value<0.001) (**Fig. 1G**).

[00026] **Fig. 2A-E**. Widespread transcriptomic alterations are seen in MDS stroma: Unsupervised hierarchical clustering of 3 primary untreated MDS stromal cells (MDS UnTx), MDS stromal cells from patients treated with 5-Azacytidine (MDS Aza) and healthy controls shows that MDS stroma has distinct gene expression profiles (**Fig. 2A**). Volcano plot shows that the majority of differentially expressed genes in untreated MDS stroma are underexpressed (**Fig. 2B**). Comparison of 5-Aza treated with Untx MDS and healthy controls shows that 5-Aza treated samples are similar to controls and do not have increased numbers of aberrantly expressed genes (**Fig. 2C, D**). Ingenuity functional pathway analysis of signaling pathways that are differentially expressed and differentially methylated between Untx MDS and control samples or UnTx and Aza treated samples (**Fig. 2E**).

[00027] **Fig. 3A-D**. Transcriptional analysis of SFRP3 and Wnt target genes in primary mesenchymal cells in human .MDS: Highly purified CD45-CD235-7AAD-CD31-CD271+CD105+ mesenchymal cells were FACS-sorted (**Fig. 3A**) and subjected to massive parallel RNA sequencing. Reduced FRZB (SFRP3) expression was seen in MDS samples when compared to Healthy controls (**Fig. 3B**) (TTest, P Value<0.05). Increased expression of downstream targets CTNNB1 and JAG1 in seen in MDS MSCs (**Fig. 3C-D**). * $p< 0.05$, ** $p<0.001$; MDS n=12, normal controls n=10.

[00028] **Fig. 4A-J**. WNT antagonist FRZB is hypermethylated and underexpressed in MDS stroma and treatment of MDS stroma with 5-Aza increases hematopoietic activity: 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 co-cultured with KG1a cells. Dark gray denotes CpG islands, while light gray denotes CpG shores (**Fig. 4A**). qRT-PCR shows decreased expression of FRZB in untreated MDS samples (n=4) when compared to control stroma (n=4) or Aza treated MDS (n=2) (T test, P value<0.05) (**Fig. 4B**). Immunohistochemistry shows increased expression of FRZB in MDS stroma treated with 5-Aza (0.5uM for 5 days) (**Fig. 4C**). siRNA mediated knockdown of FRZB was achieved in primary MSCs (**Fig. 4D**). Co-culture with FRZB knockdown MSCs led to increased nuclear β-catenin in

CD45+ cells (Representative image shown in **Fig. 4E**; T Test, P Value <0.01, N=2, **Fig. 4F**). Healthy CD34+ cells were grown with MDS stromal cells (MDS19 and MDS20) in methylcellulose media. 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 value <0.001) (**Fig. 4G**). Dysplastic colonies seen after co-culture of healthy CD34 cells with MDS stroma (**Fig. 4H**, left panel). 5-Aza pre-treatment leads to increased size of colonies (**Fig. 4H**, right panel). FACS analysis of co-cultured cells shows increase in Glycophorin A positive cells in 5-Aza treated MDS stromal co-cultures (n=2, T test, P value<0.5) (**Fig. 4I**). Increased percentages of all stages of erythroid cells are seen in 5-Aza pretreated stromal co-cultures (**Fig. 4J** right panel, compared to **Fig. 4J** left panel).

[00029] **Fig. 5A-H**. Constitutive activation of β-catenin in an MDS model accelerates myeloid disease progression. (**Fig. 5A**) Experimental scheme demonstrating donor bone marrow C57BL6; WT, S33YO/E, NHD13 and NHD13/S33YO/E mice. These cells were transplanted, allowed to engraft into congenic CD45.1 recipients for 1 month and then fed doxycycline feed (**Fig. 5B**) CD45.1 recipient mice were assessed for donor cell chimerism in the peripheral blood before doxycycline (Dox) addition (n=3 per group, TTest, *P Value<0.05, **P Value<0.01, ***P Value<0.001). (**Fig. 5C**) Representative Mac1, Gr1, and c-Kit flow cytometric staining of peripheral blood from 4 months post engraftment. Gate numbering represents Mac+Gr1+ (1), Mac+Gr1Low (2), Mac+Gr1- (3), Mac+c-Kit+ (4). (**Fig. 5D**) Quantification of flow cytometric analysis and gating as described in (**Fig. 5C**), (C57BL6; WT n=5, S33YO/E; n=5, NHD13 n=5, and NHD13/S33YO/E n=6, TTest, *P Value<0.05, **P Value<0.01, ***P Value<0.001). (**Fig. 5E**) White blood cell (WBC) counts in peripheral blood 4 months post-transplant, (C57BL6; WT n=5, S33YO/E; n=5, NHD13 n=5, and NHD13/S33YO/E n=7, TTest, *P Value<0.05, **P Value<0.01, ***P Value<0.001) (**Fig. 5F**) Kaplan-Meier curve of bone marrow transplantation described in (**Fig. 5A**), C57BL6; WT n=5, S33YO/E; n=5, NHD13 n=5, and NHD13/S33YO/E n=8, TTest, *P Value<0.05, **P Value<0.01, ***P Value<0.001). (**Fig. 5G**) Spleen weights from moribund mice or mice sacrificed at 463 day endpoint analysis, C57BL6; WT n=5, S33YO/E; n=4, NHD13 n=4, and NHD13/S33YO/E n=8, TTest, *P Value<0.05, **P Value<0.01, ***P Value<0.001) (**Fig. 5H**) WBC from moribund mice or mice sacrificed at 463 day endpoint analysis, C57BL6; WT n=5, S33YO/E; n=3, NHD13 n=4, and NHD13/S33YO/E n=6, TTest, *P Value<0.05, **P Value<0.01, ***P Value<0.001). All statistical analyses were compared to control mice or between NHD13 and NHD13/S33YO/E.

[00030] **Fig. 6A-C**. WNT activation is seen in higher risk subtypes of human MDS and is associated with worse overall survival: Expression of WNT target genes were evaluated in 183 MDS marrow derived CD34+ samples when compared to healthy controls and representative candidates are shown (**Fig. 6A**). WNT score based on degree of activation of target genes is significantly elevated in higher risk RAEB subtypes when compared to lower risk RA and RARS subtypes (TTest, * P Value<0.05)(**Fig. 6B**). MDS patients with high WNT score (above median) have a worse overall survival (median survival = 2.95 years) when compared to those with low WNT score (median survival = 5.24 years) (Log rank P Value =0.037) (**Fig. 6C**).

[00031] **Fig. 7A-B.** Proposed model of stroma mediated activation of WNT/β-Catenin signaling in MDS: 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 (**Fig. 7A**). 5-Azacytidine treated MDS stroma samples have higher FRZB levels and *in vitro* treatment can lead to increased erythroid differentiation (**Fig. 7B**). (LSC, Leukemia stem cells).

[00032] **Fig. 8.** SFRP1 methylation with co-culture. DNA methylation analysis shows hypermethylation of selected loci (marked by arrows) in the SFRP1 promoter in HS27 stromal cells that are co-cultured with KG1a cells.

[00033] **Fig. 9**. Validation of methylation changes with stroma and leukemic cell co-culture. DNA methylation analysis shows hypermethylation of selected loci (marked by arrows) in the TNF-alpha promoter in the HS27 stromal cells that are co-cultured with KG1a cells. Hypermethylation was confirmed by Massarray bisulfite epityper analysis (lower panel).

[00034] **Fig. 10.** FRZB upregulation in MDS stroma after 5-Azacytidine (5-Aza) treatment. Immunohistochemistry shows increase expression of FRZB in MDS stroma treated with 5-Aza (0.5μM) for 5 days.

[00035] **Fig. 11.** The expression of WNT signature genes in MDS and control marrow derived CD34+ cells is shown in the heatmap. The genes that are significantly differentially expressed in MDS when compared to controls are marked with asterix. Subtypes of MDS are indicated below the heatmap.

[00036] **Fig. 12A-F.** MDS disease is reversed after *in vivo* administration of a Wnt/β-catenin pathway inhibitor (Porcn C59 or LGK974). (**Fig. 12A**) Scheme for Porcn inhibitor treatment in 6 month old NHD13 MDS. (**Fig. 12B**), Placebo/untreated n=3, MDS NHD13 (n=5). Mice were bled at day 0 and then administrated oral gavage for 5mg/kg per day for six days and bled again on day 7, white blood cell (WBC), (**Fig. 12C**) Platelets, (**Fig. 12D**) Hematocrit were analyzed. (**Fig. 12E**) Flow cytometric analysis gated on Mac1+ or Gr1+. (**Fig. 12F**). Intracellular flow cytometry for β-catenin comparing bone marrow cells before treatment (pre) to the same animal after five days of Porcn inhibitor treatment. Percentage of β-catenin positive cells.

DETAILED DESCRIPTION OF THE INVENTION

[00037] The main embodiments of the present invention are described in the above Summary of the Invention section of this patent application, as well as in the Examples and Claims sections of this application. However, this Detailed Description section provides certain additional description relating to the compositions and methods of the present invention, and is intended to be read in conjunction with all other sections of the present patent application.

[00038] The headings provided throughout this patent specification are not limitations of the various aspects or embodiments of the invention, which should be understood and interpreted by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification in its entirety.

[00039] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention is related. For example, *The Dictionary of Cell and Molecular Biology* (5th ed. J.M. Lackie ed., 2013), the *Oxford Dictionary of Biochemistry and Molecular Biology* (2d ed. R. Cammack et al. eds., 2008), and *The Concise Dictionary of Biomedicine and Molecular Biology* (2d ed. P-S. Juo, 2002) can provide one of skill with general definitions of some terms used herein.

[00040] As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents, unless the context clearly dictates otherwise. The terms "a" (or "an") as well as the terms "one or more" and "at least one" can be used interchangeably.

[00041] Furthermore, "and/or" is to be taken as specific disclosure of each of the two specified features or components with or without the other. Thus, the term "and/or" as used in a phrase such as "A and/or B" is intended to include A and B, A or B, A (alone), and B (alone). Likewise, the term "and/or" as used in a phrase such as "A, B, and/or C" is intended to include A, B, and C; A, B, or C; A or B; A or C; B or C; A and B; A and C; B and C; A (alone); B (alone); and C (alone).

[00042] Units, prefixes, and symbols are denoted in their Système International de Unites (SI) accepted form. Numeric ranges are inclusive of the numbers defining the range. Where a numeric term is preceded by "about," the term includes the stated number and values ±10% of the stated number.

[00043] Wherever embodiments are described with the language "comprising," otherwise analogous embodiments described in terms of "consisting of" and/or "consisting essentially of" are included.

[00044] An "active agent" is an agent (e.g. a small molecule compound, a protein/polypeptide, or a nucleic acid molecule) as described and/or claimed herein, for example an inhibitor of a Wnt/βcatenin signaling pathway, or a DNA methylation inhibitor. Examples of specific active agents are provided in the "Summary of the Invention" and "Claims" sections of this patent application. It is also contemplated that, in each of the embodiments of the present patent disclosure that involve use of specified active agents, analogues, variants, or derivatives of each of such specified active agents can be used. One of skill in the art can readily determine whether an analogue, variant, or derivative of any of such specified active agent is suitable for use in accordance with the compositions and methods of the present invention, for example based on whether the analogue, variant, or derivative has one or more of the desired activities described herein, such as, for example, inhibition of Wnt/βcatenin signaling in bone marrow stromal cells, and/or inhibition of DNA methylation in bone marrow stromal cells.

[00045] The terms "inhibit," "block," and "suppress" are used interchangeably and refer to any detectable and/or statistically significant decrease in the specified parameter (e.g. biological activity), including full blocking of the specified parameter /biological activity. Such decreases may be measured in relation to a suitable control.

[00046] The term "inhibitor" refers to an agent (e.g. a small molecule compound, a protein/polypeptide, or a nucleic acid molecule) that can be used to achieve a statistically significant decrease in biological activity, including full blocking of the activity.

[00047] The terms "increased" or "elevated" or "higher" are used interchangeably herein and refer to any detectable and/or statistically significant increase in the recited parameter or activity. Such increases may be measured in relation to a suitable control.

[00048] The term "hyper-methylated" is used herein consistent with the usual meaning of this term in the art. In some embodiments the term hyper-methylated refers to a level of methylation that is higher, to a statistically significant degree, than a normal level of methylation and/or the level of methylation in a suitable control.

[00049] By "subject" or "individual" or "patient" is meant any subject, preferably a mammalian subject, for whom diagnosis, prognosis, or therapy is desired. Mammalian subjects include humans, domestic animals, farm animals, sports animals, and zoo animals including, *e.g.*, humans, non-human primates, dogs, cats, guinea pigs, rabbits, rats, mice, horses, cattle, and so on.

[00050] In some embodiments the subject may have or may have, be suspected of having, be at risk for developing, or have previously suffered from MDS or acute myeloid leukemia (AML).

[00051] Terms such as "treating" or "treatment" or "to treat" or "alleviating" or "to alleviate" refer to therapeutic measures that cure, slow down, lessen symptoms of, and/or halt progression of a disease or disorder, such as MDS or AML. Thus, those in need of treatment include those already with the disorder, and those that are suspected of having the disorder. In certain embodiments, a subject is successfully "treated" according to the methods provided herein if the subject shows, *e.g.*, total, partial, or transient alleviation or elimination of symptoms associated with the disease or disorder, such as MDS or AML. For example, "treating MDS" can include, but is not limited to, decreasing any of the symptoms associated with MDS.

[00052] "Prevent" or "prevention" refers to prophylactic or preventative measures that prevent and/or slow the development of a particular disease or disorder. Thus, those in need of prevention include those at risk of, or susceptible to, developing a particular disorder, such as

MDS or AML. In certain embodiments, a disease or disorder is successfully prevented according to the methods provided herein if the patient develops, transiently or permanently, *e.g.*, fewer or less severe symptoms associated with the disease or disorder, or a later onset of symptoms associated with the disease or disorder, than a patient who has not been subject to the methods of the invention.

[00053] In some embodiments the treatment and/or prevention methods described herein may be performed in combination with one or more additional MDS treatment and/or prevention methods known in the art, for example, treatment methods involving the administration of other therapeutic agents and/or treatment methods involving surgery, chemotherapy, or any other suitable treatment methods.

[00054] The various different "active agents" provided herein can be administered to a subject via any suitable route, including by systemic administration or by local administration. "Systemic administration" means that the active agent is administered such that it enters the circulatory system, for example, via enteral, parenteral, inhalational, or transdermal routes. Enteral routes of administration involve the gastrointestinal tract and include, without limitation, oral, sublingual, buccal, and rectal delivery. Parenteral routes of administration involve routes other than the gastrointestinal tract and include, without limitation, intravenous, intramuscular, intraperitoneal, intrathecal, and subcutaneous. "Local administration" means that a pharmaceutical composition is administered directly to where its action is desired (e.g., into the bone marrow). It is within the skill of one of ordinary skill in the art to select an appropriate route of administration taking into account the nature of the specific active agent being used and nature of the specific condition (e.g. MDS sub-type) to be treated.

[00055] Some embodiments of the present invention involve antibodies. As used herein, and unless otherwise specified, the term "antibody" encompasses intact polyclonal antibodies, intact monoclonal antibodies, single-domain antibody, nanobody, antibody fragments (such as Fab, Fab', F(ab')2, and Fv fragments), single chain Fv (scFv) mutants, multispecific antibodies such as bispecific antibodies generated from at least two intact antibodies, chimeric antibodies, humanized antibodies, human antibodies, fusion proteins comprising an antigen determination portion of an antibody, and any other modified immunoglobulin molecule comprising an antigen recognition site so long as the antibodies exhibit the desired biological activity. An antibody can be of any the five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, or subclasses

(isotypes) thereof (e.g. IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2), based on the identity of their heavy-chain constant domains referred to as alpha, delta, epsilon, gamma, and mu, respectively. The different classes of immunoglobulins have different and well-known subunit structures and three-dimensional configurations. Antibodies can be naked, or conjugated to other molecules.

[00056] Some embodiments of the present invention involve targeting an active agent to stromal cells in the bone marrow of a subject using a targeting agent. Such targeting agents may include, for example, antibodies that bind to antigens present on the surface of bone marrow stromal cells. Where antibodies are used, any antibody that binds to any suitable antigen expressed on the surface of bone marrow stromal cells can be used. In some embodiments an antibody-drug conjugate molecule is used that comprises both an antibody that binds to an antigen found on the surface of bone marrow stromal cells and an active agent, wherein the two components may be linked together via a linker moiety. Several such linkers are known in the art, such as those that are conventionally used in the production of antibody-drug conjugates.

[00057] An "effective amount" of a composition as disclosed herein is an amount sufficient to carry out the specifically stated purpose. An "effective amount" can be determined empirically and in a routine manner, in relation to the stated purpose, route of administration, and dosage form.

[00058] In some embodiments, administration of the active agents described herein can comprise administration at any suitable dose and/or according to any suitable dosing regimen, as determined by one of skill in the art. For example, in some embodiments, a Porcn inhibitor, such as Wnt-C59, LGK974, or an analogue, variant, or derivative thereof, is administered systemically to a subject in need thereof at a daily dose of about 0.5 mg/kg to about 500 mg/k, or about 5 mg/kg to about 50 mg/kg.

[00059] The active agents can be administered according to any suitable dosing regimen, for example, where the daily dose is divided into two or more separate doses. It is within the skill of the ordinary artisan to determine a dosing schedule and duration for any chose route of administration. For example, in some embodiments, an active agent (e.g. in a pharmaceutical composition) can be administered orally at least once a day, or at least twice a day. In some embodiments, an active agent (e.g. in a pharmaceutical composition) can be administered intravenously at least once a day, or at least twice a day.

[00060] In embodiments in which more than one active agent is administered, the agents can be administered together (for example, in the same formulation and/or at the same time), or separately (for example, in different formulations and/or at different times). Where two or more active agents are used, it may be possible to use lower dosages or amounts of each active agent, as compared to the dosages necessary when each active agent is used alone.

[00061] The term "pharmaceutical composition" refers to a preparation that is in such form as to permit the biological activity of the active ingredient to be effective, and which contains no additional components that are unacceptably toxic to a subject to which the composition would be administered. Pharmaceutical compositions can be in numerous dosage forms, for example, tablet, capsule, liquid, solution, softgel, suspension, emulsion, syrup, elixir, tincture, film, powder, hydrogel, ointment, paste, cream, lotion, gel, mousse, foam, lacquer, spray, aerosol, inhaler, nebulizer, ophthalmic drops, patch, suppository, and/or enema. Pharmaceutical compositions typically comprise a pharmaceutically acceptable carrier, and can comprise one or more of a buffer (*e.g.* acetate, phosphate or citrate buffer), a surfactant (*e.g.* polysorbate), a stabilizing agent (*e.g.* human albumin), a preservative (*e.g.* benzyl alcohol), a penetration enhancer, an absorption promoter to enhance bioavailability and/or other conventional solubilizing or dispersing agents. Choice of dosage form and excipients depends upon the active agent to be delivered and the disease or disorder to be treated or prevented, and is routine to one of ordinary skill in the art.

[00062] In those embodiments of the present invention that involve performing an assay to detect and/or measure DNA methylation in bone marrow stromal cells, or activity of Wnt/βcatenin signaling pathways in bone marrow stromal cells, or one or more of the other indicators of MDS or MDS progression or MDS prognosis described in the Examples section of this patent application, any suitable assays known in the art may be used. In addition, suitable exemplary assays are described in the Examples section of this patent application. For example, the Examples section of this patent application provides several assays that can be used to detect and/or measure DNA methylation in bone marrow stromal cells, including in specific chromosomal regions (such as chr12q15, chr5q32, chr4q21, chr7q31, chr3q13, chr2p12 and/or chr8q24) and/or outside of CpG islands.

[00063] Embodiments of the present disclosure can be further defined by reference to the following non-limiting "Examples." It will be apparent to those skilled in the art that many

modifications, both to materials and methods, can be practiced without departing from the scope of the present disclosure.

EXAMPLES

Example 1

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

[00064] Summary

[00065] Even though the marrow microenvironment plays an important role in regulation of normal and malignant hematopoiesis, the molecular alterations that facilitate its role in leukemogenesis have not been elucidated. As described herein, DNA methylome analysis of marrow derived stromal cells from myelodysplastic syndrome patients was performed and widespread aberrant hypermethylation that occurred preferentially outside of CpG islands was observed. Stroma derived from 5-Azacytidine-treated patients demonstrated abrogation of aberrant methylation and *in vitro* treatment of primary MDS stroma enhanced its ability to support erythroid differentiation. Integrative expression analysis revealed that the WNT pathway antagonist FRZB was aberrantly hyper-methylated and under-expressed in MDS stroma. Significant under-expression of FRZB and transcriptional activation of the Wnt pathway and its targets was confirmed in an independent set of sorted, primary MDS mesenchymal cells. A WNT/β-catenin activation signature was seen in CD34+ cells from advanced cases of MDS and was associated with adverse prognosis. Constitutive activation of β-catenin in hematopoietic cells, led to development of a lethal myeloid disease in the NUP98-HOXD13 MDS mouse model, demonstrating its role in disease progression *in vivo*. These results reveal novel widespread aberrant epigenetic changes in the MDS marrow microenvironment and demonstrate the epigenetically regulated β-catenin activation is associated with disease progression.

[00066] Epigenetic alterations are believed to be important in regulating gene transcription in hematopoietic cells in MDS. However, the hypomethylating activity of the DNA methyltransferase inhibitor, 5-Azacytidine (Aza) (which is approved for therapy of MDS) fails to show a strong correlation with therapeutic responses. The present inventors

hypothesized that epigenetic alterations, such as methylation, of stromal cells - rather than (or in addition to) hematopoietic cells, might play a role in MDS.

[00067] To evaluate the epigenome of the MDS marrow microenvironment, the HELP (HpaII tiny fragment Enrichment by Ligation-mediated PCR) assay was used to study cytosine methylation patterns in primary stromal cells from patients with MDS. The HELP assay relies on differential digestion by a pair of isoschizomer enzymes, HpaII and MspI, which differ on the basis of their methylation sensitivity. The HpaII and MspI genomic representations can be co-hybridized 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]. As described herein it has now been demonstrated, using the HELP assay, that MDS stromal cells contain aberrant hypermethylation that affects the Wnt/β-catenin pathway. It has also now been demonstrated that β-catenin activation cooperates and results in a lethal MDS or myeloid leukemia in an MDS mouse model, and that treatment with 5-Aza leads to abrogation of hypermethylation in stromal cells and enhances the ability of MDS stromal cells to support hematopoiesis (of healthy donor cells) *in vitro*. Taken together, these results show that aberrant epigenetic marks in MDS stroma contribute to MDS disease progression and can be targeted for therapeutic interventions, for example using DNMT inhibitors.

[00068] Materials & Methods

[00069] 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 papilloma virus E6/E7 construct[15]. The KG1a cell line was obtained from ATCC (Manasses, VA). BM specimens were obtained from patients diagnosed with MDS and from controls after obtaining informed consent and Institutional Review Board approvals (**Table 1**).

[00070] BM mononuclear cells (MNC) were isolated using ficoll-paque gradient separation. Bone marrow cells, 25–30 × 10^6 cells per 75-ml cell flasks were cultured in non-hematopoietic expansion medium (Miltenyi Biotech Inc. Auburn, CA) 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 immune-magnetically

(Miltenyi). The resulting CD45-ve, low passage, BM-stromal cells were used for DNA and RNA extraction.

[00071] DNA Methylation Analysis by HELP Assay. The HELP assay was carried out as described previously[14,16] to determine methylome of 50,000 CpGs corresponding to 14,000 genes (GEO (GSE60233). Pathway analysis was performed using the IPA software (Redwood City, CA).[17] The list of hyper-methylated genes was examined for enrichment of conserved gene-associated regions using the Molecular Signatures Database (MSigDB).[18] Transcription factor binding sites in the differentially methylated regions were determined using the HOMER algorithm.[19]

Table 1 - Patient Disease Characteristics

Sample ID	Type	Age	Sex	HGB	WBC	PLTs x 10^3	Disease Stage
1	MDS	80	M	10.2	730	27	RCMD
2	Control	29	M	Healthy donor			Healthy donor
3	Control	62	F	9.5	980	134	AML in CR, AlloSCT
4	Control	65	M	9.5	10960	498	NHL
5	MDS	70	F	10.4	10390	37	CMML
7	MDS	86	M	10.2	2080	47	RAEB-2
11	MDS	91	M	8.8	910	7	RAEB-1
14	MDS	71	M	8.6	2810	36	RAEB-1
15	MDS	87	M	6.3	2100	179	RA
16	MDS	90	F	8	4010	96	RAEB-2
18	MDS	34	M	8.7	2400	7	Hypoplastic MDS
19	MDS	53	M	11.4	2900	174	RAEB-1
20	MDS	66	F	12.7	1470	151	RCUD
21	MDS	41	F	11.5	4000	150	RA
22	MDS	69	M	8.5	3050	40	RAEB-2
23	MDS	55	M	10.2	3450	70	RCMD

[00072] 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 (Sequenom, CA) on bisulfite-converted DNA as described previously.[20,21]

[00073] HELP-tagging Analysis for HS27 Stromal Cells. HS27a stromal cells were grown to 80% confluency in RPMI1640 and then co-cultured with KG1a cells (CD45+) at a ratio of 1:3 for 48hrs. Adherent cells were washed, trypsinized, and depleted of residual KG1a cells by CD45 MicroBeads (Miltenyi Biotech Inc. Auburn, CA). 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.

[00074] 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]

[00075] Hematopoietic Progenitor Cell Assays and Flow Cytometry. Hematopoietic progenitor colony formation was determined by clonogenic assays in methylcellulose, as in previous studies [25,26]. BM-stroma cells from controls and MDS patients were expanded and treated with either 0.5μM 5-Aza in alpha-minimal essential medium (MEM) and 10% FBS daily for 5 days. After treatment (~ 40% confluency), 1ml of Methocult H4434 containing 5000 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, Glycophorin A - PE-Cy5, CD71 – FITC antibodies.

[00076] Immunohistochemistry for FRZB. MDS patient stromal cells were cultured on 4-chambered slides with two chambers having received no 5-AZA treatment and two chambers receiving 5-AZA (0.5μM for five days). Immunohistochemistry for FRZB was performed with FRP-3(H-170) rabbit monoclonal antibody (Cat# sc-13941, Santa Cruz Biotechnology, San Diego, CA) and matched isotype control diluted to 1:100, for 30 min.

[00077] Mice. Doxycycline-inducible constitutively active β-catenin mice (*KH2-Col1A1-tetO-CTNN1S33Y/Rosa-rtTA; S33Y*) were generated. [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* (rtTA) loci and for the *NUP98-HOXD13* transgene. Transgenic primary *NUP98-HOXD13* mice aged 14-18 months were used for analysis and were verified to display clinical hallmarks of MDS and cytopenias.

[00078] Analysis of WNT signature in MDS cohort. WNT target genes obtained from a comprehensive web 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..

[00079] Primary MDS mesenchymal cell isolation and RNA-seq analysis. Control marrow was obtained from donors for allogeneic transplantation (median age: 45 (35-61), after

IRB approval. Mesenchymal cells from human MDS patients were FACS sorted using the FACSAria III systems (BDBioscience) with the following antibodies using optimized dilutions: CD45-PE-Cy7 (1:200), CD235a-BV421-A (1:100), CD271-PE (1:100), CD105-APC (1:50), CD31-APC-CY7 (1:50). Sorted cells were kept in TRIzol (Ambion). Smarter Ultra Low RNA kit for Illumina Sequencing (Clonetech) was used for cDNA synthesis according to the manufacturer's protocol. Sample preparation, sequencing, de-multiplexing and alignment were performed as previously described [29] with modifications specific to the application of Smarter kit.

[00080] Results

[00081] Primary stromal cells in MDS are characterized by aberrant hypermethylation. Primary cultures of stromal cells were established from 6 MDS bone marrow samples and 3 controls (Supplemental Table 1 with clinical characteristics). The MDS samples included 3 patients who had not been treated with the DNMT inhibitor 5-Azacytidine (5-Aza) (MDS UnTX), and 3 patients who had been treated with 5-Aza (MDS Tx). Controls were age matched and had blood counts in the normal range. CD45-negative non-hematopoietic cells from the cultures were immune-magnetically sorted and used for DNA/RNA extraction after low passage numbers (up to 3 passages). Genome wide cytosine methylation was analyzed by the HELP assay, that 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 CpGs loci covering 14,000 promoters [13,30] Unsupervised clustering based on cytosine methylation profiles demonstrated that untreated MDS stromal cells were epigenetically distinct from healthy controls, (**Fig. 1A**), while MDS stromal cells from 5-Aza treated patients clustered closer to healthy controls. Next, to determine the qualitative epigenetic differences between these groups, a supervised analysis of the respective DNA methylation profiles was performed. 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**. It was observed that MDS stromal cells were characterized by aberrant hypermethylation when compared to controls (3626 hyper-methylated vs 306 hypo-methylated 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-D**).

[00082] Even though aberrant methylation in MDS stromal samples occurred genome wide (**Fig. 1E**), there was a significant enrichment at chromosomal regions chr12q15, chr5q32, chr4q21, chr7q31, chr3q13, chr2p12 and chr8q24 when compared with the genomic distribution of all HpaII fragments from the HELP array (P value <0.05; MDSig Program). Further, to determine whether these hyper-methylated loci shared any common DNA elements, a search was performed for transcription factor-binding sites enriched in these regions. A significant over-representation of binding sites for Sp1, NFY, MYB, and other transcription factors was observed in differentially methylated regions in MDS stroma (P value <0.05; HOMER Program) (**Fig. 1F**). Finally, experiments were performed to determine if CpG islands were predominantly affected by differential methylation in MDS stromal cells. It was observed that a large proportion of aberrantly methylated loci were located outside of CpG islands (Test of Proportions, P value <0.01, **Fig. 1G**), consistent with recent data demonstrating epigenetic changes at less CpG dense regions of the genome[31].

[00083] 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 transcriptomic changes were seen in the untreated MDS stromal samples and consisted of aberrantly under-expressed genes (**Fig. 2B**). Due to cell limitations associated with low passage numbers, it was not possible to obtain sufficient 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 2.C,D**). Thus, these data demonstrated both methylomic 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, as shown in **Table 2**, below.

[00084] Determination of epigenetically regulated signaling pathways included those controlling WNT/Beta catenin signaling, Integrin signaling and other metabolic pathways and included WNT antagonists FRZB (SFRP3) and SFRP1 and cellular receptors belonging to the integrin and ephrin families (**Fig. 2E**).

[00085] FRZB is repressed and the Wnt/Beta catenin pathway activated in highly purified mesenchymal cells from human MDS patients.

[00086] Next, studies were performed to confirm the relevance of these epigenetic and transcriptional aberrancies in *ex vivo* expanded stromal cells in primary highly purified unexpanded mesenchymal cells. 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 was interrogated (**Fig. 3A**). 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.

Table 2 - Pathways affected by hypermethylation in MDS stroma

ID	Top Diseases and Functions	Molecules in Network
1	Cellular Assembly and Organization, Cellular Function and Maintenance, Cancer	ABL1,ADAM19,AFAP1,AKT2,ARHGAP17,ARRDC1,COL14A1,HTT,ICAM1,IRF2BP1,JUNB,KEAP1,MAP1LC3A,MARK2,NF2,SEMA7A,SFRP1,SMARCB1,SYNPO2,TADA2B,TBC1D16,ZFYVE19
2	Developmental Disorder, Hematological Disease, Hereditary Disorder	C16orf58,C19orf47,CECR5,CPNE2,PIEZO1,SH3RF3,SYDE1,THG1L,TMEM132A,TTC28,TTYH3,ZZEF1
3	DNA Replication, Recombination, and Repair, Energy Production, Nucleic Acid Metabolism	ANKRD6,ATF5,CHD1L,CPSF1,CST6,DHX30,DHX34,DNA2,DOCK1,EPHB3,FXR2,GIPC1,ITGA7,PEX6,TIMM44,TUFM,WIZ
4	Cellular Assembly and Organization, Endocrine System Development and Function, Protein Synthesis	ACADS,CCDC8,ECE1,FBXO42,GBF1,PES1,PPIL2,RBM42,RGS16,RRS1,SGK223,SLC1A5,SNX18,TALDO1
5	Protein Degradation, Protein Synthesis, Organ Morphology	AHCY,ANKS6,AP5B1,BEND5,C21orf33,COL5A3,ENKD1,LIMS2,LMCD1,PDLIM7,SCN8A,SEPT9,SH3BP5L

[00087] FRZB (SFRP3) was significantly under-expressed in mesenchymal cells from MDS (n=12) in comparison to normal controls (n=10) (**Fig. 3B**, TTest, P =0.02). This was further corroborated by significantly increased expression of the gene encoding β-catenin (CTNNB1) and increased expression of Jagged-1 (JAG1) (**Fig. 3C,D**) (TTest, P value <0.001), in line with the recent finding that mesenchymal/osteoblastic activation of β-catenin resulted in overexpression of *Jag1* in a mouse model of niche-induced myelodysplasia [9]. Together, this data shows that reduced expression of SFRP3, and its concomitant activation of Wnt signatures in expanded stromal cells, is of relevance to mesenchymal biology in human disease.

[00088] WNT pathway antagonists are epigenetically silenced in MDS stroma and can lead to activation of β-catenin in co-cultured HSCs. Next, to further validate the hyper-methylation and under-expression of FRZB, studies were performed to evaluate whether the epigenetic changes observed in primary cells could be replicated *in vitro*. Human stromal HS-27 cells were co-cultured with the leukemic cell line KG1a, and cytosine methylation changes were analyzed at a high resolution by a "next-gen" sequencing-based HELP-Tagging assay. Co-culture led to hyper-methylation of various CpGs in the FRZB and SFRP1 promoters in stromal cells (**Fig. 4A, Fig. 8**). The differentially methylated CpGs were located in canonical CpG islands as well as 2KB flanking regions (CpG shores) in the promoter region. Other differentially methylated sites were also validated by sequencing and quantitative mass array epityper analysis (**Fig. 9**). Under-expression of the WNT antagonist FRZB was also validated by qRTPCR analysis in primary expanded MDS stromal samples (**Fig. 4B**). FRZB protein levels were shown to be epigenetically downregulated in an independent set of 3 MDS stromal cultures (MDS 21-23) and increased after *in vitro* treatment with 5-Aza (**Fig. 4C, Fig. 10**). Having shown under-expression of secreted WNT antagonist FRZB in MDS stroma, studies were performed to determine whether this could lead to activation of WNT/β-catenin in surrounding HSCs. Specific siRNAs were used to knock down FRZB in primary human MSCs (**Fig. 4D**) which were co-cultured with primary marrow derived CD34+ cells in methylcellulose. Intracellular flow cytometry demonstrated that co-culture with FRZB knockdown stromal cells led to activation of nuclear β-catenin in CD34+, CD45+ hematopoietic cells (**Fig. 4E,F**).

[00089] Treatment with 5-Azacytidine improves the ability of MDS stroma to support erythropoiesis. Having shown that MDS stroma contains hyper-methylated loci that are not seen in 5-Aza treated samples, studies were performed to test the efficacy of Aza treatment on the stroma. Primary MDS stromal samples (MDS19, 20) were pre-treated with 5-Aza for 5 days and then co-cultured with healthy human CD34+ cells. Co-culture with MDS stromal cells from 2 patients (mock-treated, not exposed to 5-Aza) led to dysplastic colony formation (**Fig. 4H**). When primary MDS stroma was pre-treated with 5-Aza the numbers of hematopoietic colonies increased, and colonies were of larger size than in mock-treated controls (**Fig 4. G,H**). Next, co-cultured hematopoietic cells were collected and examined for differentiation markers by FACS. There was a significant increase in erythroid differentiation after co-culture with Aza-treated MDS stroma, as evident from glycophorin A positivity (**Fig. 4I,J**). Increased erythroid

differentiation was seen in all stages as evident from increased percentages of pro-erythroblasts, basophilic and mature erythrocytes (**Fig. 4J**).

[00090] 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. In order to test the role of activated β-catenin in the context of MDS, an established murine model of MDS, the *NUP98-HOXD13* transgenic model (*NHD13*), was used. This model recapitulates many of the salient features of MDS including pancytopenias accompanied by hypercellular or normocellular bone marrow at 4-7 months[32-35]. Also, 12-17% of the marrow contains dysplastic erythroid, myeloid and rare megakaryocytic cell types[32]. 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[34]. Although the *NHD13* transplanted bone marrow cells engraft poorly, they still retain the clinical features of MDS (~10-20% chimerism)[34]. 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)* over-expression mouse model described previously[27].

[00091] Bone marrow transplants of C57BL6 (WT), S33Y, NHD13 or compound NHD13/S33Y cells into lethally irradiated CD45.1+ recipients were performed (**Fig. 5A**). After equivalent engraftment was verified in the peripheral blood (**Fig. 5B**), mice were treated with doxycycline to constitutively activate β-catenin ($S33Y^{O/E}$ and NHD13/$S33Y^{O/E}$) and were then followed for disease progression. At four months, flow cytometric analysis revealed that the NHD13/S33Y mice had an increase in mature and immature myeloid cells among the donor cells (**Fig. 5C-D**). Additionally, the $S33Y^{O/E}$, NHD13 and NHD13/$S33Y^{O/E}$ animals all had reduced white blood cell counts compared to the control mice (**Fig. 5E**). Surprisingly, the NHD13/$S33Y^{O/E}$ mice succumbed to a lethal myeloid disease with a median latency of 388 days while other mice were followed until 451 days and then sacrificed to assess their phenotype (**Fig. 5F**). The NHD13/$S33Y^{O/E}$ mice mainly died of a myeloid leukemia (5 out 8) with enlarged spleens and increased white blood cell counts while the other mice died of an MDS-like phenotype (3 out 8), (**Fig. 5G, H**). These data suggest that activation of β-catenin drives a lethal and aggressive myeloid disease with an increased likelihood of transformation.

[00092] WNT activation signature is present in MDS and is marker of adverse prognosis. Next, studies were performed to determine whether WNT pathway activation was seen in an independent cohort of MDS samples. The expression patterns of known WNT pathway targets, (Nusse et al.) 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, Fig. 11**) were found to be overexpressed in MDS samples (18/39 overexpressed, **Fig. 11**) when compared to healthy controls and demonstrated a trend towards elevated mean expression in higher risk pre-leukemic RAEB (Refractory anemia with excess of blasts) subtypes. Since the WNT/β-catenin pathway controls numerous downstream genes, a composite signature based on the degree of activation of all expressed downstream targets was developed and correlated with clinical subtypes of MDS. It was 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 RA (refractory anemia) and RARS (refractory anemia with ringed sideroblasts) subtypes (**Fi.g 6B**, TTest, P Value<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 yrs in patients with high levels of WNT activation vs 5.24 yrs in patients with low WNT expression (**Fig. 6C**, Log Rank P =0.037). Association with mutations in a subset of genotyped samples (n=100) revealed that SF3B1 mutant samples had significantly lower scores (P Value=0.002, Correlation Coefficient =-0.3), while no other significant associations were seen with any other mutations. These data, taken together (**Fig. 7**) with *in vivo* murine data, demonstrate that stroma mediated WNT activation is a pathogenic and prognostic event in MDS progression.

[00093] Discussion

[00094] The data presented in the present Example shows that marrow stroma in patients with MDS is aberrantly hyper-methylated 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. This data supports the possibility of targeting the marrow niche with epigenetically active drugs (e.g. DNMT inhibitors) in MDS.

[00095] 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 [21,36-38]. 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 [39]. The findings presented herein regarding epigenetic alterations that affect important regulatory pathways provide a molecular basis to explain micro-environmental 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.

[00096] 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] The results presented herein elucidate a mechanism for β-catenin activation in the niche. In particular, the results presented herein demonstrate reduced expression of FRZB (SFRP3) via epigenetic silencing and consequent activation of the Wnt/β-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 supports the view that a small population of mesenchymal colony forming stem and progenitor cells (CFU-F, comprising <2% of CD271+ cells in MDS) is epigenetically altered in human MDS and maintains the $CD271^+$ reticular network in the bone marrow.

[00097] Additionally, the results presented herein 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**). This data 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 [40,41]. Another study demonstrated that haploinsufficiency of del(5q) genes, Egr1 and Apc, cooperate with Tp53 loss to induce acute myeloid leukemia in mice[42]. The present 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[43]. 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. [44,45] In contrast to these prior studies, the model presented herein failed to alter hematopoietic differentiation or cause lethality, unless it was combined with the NHD13 model. One potential explanation for these disparate findings is that the present model utilizes a different promoter to activate β-catenin (Col1A1/Rosa versus the endogenous promoter). It could be that this mouse model allows for the study of β-catenin activation without the toxicity that was previously observed. The NHD13 model used in the present studies demonstrates dysplasia and cytopenias coupled with progression to accelerated disease, thus serving as a representative model of human MDS.[35,46,47] Taken together, the results presented herein elucidate 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.

[00098] 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 prior studies have examined hematopoietic cells. However, the data presented herein suggest that *stromal* methylation may be partly responsible for the therapeutic efficacy of these drugs. Overall, the results presented herein demonstrate that MDS stromal cells have widespread epigenetic alterations that modify MDS disease pathophysiology and that can be targeted therapeutically, for example using DNMT inhibitor treatment.

Example 2

WNT/β-catenin pathway inhibitors reverse disease in a mouse model of MDS

[00099] Porcupine (Porcn) is a critical enzyme that modifies all WNTs through palmitylation and this modification is essential for the secretion and binding to cell surface to Wnt ligand receptors. LGK974 (Novartis) is a potent and selective PORCN inhibitor and is currently in a Phase I clinical trial for various diseases associated with β-catenin activation (NCT01351103), but MDS is not currently a disease indication (Madan et al. "Targeting Wnts at the source--new mechanisms, new biomarkers, new drugs." Mol. Cancer Ther. 14, 1087-1094 (2015); Liu et al., "Targeting Wnt-driven cancer through the inhibition of Porcupine by LGK974." Proc. Natl. Acad. Sci. U.S.A. 110, 20224-20229 (2013)). A recent study found that Porcn deletion or PORCN enzyme inhibition had no effect on HSPC function and no overt toxicity suggesting tolerability to the hematopoietic compartment (Kabiri et al., "Wnts are dispensable for differentiation and self-renewal of adult murine hematopoietic stem cells." Blood 126, 1086-1094 (2015)).

[000100] Experiments were performed to test the effect of Porcn inhibitor treatment in animal models of MDS. The experimental scheme used is presented in **Fig. 8A.** Briefly, 6 month old NHD13 MDS mice were treated with a Porcn inhibitor or placebo. Mice were bled at day 0 and then the Porcn inhibitor or placebo was administrated orally (by oral gavage) for six days. The dose of the Porcn inhibitor (Porcn C59 (Wnt-C59) or LGK974) was 5mg/kg per day. Following the treatment period mice were bled again on day 7. White blood cells (WBC) counts, platelet counts, and hematocrit levels were analyzed. The results of these experiments are presented in **Fig. 12**. Porcn inhibitor treatment was found reverse progression of MDS disease - increasing white blood cell counts, platelet counts, and hematocrit levels, as well as increasing myeloid differentiation – concomitant with reduced β-catenin positivity.

Example 3

Further testing of Porcn Inhibitors in MDS

[000101] The above studies on Porcn inhibitor are expanded to test a variety or *in vitro* and *in vivo* effects.

[000102] A large cohort of human MDS primary stem/progenitor cells are cocultured with stromal cells (100 samples). *In vitro* responses of Porcn inhibitor are correlated with clinical and molecular characteristics.

[000103] The NHD13 mouse model of MDS described above is used to perform additional tests *in vivo*. These mice are treated with LGK974 (Wnt-C59) to further assess its efficacy in reversing MDS disease and/or progression to AML. In primary NHD13 animals the MDS disease becomes more severe by 5 months (Raza-Egilmez et al., "NUP98-HOXD13 gene fusion in therapy-related acute myelogenous leukemia." Cancer Res 58, 4269-4273 (1998); Lin et al. "NUP98-HOXD13 transgenic mice develop a highly penetrant, severe myelodysplastic syndrome that progresses to acute leukemia." Blood 106, 287-295 (2005)). Thus, experiments can be performed over such time frames, for example to study the effects of treatment on severe MDS. Blood counts and bone marrow aspirates are obtained prior to drug administration to obtain a base line analysis of the disease. Up to 50mg/Kg LGK974 (Wnt-C59) can be administrated by oral gavage for 20 days without overt toxicity to the animals or to blood cells (Kabiri et al., "Wnts are dispensable for differentiation and self-renewal of adult murine hematopoietic stem cells. Blood 126, 1086-1094 (2015)). To further assess the effects of inhibiting the Wnt/β-catenin axis with a Porcupine inhibitor on survival, two treatment protocols are used (n=10 mice per prtocol). In one protocol 1mg/Kg LGK974 (Wnt-C59) is administered by daily oral gavage for three weeks. In the other protocol 5mg/Kg LGK974 (Wnt-C59) is administered – also by daily oral gavage for three weeks. Blood counts and bone aspirates are obtained and to assess the duration of the response. Studies are performed to determine if β-catenin levels are reduced in these animals, as well as to assess downstream target genes. Mice are assessed bi-monthly and survival is assessed after treatment. β-catenin levels may be reactivated after the inhibitor is removed. Global gene expression profiling is performed on HSPCs from mice that have been acutely treated *in vivo* (e.g. treated for 24 hours or until reduced β-catenin levels are first detected). The effects of β-catenin inhibition on a variety of MDS disease features are assessed. The results of these studies can inform potential dosing strategies and other clinical protocol parameters for human clinical trials of Porcn inhibitors in MDS.

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[000104] The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art, readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention.

Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance.

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The present invention is further described by the following claims.

CLAIMS

1. A method of treating or preventing myelodyplastic syndrome (MDS) in a subject in need thereof, the method comprising contacting the subject's bone marrow stromal cells with an effective amount of an active agent that inhibits the Wnt/βcatenin signaling pathway.

2. A method of treating or preventing MDS, the method comprising: administering to a subject in need thereof an effective amount of an active agent that inhibits the Wnt/βcatenin signaling pathway in bone marrow stromal cells.

3. A method of preventing AML in a subject that has, or that is suspected of having, MDS, the method comprising contacting the subject's bone marrow stromal cells with an effective amount of an active agent that inhibits the Wnt/βcatenin signaling pathway.

4. A method of preventing acute myeloid leukemia (AML) in a subject in need thereof, the method comprising: administering to a subject that has, or that is suspected of having, MDS, an effective amount of an active agent that inhibits the Wnt/βcatenin signaling pathway in bone marrow stromal cells.

5. A method of treating or preventing myelodyplastic syndrome (MDS) in a subject in need thereof, the method comprising (a) performing an assay to determine if bone marrow stromal cells obtained from a subject comprise hyper-methylated DNA, and (b) if the subject's bone marrow stromal cells comprise hyper-methylated DNA, subsequently contacting the subject's bone marrow stromal cells with an effective amount of an active agent that inhibits the Wnt/βcatenin signaling pathway.

6. A method of treating or preventing myelodyplastic syndrome (MDS) in a subject in need thereof, the method comprising (a) performing an assay to determine if bone marrow stromal cells obtained from a subject comprise hyper-methylated DNA, and (b) if the subject's bone marrow stromal cells comprise hyper-methylated DNA, subsequently administering to the subject an effective amount of an active agent that inhibits the Wnt/βcatenin signaling pathway in bone marrow stromal cells.

7. The method of claim 1, claim 3, or claim 5, wherein step of contacting the subject's bone marrow stromal cells is performed *ex vivo*.

8. The method of claim 1, claim 3, or claim 5, wherein step of contacting the subject's bone marrow stromal cells is performed *in vivo*.

9. The method of any of any one of claims 1-6, wherein the active agent is selected from the group consisting of Wnt-C59, LGK-974, X-AV-939, ICG-001, IWR-1-endo, IWR-1-exo. KY02111, IWP-2, FH535, WIK14, IWP-L6, PNU-74654, CCT036477, OMP-18R5, dimethoxy curcumin, Dickkopf(DKK), Axin, FRZB, SFRP1, and SFRP3.

10. The method of any of any one of claims 1-6, wherein the active agent is a Porcn inhibitor.

11. The method of any of any of any one of claims 1-6, wherein the active agent is a DNA methyltransferase (DNMT) inhibitor.

12. The method claim 11, wherein the DNMT inhibitor is 5-azacytidine or decitabine.

13. The method of any of claims 1-6, wherein the active agent is targeted to stromal cells in the bone marrow of the subject by localized delivery to the bone marrow or by using a targeting agent.

14. The method of claim 13, wherein the targeting agent binds to bone marrow stem cells.

15. The method of claim 13, wherein the targeting agent comprises an antibody that binds to an antigen on the surface of bone marrow stromal cells.

16. The method of any of claims 1-6, wherein the subject is a mammal.

17. The method of any of claims 1-6, wherein the subject is a human.

18. The method of any of claims 1-6, wherein the subject is determined to be at high risk of progression to AML.

19. The method of any of claims 1-6, wherein the MDS comprises: refractory cytopenia with unilineage dysplasia (RCUD), refractory anemia with ringed sideroblasts (RARS), refractory cytopenia with multilineage dysplasia (RCMD), refractory anemia with excess blasts-1 (RAEB-1), refractory anemia with excess blasts-2 (RAEB-2), myelodysplastic

syndrome associated with isolated del(5q), or unclassified myelodysplastic syndrome (MDS-U).

20. The method of any of claims 1-6, wherein the subject has refractory anemia with excess blasts-1 (RAEB-1) or refractory anemia with excess blasts-2 (RAEB-2).

21. The method of any of claims 1-6, wherein the active agent is LGK974 or Wnt-C59, and wherein the active agent is administered to the subject at a dose from about 0.5mg/kg/day to about 5 mg/kg/day.

22. The method of any of claims 1-6, wherein the active agent is provided in a pharmaceutical composition.

23. A method of any of claims 1-6, further comprising administering to the subject one or more additional agents useful in the treatment of MDS.

24. The method claim 23, wherein the additional agent is a DNA methyltransferase (DNMT) inhibitor.

25. The method claim 23, wherein the additional agent is selected from the group consisting of cytarabine (ara-C), idarubicin, topotecan, fludarabine, 5-azacytidine (Vidaza), decitabine (Dacogen), thalidomide, lenalidomide (Revlimid), anti-thymocyte globulin (ATG), cyclosporine, granulocyte colony stimulating factor (G-CSF, Neupogen, or filgrastim), granulocyte macrophage-colony stimulating factor (GM-CSF, Leukine, or sargramostim), pegfilgrastim (Neulasta), erythropoietin (Epo or Procrit), darbepoetin alfa (Aranesp), and oprelvekin (Neumega, interleukin-11, or IL-11).

26. A method according to any of claims 1-6, the method further comprising treating the subject with an additional treatment regimen useful in the treatment of MDS.

27. The method claim 26, wherein the additional treatment regimen is selected from the group consisting of bone marrow transplantation, stem cell transplantation, and chemotherapy.

28. The method of claim 5 or claim 6, comprising performing an assay to determine if the bone marrow stromal cells obtained from the subject comprise hyper-methylated DNA at

one or more chromosomal regions selected from the group consisting of chr12q15, chr5q32, chr4q21, chr7q31, chr3q13, chr2p12 and chr8q24.

29. The method of claim 5 or claim 6, comprising performing an assay to determine if the bone marrow stromal cells obtained from the subject comprise hyper-methylated DNA outside of CpG islands.

30. A pharmaceutical composition for use in a method according to any of claims 1-6, wherein the pharmaceutical composition comprises an active agent selected from the group consisting of Wnt-C59, LGK-974, X-AV-939, ICG-001, IWR-1-endo, IWR-1-exo. KY02111, IWP-2, FH535, WIK14, IWP-L6, PNU-74654, CCT036477, OMP-18R5, dimethoxy curcumin, Dickkopf(DKK), Axin, FRZB, SFRP1, and SFRP3.

31. A pharmaceutical composition comprising: (a) an active agent, wherein the active agent is a Wnt/βcatenin signaling pathway inhibitor, and (b) a targeting agent that binds to the surface of bone marrow stromal cells.

32. A pharmaceutical composition according to claim 31, wherein the active agent is selected from the group consisting of Wnt-C59, LGK-974, X-AV-939, ICG-001, IWR-1-endo, IWR-1-exo. KY02111, IWP-2, FH535, WIK14, IWP-L6, PNU-74654, CCT036477, OMP-18R5, dimethoxy curcumin, Dickkopf(DKK), Axin, FRZB, SFRP1, and SFRP3.

33. A pharmaceutical composition according to claim 31, wherein the active agent is a DNA methyltransferase (DNMT) inhibitor.

34. The pharmaceutical composition according to claim 33, wherein the DNMT inhibitor is 5-azacytidine or decitabine.

35. A pharmaceutical composition according to claim 31, wherein the targeting agent comprises an antibody or antibody-fragment that binds to an antigen on the surface of bone marrow stromal cells.

36. A pharmaceutical composition for use in treating or preventing MDS in a subject in need thereof, wherein the pharmaceutical composition comprises an active agent selected from the group consisting of nt-C59, LGK-974, X-AV-939, ICG-001, IWR-1-endo, IWR-1-

exo. KY02111, IWP-2, FH535, WIK14, IWP-L6, PNU-74654, CCT036477, OMP-18R5, dimethoxy curcumin, Dickkopf(DKK), Axin, FRZB, SFRP1, and SFRP3.

37. A pharmaceutical composition for use in inhibiting Wnt/βcatenin signaling in bone marrow stromal cells in a subject that has, or is suspected of having, MDS, wherein the pharmaceutical composition comprises an active agent selected from the group consisting of Wnt-C59, LGK-974, X-AV-939, ICG-001, IWR-1-endo, IWR-1-exo. KY02111, IWP-2, FH535, WIK14, IWP-L6, PNU-74654, CCT036477, OMP-18R5, dimethoxy curcumin, Dickkopf(DKK), Axin, FRZB, SFRP1, and SFRP3.

38. A pharmaceutical composition for use in preventing AML in a subject that has, or is suspected of having MDS, wherein the pharmaceutical composition comprises an active agent selected from the group consisting of W nt-C59, LGK-974, X-AV-939, ICG-001, IWR-1-endo, IWR-1-exo. KY02111, IWP-2, FH535, WIK14, IWP-L6, PNU-74654, CCT036477, OMP-18R5, dimethoxy curcumin, Dickkopf(DKK), Axin, FRZB, SFRP1, and SFRP3.

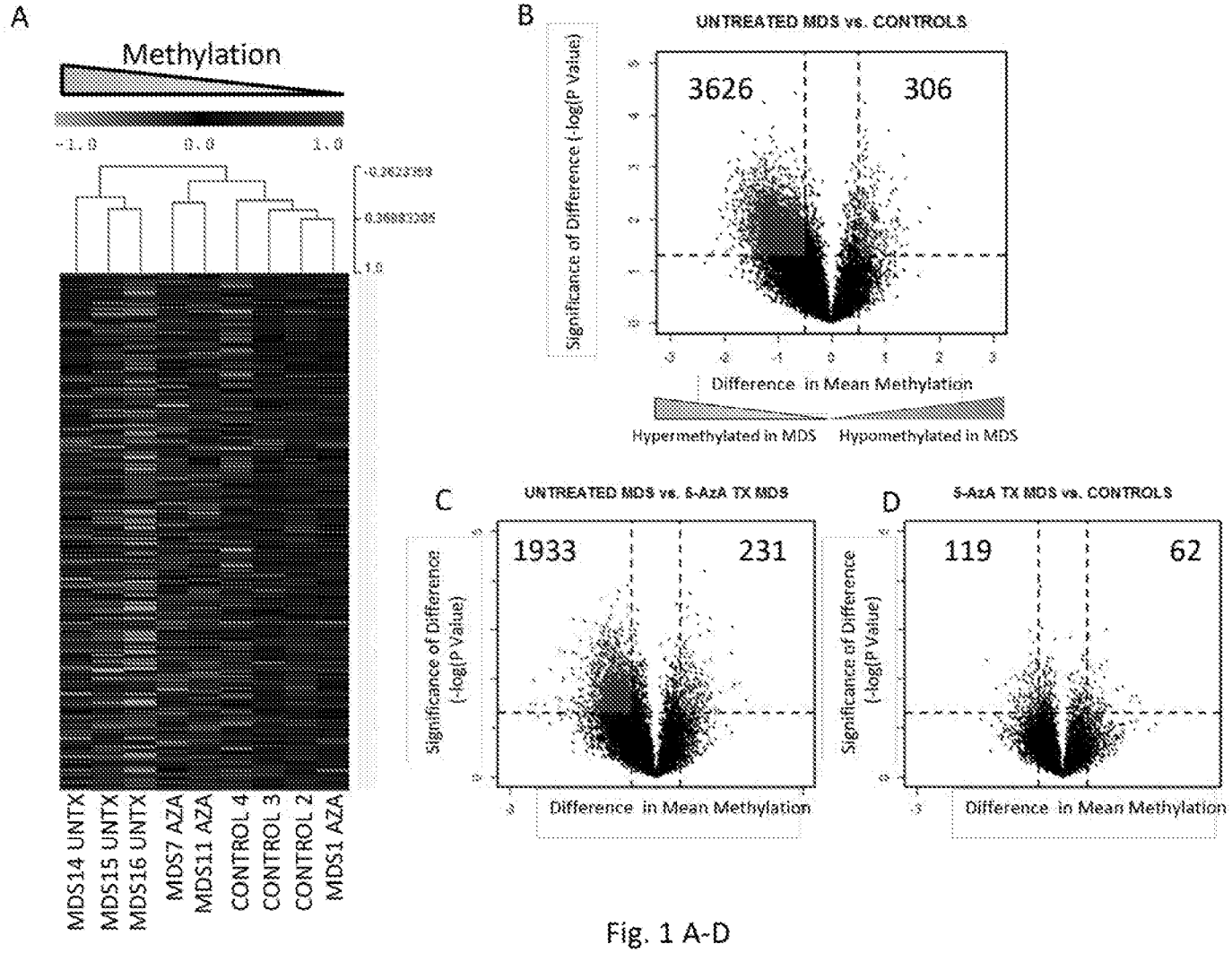
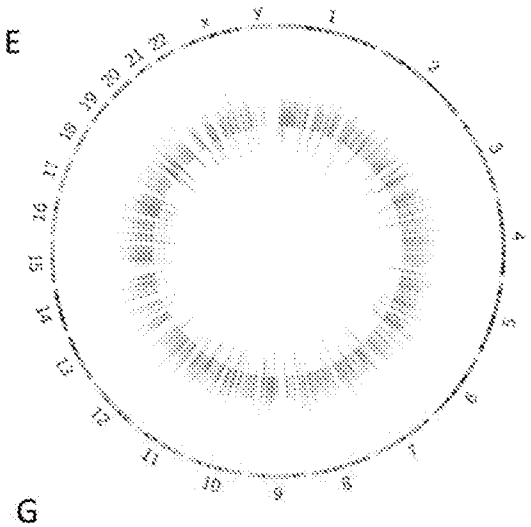

A
Methylation
-1.0
0.0
1.0
MDS14 UNTX
MDS15 UNTX
MDS16 UNTX
MDS7 AZA
MDS11 AZA
CONTROL 4
CONTROL 3
CONTROL 2
MDS1 AZA
B
UNTREATED MDS vs. CONTROLS
3626
306
Significance of Difference (-log(P Value)
Difference in Mean Methylation
Hypermethylated in MDS
Hypomethylated in MDS
C
UNTREATED MDS vs. 5-AzA TX MDS
1933
231
Significance of Difference (-log(P Value)
Difference in Mean Methylation
D
5-AzA TX MDS vs. CONTROLS
119
62
Significance of Difference (-log(P Value)
Difference in Mean Methylation

Fig. 1 A-D

E



F

	Motif Name	q-value (Benjamini)	Binding Motif
1	Sp1	0	
2	NFY	0.0002	
3	BMYB	0.0015	
4	AMYB	0.0024	
5	MYB	0.0024	
6	NF1	0.0024	
7	Egr1	0.0024	
8	Maz	0.0126	
9	E2F4	0.0175	
10	AP-2gamma	0.0479	

G

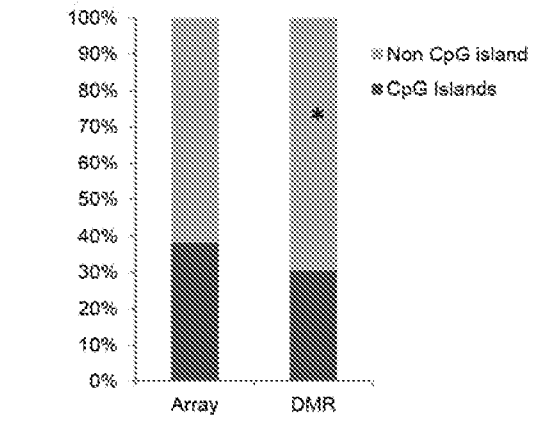


Fig.1 E-G

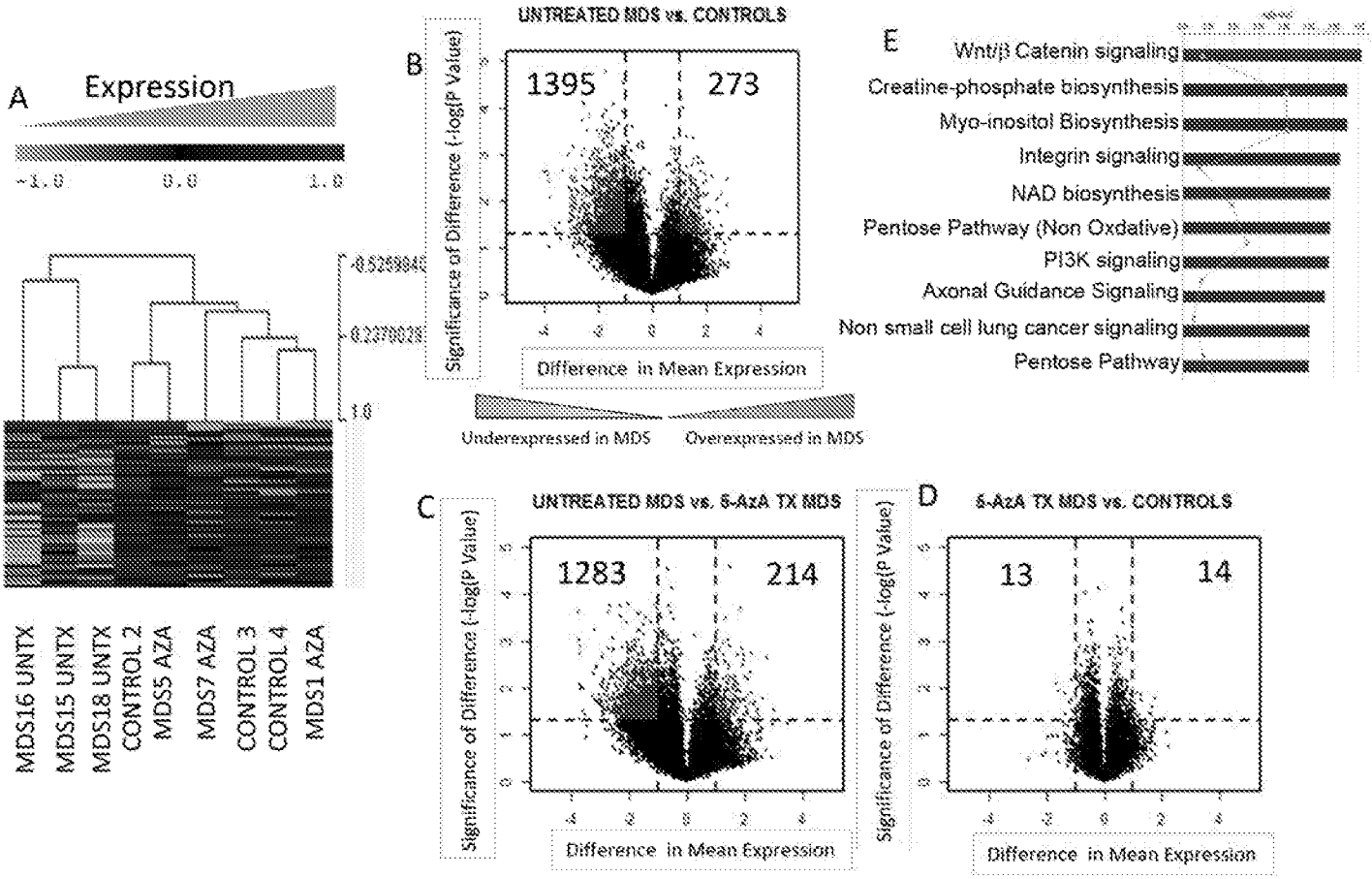

A
Expression
-1.0
0.0
1.0
MDS16 UNTX
MDS15 UNTX
MDS18 UNTX
CONTROL 2
MDS5 AZA
MDS7 AZA
CONTROL 3
CONTROL 4
MDS1 AZA
B
UNTREATED MDS vs. CONTROLS
1395
273
Significance of Difference (-log(P Value)
Difference in Mean Expression
Underexpressed in MDS
Overexpressed in MDS
C
UNTREATED MDS vs. 5-AzA TX MDS
1283
214
Significance of Difference (-log(P Value)
Difference in Mean Expression
D
5-AzA TX MDS vs. CONTROLS
13
14
Significance of Difference (-log(P Value)
Difference in Mean Expression
E
Wnt/β Catenin signaling
Creatine-phosphate biosynthesis
Myo-inositol Biosynthesis
Integrin signaling
NAD biosynthesis
Pentose Pathway (Non Oxdative)
PI3K signaling
Axonal Guidance Signaling
Non small cell lung cancer signaling
Pentose Pathway

Fig. 2A-E

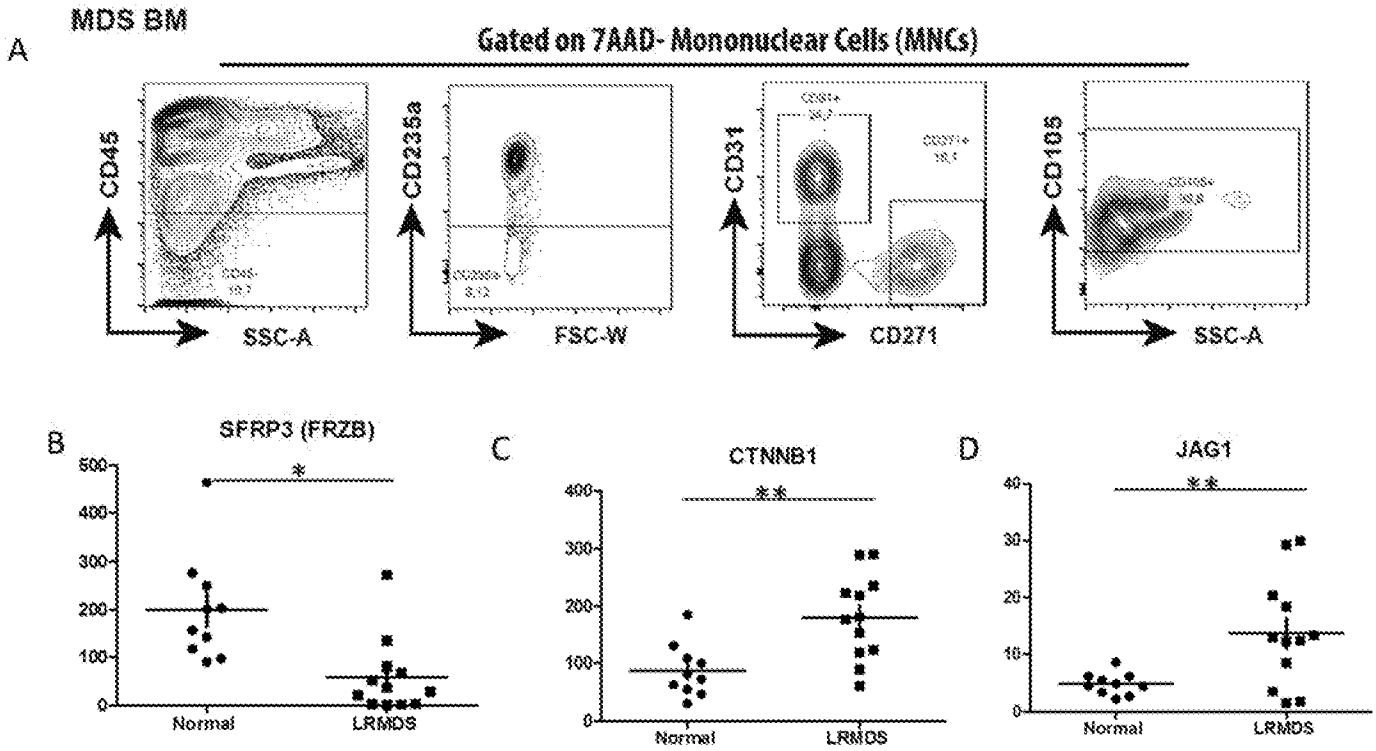

A
MDS BM
Gated on 7AAD- Mononuclear Cells (MNCs)
CD45
SSC-A
CD235a
FSC-W
CD31
CD271
CD105
SSC-A
B
SFRP3 (FRZB)
*
500
400
300
200
100
0
Normal
LRMDS
C
CTNNB1
**
400
300
200
100
0
Normal
LRMDS
D
JAG1
**
40
30
20
10
0
Normal
LRMDS

Fig. 3A-D

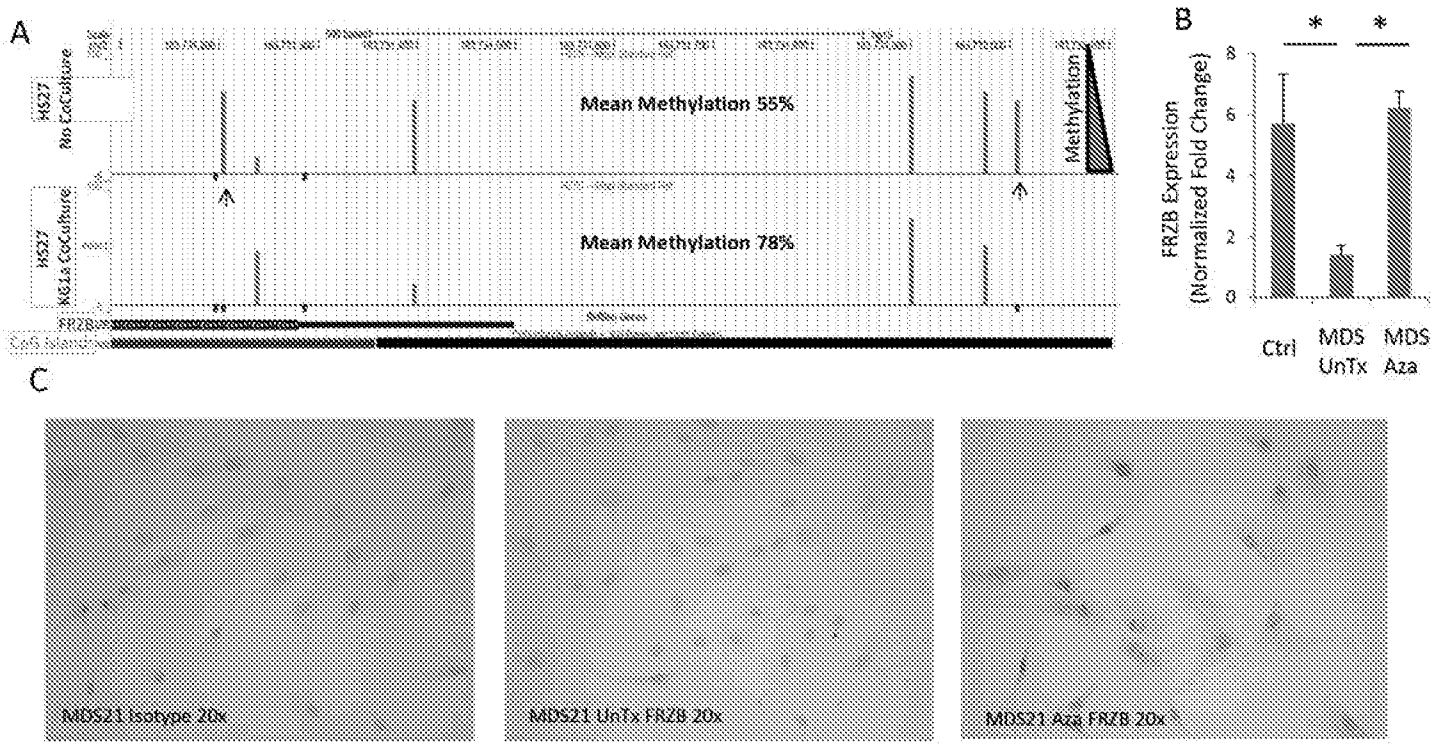

A
MS27 No CoCulture
MS27 KG1a CoCulture
Mean Methylation 55%
Mean Methylation 78%
Methylation
FRZB
CpG Islands
B
FRZB Expression (Normalized Fold Change)
Ctrl
MDS UnTx
MDS Aza
C
MDS21 Isotype 20x
MDS21 UnTx FRZB 20x
MDS21 Aza FRZB 20x

Fig. 4A-C

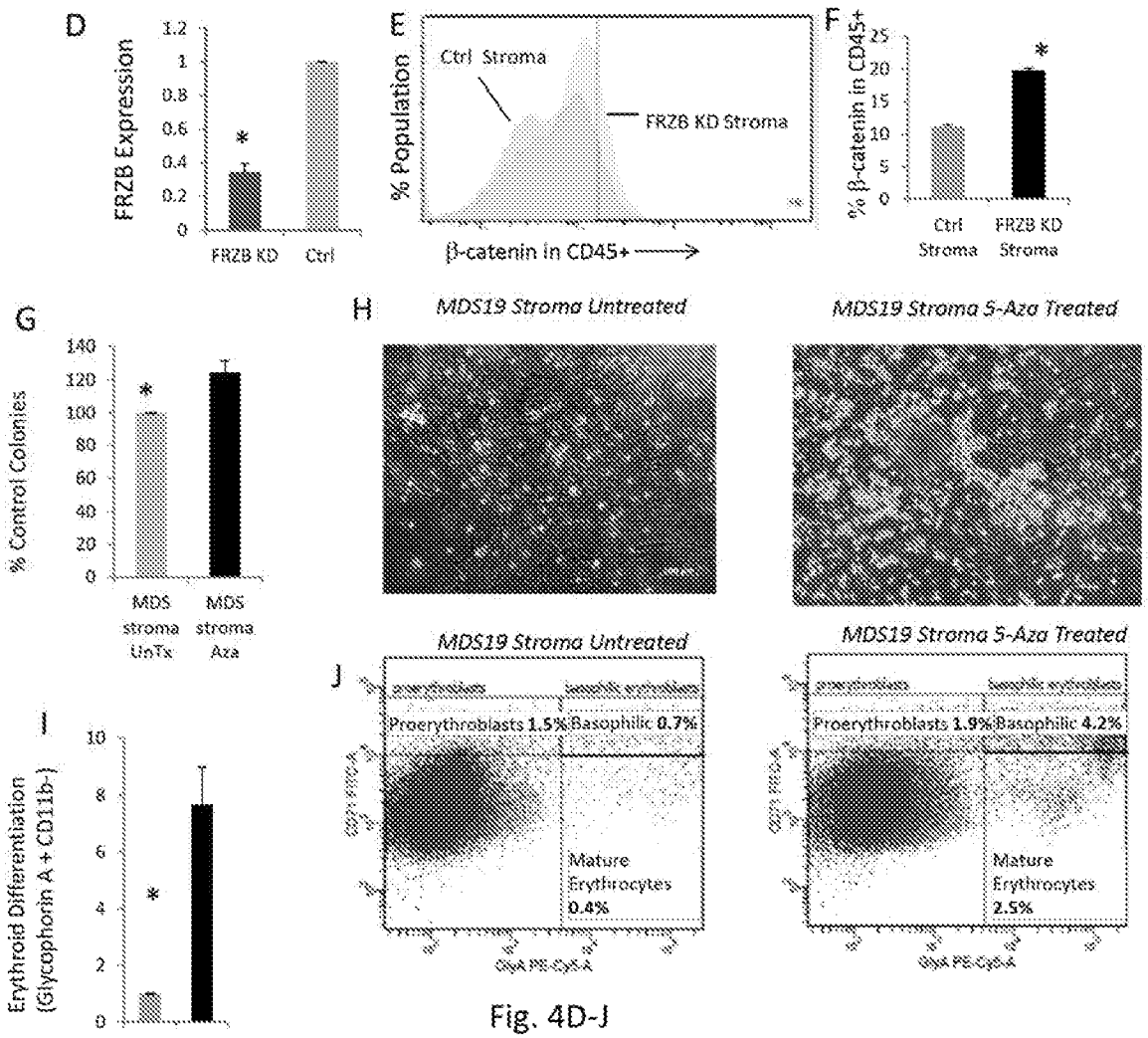

D
FRZB Expression
1.2
1
0.8
0.6
0.4
0.2
0
*
FRZB KD
Ctrl
E
% Population
Ctrl Stroma
FRZB KD Stroma
β-catenin in CD45+
F
% β-catenin in CD45+
25
20
15
10
5
0
*
Ctrl Stroma
FRZB KD Stroma
G
% Control Colonies
140
120
100
80
60
40
20
0
*
MDS stroma UnTx
MDS stroma Aza
H
MDS19 Stroma Untreated
MDS19 Stroma 5-Aza Treated
I
Erythroid Differentiation (Glycophorin A + CD11b-)
10
8
6
4
2
0
*
J
MDS19 Stroma Untreated
Proerythroblasts 1.5%
Basophilic 0.7%
Mature Erythrocytes 0.4%
MDS19 Stroma 5-Aza Treated
Proerythroblasts 1.9%
Basophilic 4.2%
Mature Erythrocytes 2.5%

Fig. 4D-J

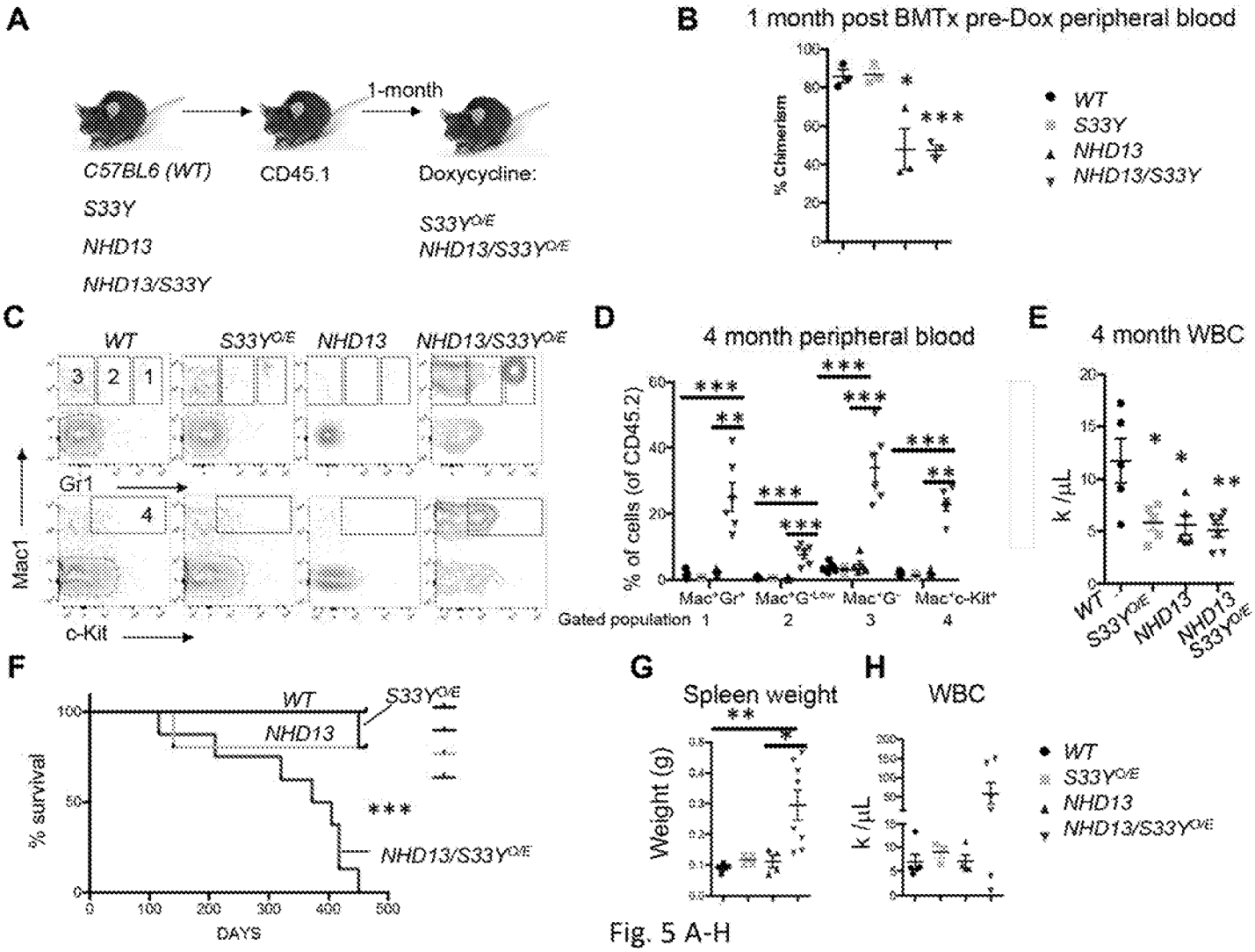

A
1-month
C57BL6 (WT)
S33Y
NHD13
NHD13/S33Y
CD45.1
Doxycycline:
S33Y^O/E
NHD13/S33Y^O/E
B
1 month post BMTx pre-Dox peripheral blood
% Chimerism
WT
S33Y
NHD13
NHD13/S33Y
C
WT
S33Y^O/E
NHD13
NHD13/S33Y^O/E
Gr1
Mac1
c-Kit
D
4 month peripheral blood
% of cells (of CD45.2)
Mac+Gr+
Mac+G+Low
Mac+G-
Mac+c-Kit+
Gated population
E
4 month WBC
k /μL
F
% survival
DAYS
G
Spleen weight
Weight (g)
H
WBC
k /μL

Fig. 5 A-H

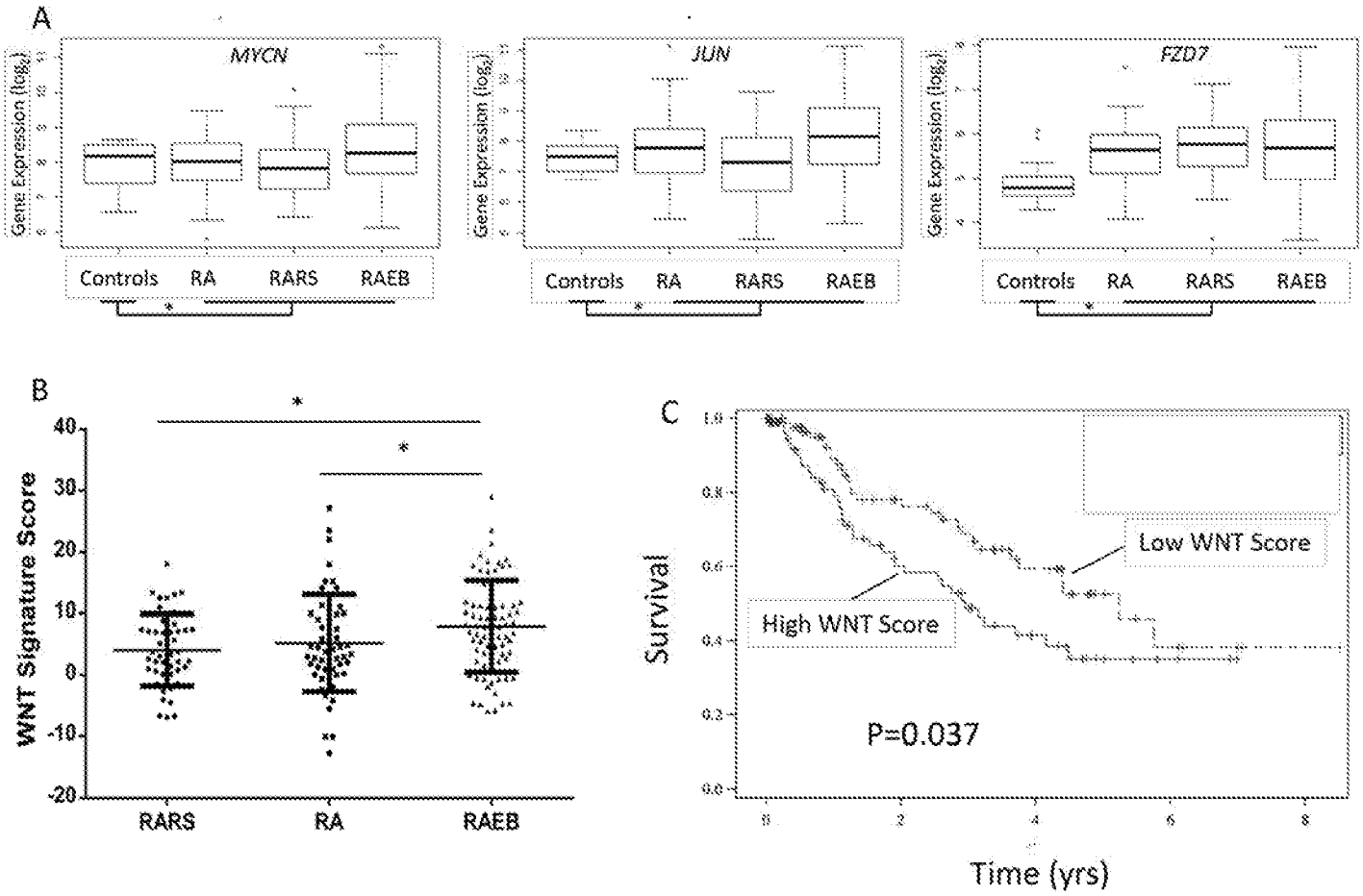

A
MYCN
JUN
FZD7
Gene Expression (log2)
Controls
RA
RARS
RAEB
B
WNT Signature Score
RARS
RA
RAEB
C
Survival
Low WNT Score
High WNT Score
P=0.037
Time (yrs)

Fig. 6 A-C

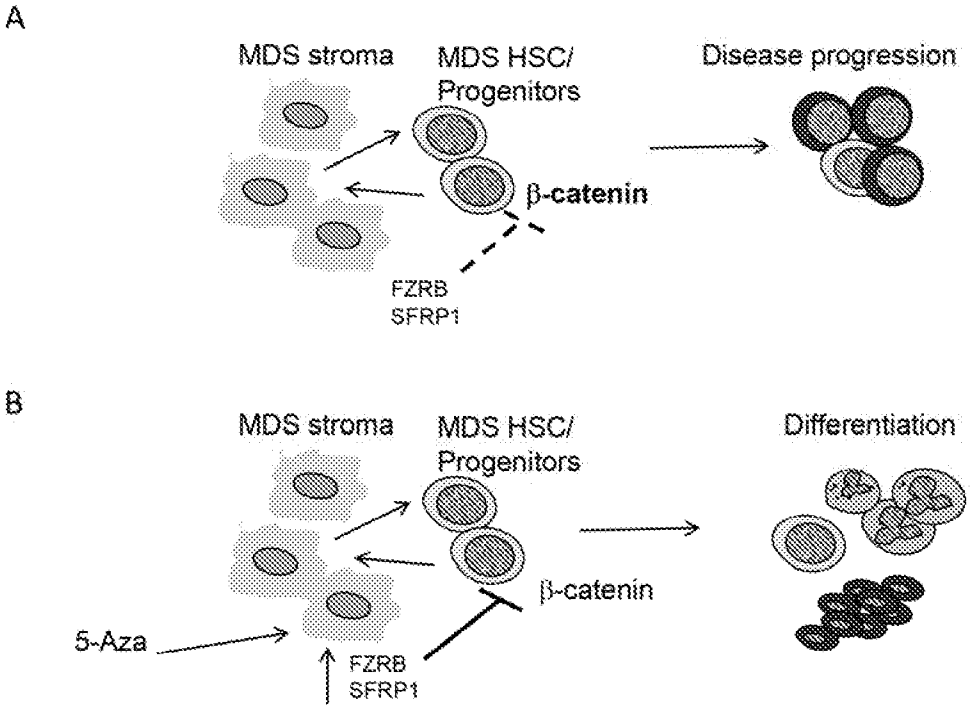

A
MDS stroma
MDS HSC/
Progenitors
Disease progression
β-catenin
FZRB
SFRP1
B
MDS stroma
MDS HSC/
Progenitors
Differentiation
β-catenin
5-Aza
FZRB
SFRP1

Fig. 7 A-B

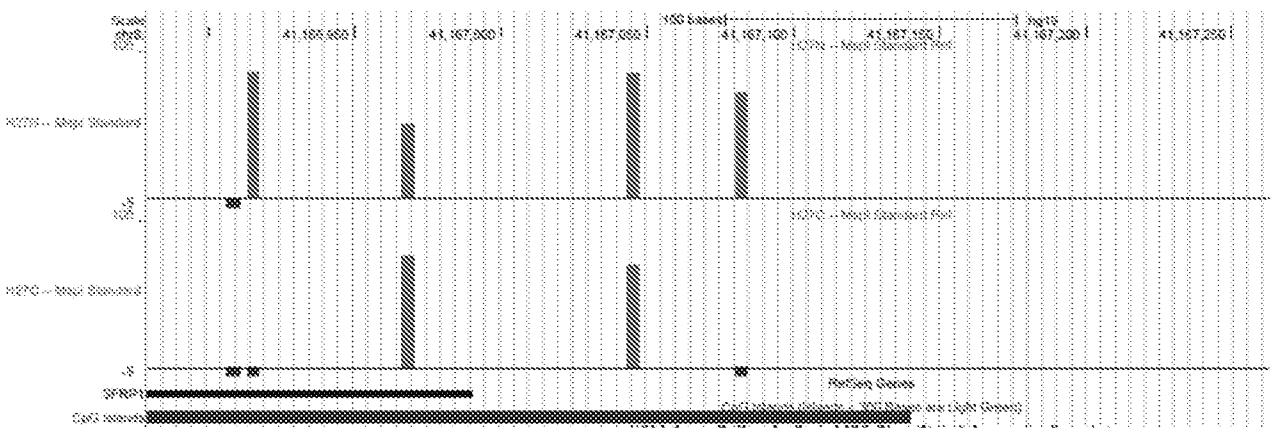


Fig. 8

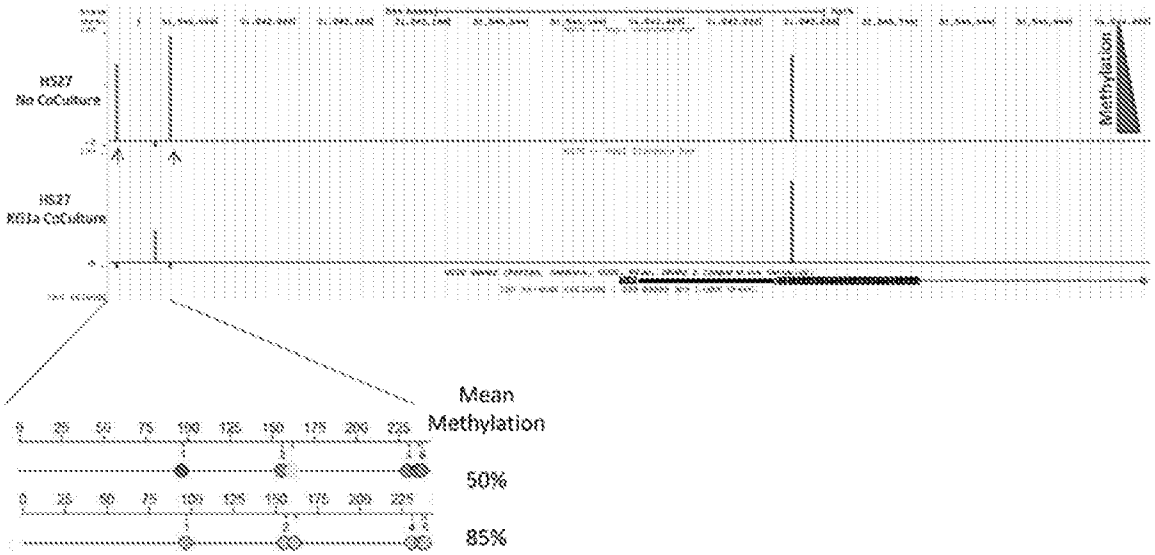


Fig. 9

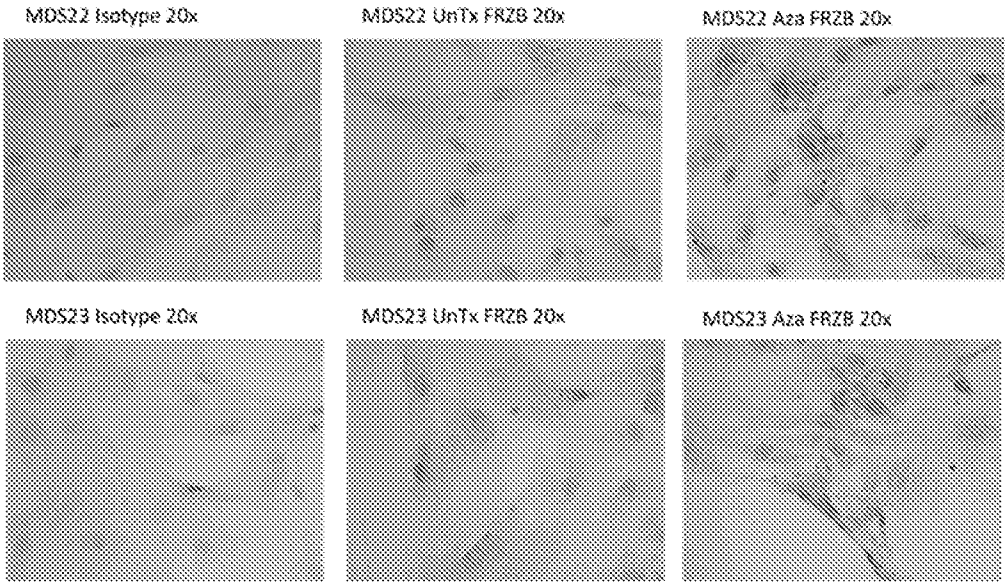

MDS22 Isotype 20x
MDS22 UnTx FRZB 20x
MDS22 Aza FRZB 20x
MDS23 Isotype 20x
MDS23 UnTx FRZB 20x
MDS23 Aza FRZB 20x

Fig. 10

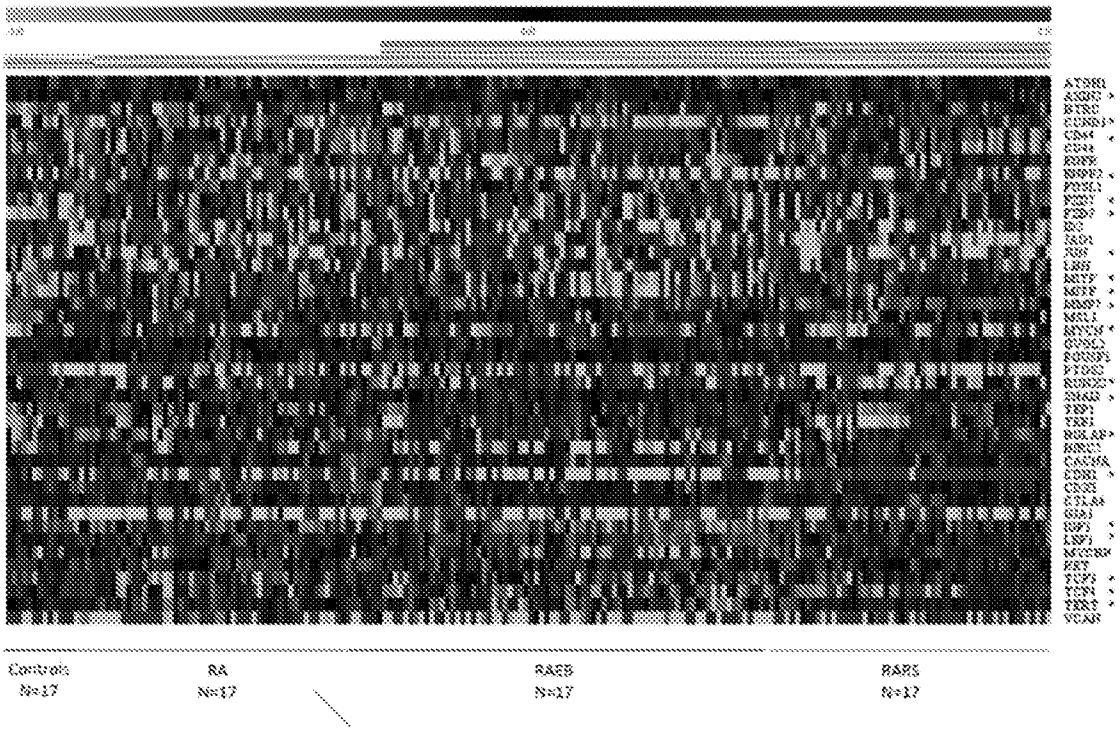


Fig. 11

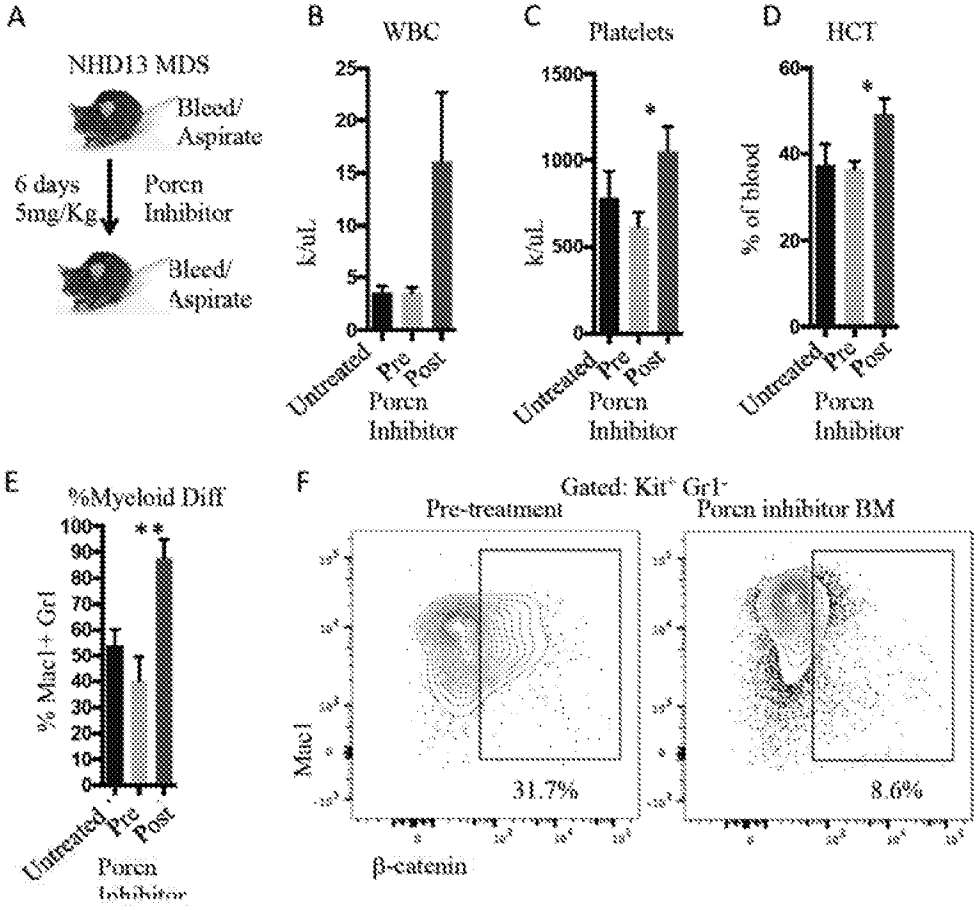

A
NHD13 MDS
Bleed/
Aspirate
6 days
5mg/Kg
Porcn
Inhibitor
Bleed/
Aspirate
B
WBC
k/uL
Untreated
Pre
Post
Porcn
Inhibitor
C
Platelets
k/uL
Untreated
Pre
Post
Porcn
Inhibitor
D
HCT
% of blood
Untreated
Pre
Post
Porcn
Inhibitor
E
%Myeloid Diff
% Mac1+ Gr1
Untreated
Pre
Post
Porcn
F
Gated: Kit+ Gr1-
Pre-treatment
Porcn inhibitor BM
Mac1
31.7%
8.6%
β-catenin

Fig. 12 A-F

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/029513

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/00; A61K 38/00; A61K 38/17; A61K 39/00; A61K 39/395 (2017.01)
CPC - A61K 38/00; A61K 45/06; C07K 7/06; C07K 14/47; C07K 16/18; C07K 16/28 (2017.02)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/172.1; 424/178.1; 506/9; 514/19.3; 530/387.9; 530/387.1 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2014/110506 A2 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 17 July 2014 (17.07.2014) entire document	1-4, 7-9, 13, 14, 16, 17, 22, 30, 36-38
--		----------
Y		5, 6, 10-12, 15, 18-21, 23-27, 31-35
Y	WO 2014/089241 A9 (CARIS MPI, INC.) 20 August 2015 (20.08.2015) entire document	5, 6
Y	WO 2016/055790 A1 (REDX PHARMA PLC) 14 April 2016 (14.04.2016) entire document	10
Y	US 2010/0311683 A1 (BEACH et al) 09 December 2010 (09.12.2010) entire document	11, 12, 18-20, 33, 34
Y	US 2016/0002354 A1 (OXFORD BIOTHERAPEUTICS LTD) 07 January 2016 (07.01.2016) entire document	15, 31-35
Y	WO 2016/007775 A1 (GENENTECH, INC. et al) 14 January 2016 (14.01.2016) entire document	21
Y	WO 2016/023017 A1 (BAYLOR COLLEGE OF MEDICINE) 11 February 2016 (11.02.2016) entire document	23-25
Y	US 2007/0105792 A1 (DIMARTINO) 10 May 2007 (10.05.2007) entire document	26, 27

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance
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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 July 2017	04 AUG 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774