Massive parallel RNA sequencing of highly purified mesenchymal elements in low-risk MDS reveals tissue-context dependent activation of inflammatory programs

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Myelodysplastic syndromes (MDS) have long been considered hematopoietic cell-autonomous disorders in which disease initiation and progression is exclusively driven by hematopoietic cell intrinsic genetic events. Recent experimental findings have challenged this view, implicating mesenchymal elements in the bone marrow microenvironment in disease pathogenesis. Specifically, genetic perturbation of mesenchymal cells has the ability to induce MDS and acute myeloid leukemia (AML), establishing an experimental concept of 'niche-induced' oncogenesis.[1, 2] Alternatively, primary alterations in hematopoietic cells have the ability to alter mesenchymal niche components such that niche cells facilitate disease propagation in the context of xenograft transplantation.[3] Together, these observations challenge the view that ineffective hematopoiesis and leukemic progression is exclusively driven by hematopoietic-cell autonomous events in human MDS. Translation of experimental findings to human disease is complicated by a lack of insight in the molecular wiring of primary, non-expanded, mesenchymal cells in MDS. Insights into the biology of mesenchymal elements in human MDS, and other hematopoietic disorders, thus far, have been derived from studies investigating *ex vivo* expanded mesenchymal cells derived from the diseased bone marrow. The hierarchic, biologic and molecular relationship between these *ex vivo* expanded cells and their in situ counterparts, however, has remained largely unknown. Here, we describe massive parallel transcriptome sequencing of prospectively isolated mesenchymal elements from human low risk MDS (LR-MDS), revealing a common molecular signature, distinct from both normal and *ex vivo* expanded cells, characterized by cellular stress and upregulation of genes encoding inflammation-associated secreted factors with established inhibitory effects on hematopoiesis.

Mesenchymal cells were prospectively FACS-sorted from bone marrow aspirates of LR-MDS patients (n=12, Supplementary Table S1) and normal controls (n=10) using previously established markers of primary bone marrow mesenchymal cells (Figure 1A).[4] The frequency of $CD45^-/7AAD^-/CD235a^-/CD31^-/CD271^+/CD105^+$ mesenchymal cells in LR-MDS was not

significantly different from normal bone marrow (Figure 1B) (0.019%±0.0086% vs. 0.022%±0.0066% of mononuclear cells (MNCs), p=0.819 by unpaired student t-test), and these cells comprised a small subset of $CD45^-/7AAD^-/CD235a^-$ 'niche' cells (10.41%±4.086% vs. 12.30%±5.052, p=0.771) with the major constituent being $CD31^+$ endothelial cells (43.80%±7.243% vs. 38.28%±9.424%, p=0.816).

RNA was extracted from highly purified mesenchymal elements and cDNA synthesis was performed using the SMARTer Ultra Low RNA kit for Illumina Sequencing (Clonetech) (Supplementary Methods). Quality of RNA-sequencing data was shown to be similar for normal and LR-MDS derived samples using various quality parameters including the number of aligned bases, base composition, coverage coefficient and full-length transcript coverage (from 5' end to 3' end) reflecting no systematic 5'-end or 3'-end bias (Supplementary Figure S1). The mesenchymal identity of $CD45^-/7AAD^-/CD235a^-/CD31^-/CD271^+/CD105^+$ cells was confirmed molecularly by whole transcriptome analysis demonstrating significant abundance of transcripts encoding defining membrane proteins (Figure 1C), established markers of mesenchymal stem cells (Figure 1D)[4, 5], essential 'niche' factors governing the behavior of hematopoietic stem and progenitor cells (HSPCs) (Figure 1E) and osteolineage markers (Figure 1F) compared to endothelial cells. Collectively, the findings demonstrate the feasibility of prospective isolation and molecular characterization of highly purified primary mesenchymal elements in LR-MDS by massive parallel transcriptome sequencing.

Principle component analysis (PCA) of all transcriptomes demonstrated uniform clustering of normal mesenchymal cells, implying transcriptional homogeneity (Figure 2A). Strikingly, distinct and more heterogeneous clustering of mesenchymal transcriptomes was found in LR-MDS revealing that these cells are transcriptionally distinct from their normal counterparts. Gene set enrichment analysis (GSEA) was subsequently performed to define the molecular networks

underlying the distinct transcriptional landscape of LR-MDS. Gene sets associated with inflammatory response and cellular stress were remarkably enriched in LR-MDS (Figure 2B-C; Supplementary Table S2). Cellular stress was reflected by a reduced capacity of the $CD271^+$ mesenchymal population in LR-MDS to form colonies (Figure 2D) with morphologic features reminiscent of cellular senescence (Figure 2E), as described earlier for expanded stromal cells in LR-MDS.[6, 7] Distinct hierarchical clustering and the signatures of cellular stress were not age-dependent, as these signatures remained statistically significant when examined in an age-matched sub-cohort of patients and controls (Supplementary Figure S2). Together, the data indicate that mesenchymal cells in LR-MDS are molecularly and functionally distinct from their normal counterparts, characterized by cellular stress, reflected by a reduced, *ex vivo,* capacity to form fibroblast colonies.

Thus far, molecular and biologic insights into the role of mesenchymal cells in the pathogenesis of human MDS have been derived from studies using *ex vivo* expanded, plastic adherent stromal cells. The molecular relationship between these expanded cells and their *in situ* mesenchymal counterparts has remained largely unknown. Elucidation of the transcriptome of mesenchymal elements in the MDS marrow allows us to compare our transcriptional data to sequencing data obtained from expanded cells in an age-matched cohort of LR-MDS published earlier (Supplementary Figure S3).[3]

Comparison of FDR-significant differentially expressed transcripts between the two datasets demonstrated limited overlap (Figure 2F), suggesting distinct molecular wiring between the two mesenchymal cell sources. To obtain insight into the biologic processes underlying differential gene expression, GO (gene ontologies) term analysis was performed focusing on cellular biologic processes. To correct for potential experimental differences affecting FPKM values, we normalized expression of all genes in LR-MDS to the expression of the controls in the

respective data sets as detailed in the Supplementary Methods section. Normalized expression was subsequently used to perform GO term analysis and GSEA, comparing sorted to expanded cells.

25 GO terms were significantly (FDR < 0.25) enriched in primary CD271$^+$ mesenchymal cells (while no signatures were enriched in the *ex vivo* expanded mesenchymal cells), many of which (8/25) reflected response to external stimuli, chemokine activity and immune regulation (Figure 2G). Transcript abundance analysis in CD271$^+$ cells in comparison to their normal counterparts indeed revealed significant upregulation of numerous cytokines (Supplementary Table S3), including a large number of inflammatory factors, such as IL6 and IL8, and a wide variety of factors previously demonstrated to be negative regulators of hematopoiesis, in particular erythropoiesis and B-lymphopoiesis, cell lineages that are typically affected in LR-MDS (Supplementary Table S3).

To obtain insight into the molecular pathways underlying the biologic processes identified, transcriptional network analysis (GSEA) was performed. This identified 504 gene sets that were significantly (FDR < 0.25) enriched in primary sorted LR-MDS stromal cells, whereas 16 signatures were enriched in expanded LR-MDS stromal cells. Again, gene signatures related to inflammation and cellular stress were enriched in CD271$^+$ cells with a remarkable abundance of signatures related to EGF, TGFß and TNF signaling (Supplementary Figure S4, Table S4).

Collectively, the data comprise, to our knowledge, the first comprehensive transcriptional network analysis of highly purified mesenchymal elements directly isolated from the marrow in human hematopoietic disease. They support the view that these cells are intricately implicated in MDS disease pathogenesis, stressing the relevance of considering the tissue context in generating a comprehensive understanding of the disease. The data further support the notion

that inflammatory signaling is an important pathophysiologic factor in LR-MDS and implicate the mesenchyme in this process. Finally, the data complement findings derived from *ex vivo* stromal cells in this disease revealing preferential overexpression of inflammatory pathways and secreted factors in FACS-purified CD271$^+$ cells. This likely reflects active cross-talk with other cellular elements within the inflammatory bone marrow environment in LR-MDS[8], eliciting or maintaining these transcriptional programs, which may not be fully appreciated in *ex vivo* cultures.

The finding that secretory programs implicated in negative regulation of hematopoiesis are activated in CD271$^+$ cells may be of particular relevance given their close anatomical proximity with CD34$^+$ cells[9], potentially harboring the MDS initiating population.[10] The data warrant future investigations unraveling the signaling between cellular elements in the MDS marrow driving these secretory programs. We anticipate that elucidation of the transcriptome of highly purified mesenchymal elements in MDS will thus be a valuable resource to the community, instructing the validation and discovery of novel pathophysiologic factors and putative therapeutic targets.[11]

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Authorship

S.C and M.H.G.P.R designed studies; S.C, N.A.Z, K.K, A.M.M, and N.M.A performed experiments and acquired data; S.C, R.M.H, E.M.J.B, and M.A.S provided technical guidance and bioinformatical analysis; E.M.P.C and A.vd.L provided patient material and clinical data;

J.H.J performed mutational studies and provided molecular data of the patients; S.C and M.H.G.P.R. wrote the manuscript; all authors were involved in data interpretation and manuscript reviewing, M.H.G.P.R supervised the study.

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Figure legends

Figure 1. Prospective isolation and molecular characterization of mesenchymal cells in LR-MDS. (A) Gating strategy to identify and isolate $7AAD^-/CD45^-/CD235a^-/CD271^+/CD105^+$ mesenchymal cells. (B) Frequency of mesenchymal cells in normal and MDS samples. (C-F) Transcriptional validation of the mesenchymal identity of $7AAD^-/CD45^-/CD235a^-/CD271^+/CD105^+$ cells, revealing differential expression in comparison to endothelial subsets of (C) defining cell surface markers (CD271, CD105, CD31), (D)

known mesenchymal markers (CD73, CD90, CD146), (E) established hematopoiesis-supporting cytokines (*CXCL12, ANGPT1, KITL*) and (F) bone lineage markers (*BGLAP, RUNX2, SPP1* and *ALPL*).

FPKM: fragments per kilobase of exon per million fragments mapped. CD73 (*NT5E*: ecto-5'-nucleotidase); CD90 (*THY1*: Thy-1 T-Cell Antigen); CD146 (*MCAM*: melanoma cell adhesion molecule); *CXCL12* (stromal cell-derived factor 1); *ANGPT1* (angiopoietin 1); *KITL* (KIT ligand); *BGLAP* (osteocalcin); *RUNX2* (runt-related transcription factor 2); *SPP1* (osteopontin); *ALPL* (alkaline phosphatase, liver/bone/kidney). Figure C to figure F: Normal samples (n=10); MDS samples (n=12). Black bar: CD271+ mesenchymal cells; white bar: CD31+ endothelial cells. ** FDR < .01; *** FDR < .001.

Figure 2. Mesenchymal cells in LR-MDS display a distinct molecular signature characterized by cellular stress and inflammation. (A) Principle component analysis (PCA) on the transcriptomes of normal and LR-MDS mesenchymal cells. Patient numbers in Figure 2A refer to LR-MDS patient IDs (Table S1). (B) Example of GSEA plot revealing inflammatory response in the mesenchymal cells from LR-MDS. (C) Representative GSEA plot demonstrating deregulation of the gene set associated with cellular stress in response to UV in LR-MDS mesenchymal cells. Gene set size, NES and FDR value of each gene set is listed. GSEA: gene sets enrichment analysis. NES: normalized enrichment score. FDR: false discovery rate. (D) Number of CFU-F colonies formed by normal (n=3) or LR-MDS (n=3) CD271+ mesenchymal cells. (E) Representative images of cell clusters and colonies formed by mesenchymal cells from healthy control (left panel) and LR-MDS patients (right panel). (F) Comparison of significantly differentially expressed genes in FACS-purified CD271+ versus culture-expanded mesenchymal cells in LR-MDS. The total number of differentially regulated transcripts in each data set is indicated and the overlapping differentially regulated genes in the two datasets are listed. (G) Biologic processes significantly enriched (FDR < 0.25) in FACS-purified CD271+ LR-MDS mesenchymal cells in comparison to expanded stromal cells defined by GO term analysis. ** P < .01

Figure 1

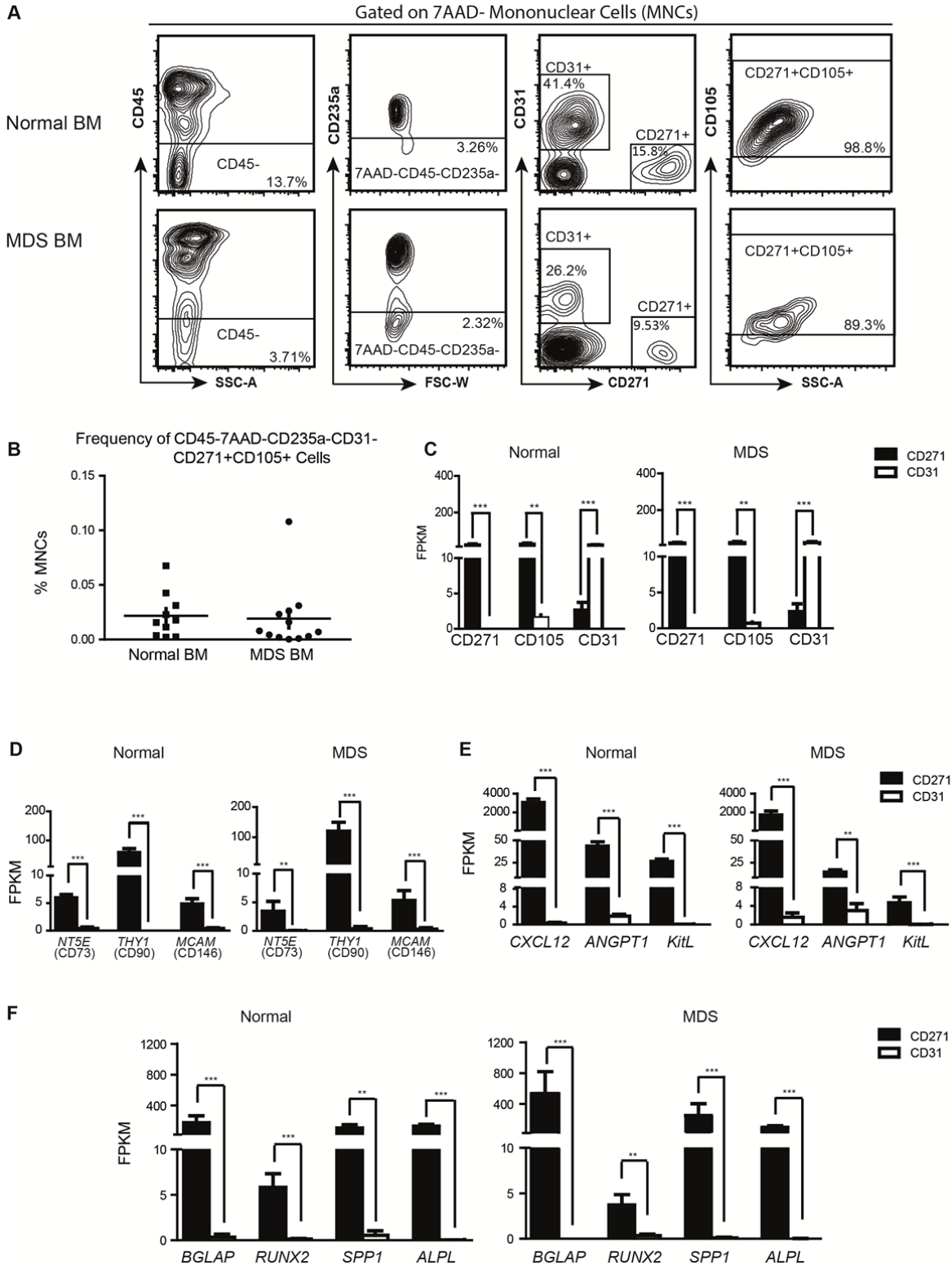
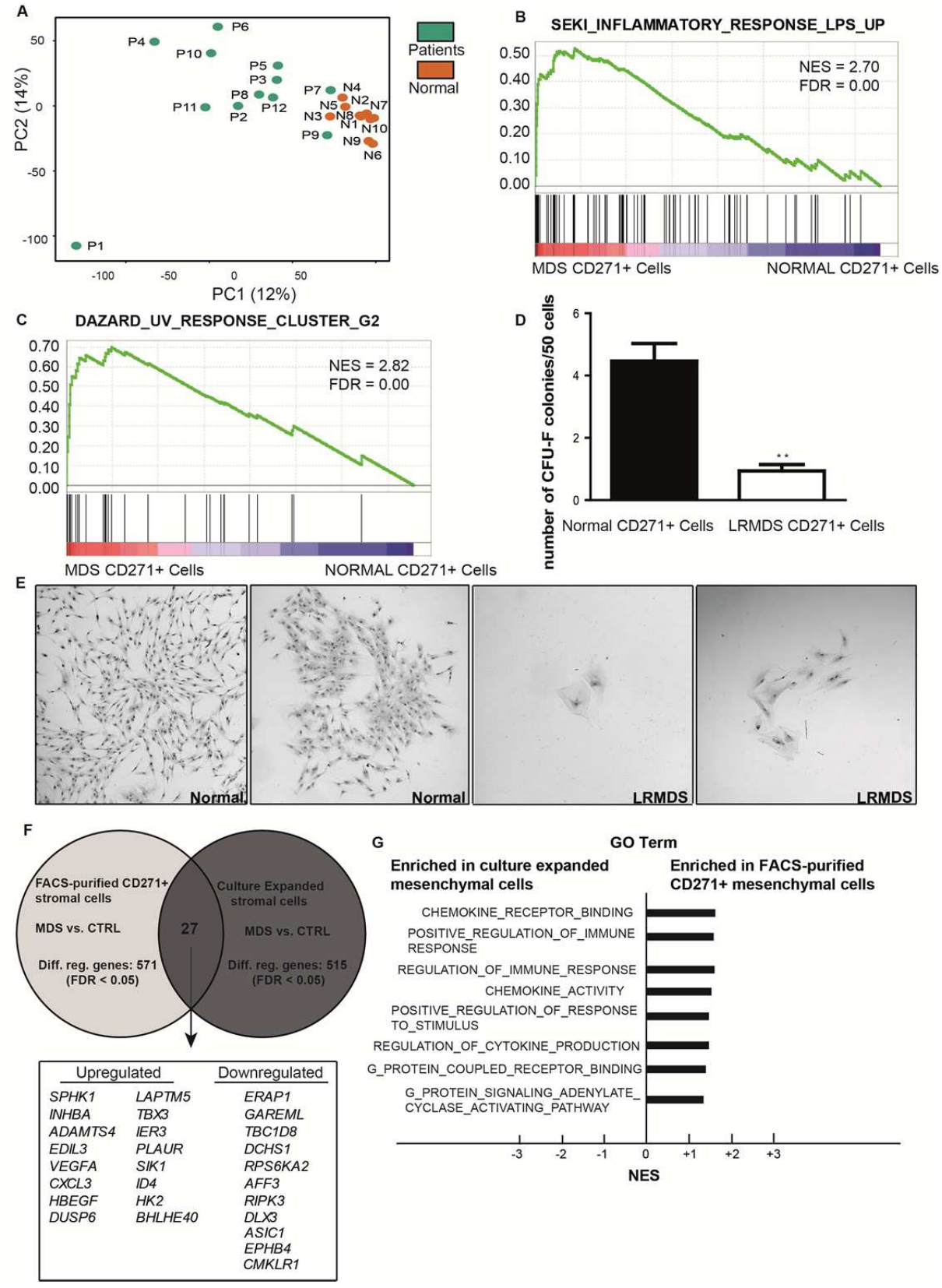

A
Gated on 7AAD- Mononuclear Cells (MNCs)
Normal BM
MDS BM
CD45
CD45-
13.7%
CD45-
3.71%
SSC-A
CD235a
3.26%
7AAD-CD45-CD235a-
2.32%
7AAD-CD45-CD235a-
FSC-W
CD31
CD31+
41.4%
CD271+
15.8%
CD31+
26.2%
CD271+
9.53%
CD271
CD105
CD271+CD105+
98.8%
CD271+CD105+
89.3%
SSC-A
B
Frequency of CD45-7AAD-CD235a-CD31-CD271+CD105+ Cells
% MNCs
0.15
0.10
0.05
0.00
Normal BM
MDS BM
C
Normal
MDS
CD271
CD31
FPKM
400
200
10
5
0
***
**
CD271
CD105
CD31
D
Normal
MDS
FPKM
200
100
10
5
0
NT5E (CD73)
THY1 (CD90)
MCAM (CD146)
E
Normal
MDS
CD271
CD31
FPKM
4000
2000
50
25
8
4
0
CXCL12
ANGPT1
KitL
F
Normal
MDS
CD271
CD31
FPKM
1200
800
400
10
5
0
BGLAP
RUNX2
SPP1
ALPL

Figure 2


A
PC2 (14%)
PC1 (12%)
Patients
Normal
B
SEKI_INFLAMMATORY_RESPONSE_LPS_UP
NES = 2.70
FDR = 0.00
MDS CD271+ Cells
NORMAL CD271+ Cells
C
DAZARD_UV_RESPONSE_CLUSTER_G2
NES = 2.82
FDR = 0.00
MDS CD271+ Cells
NORMAL CD271+ Cells
D
number of CFU-F colonies/50 cells
Normal CD271+ Cells
LRMDS CD271+ Cells
E
Normal
Normal
LRMDS
LRMDS
F
FACS-purified CD271+ stromal cells
MDS vs. CTRL
Diff. reg. genes: 571 (FDR < 0.05)
27
Culture Expanded stromal cells
MDS vs. CTRL
Diff. reg. genes: 515 (FDR < 0.05)
Upregulated
SPHK1 LAPTM5
INHBA TBX3
ADAMTS4 IER3
EDIL3 PLAUR
VEGFA SIK1
CXCL3 ID4
HBEGF HK2
DUSP6 BHLHE40
Downregulated
ERAP1
GAREML
TBC1D8
DCHS1
RPS6KA2
AFF3
RIPK3
DLX3
ASIC1
EPHB4
CMKLR1
G
GO Term
Enriched in culture expanded mesenchymal cells
Enriched in FACS-purified CD271+ mesenchymal cells
CHEMOKINE_RECEPTOR_BINDING
POSITIVE_REGULATION_OF_IMMUNE RESPONSE
REGULATION_OF_IMMUNE_RESPONSE
CHEMOKINE_ACTIVITY
POSITIVE_REGULATION_OF_RESPONSE TO_STIMULUS
REGULATION_OF_CYTOKINE_PRODUCTION
G_PROTEIN_COUPLED_RECEPTOR_BINDING
G_PROTEIN_SIGNALING_ADENYLATE_ CYCLASE_ACTIVATING_PATHWAY
NES