Characterization of freshly isolated bone marrow mesenchymal stromal cells from healthy donors and patients with multiple myeloma: transcriptional modulation of the microenvironment

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Supplementary Materials

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Participants: Patients and healthy donors bone marrow samples.

A total of 56 patients with newly diagnosed MM and 16 paired diagnosis-MRD cases were included in this study (median age 72 years old, range of 34-89). Control samples were obtained from 21 healthy individuals (median age 22 years old, range of 18-86). The use of human samples was approved by the Institutional Review Board of the Clinica Universidad de Navarra, in accordance with the declaration of Helsinki.

Human MSCs: Isolation and Characterization.

For MSCs isolation, red blood cells (RBC) were removed from BM aspirates using 1X RBC Lysis buffer for 15 minutes at room temperature. Cells were immunophenotyped and FACS-sorted (FACSAria II, BDB; purity ≥99%) using an 6-color direct immunofluorescence stain with a combination of monoclonal antibodies (MoAb): fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein-cyanin 5.5 (PerCP-Cy5.5), PE-cyanin 7 (PE-Cy7), violet 500 (V500) and Brilliant Violet 421 (V421): CD73, CD105, CD271, CD13, CD45 and CD90, respectively. MSCs were directly collected in Lysis/Binding Buffer (Invitrogen, A33562) for mRNA isolation.

***In vitro*-expanded MSCs**

In vitro-expanded MSCs from BM samples of healthy donors (n=11) and MM patients (n=10) were generated as described by Garayoa et al, 2009. Briefly, mononuclear cells from bone marrow samples were isolated using Ficoll-Paque density gradient centrifugation (ρ 1.073; GE Healthcare, Uppsala, Sweden), cultured in DMEM with 10% FBS, 100U/mL penicillin, 100μg/mL streptomycin and 2mM L-glutamine for four days and selected by their adherence to plastic. The culture medium was replaced twice weekly until MSC cultures were approximately 90%

confluent or had been in culture for a maximum of 21 days, at which point, cells were trypsinized (0.05% Trypsin-EDTA) and expanded in a 1:3 ratio. At passage 3, selected MSCs from both origins were tested to meet definition criteria according to the recommendations of the International Society for Cellular Therapy[1] and 100ul of Lysis/Binding Buffer (Invitrogen, A33562) were added for RNA isolation.

Differentiation to osteoprogenitor cells and alizarin red staining

To induce *ex vivo* differentiation to osteoblasts (OBs), the growth medium of MSCs at 80-90% confluence was replaced by an osteogenic differentiation medium consisting of α-Minimal Essential Medium (α-MEM) supplemented with 10% FBS, 10mM β-glycerol phosphate, 50μg/mL ascorbic acid and 10nM dexamethasone (all additives from Sigma-Aldrich, St. Louis, MO, USA). MSCs were grown in the osteogenic medium for 7-early stage of OB differentiation, 14-pre-OB stage or 21 days -fully differentiated OB, replacing the medium every 3 or 4 days.

For alizarin red staining, MSCs differentiated in osteogenic media were fixed for 15 minutes in 1% formaldehyde, rinsed with water, stained with Alizarin Red Solution (Sigma-Aldrich) (2% wt/vol, pH 4.2) for 10 minutes, rinsed 3 times with water, and then imaged. Staining was quantified by dissolving Alizarin Red Solution stain from wells in 6-well plates in 1mL of decalcification solution (Cal-EX Decalcifier; Fisher Scientific) and reading absorbance of the solution at 405nm (200μL per well, 96-well plates in a FLUOstar Optima plate reader). Microscopy was performed using an Olympus CKX41 microscope with appropriate filter cubes and an Olympus DP72 Camera with x10, x20 and x60 objectives.

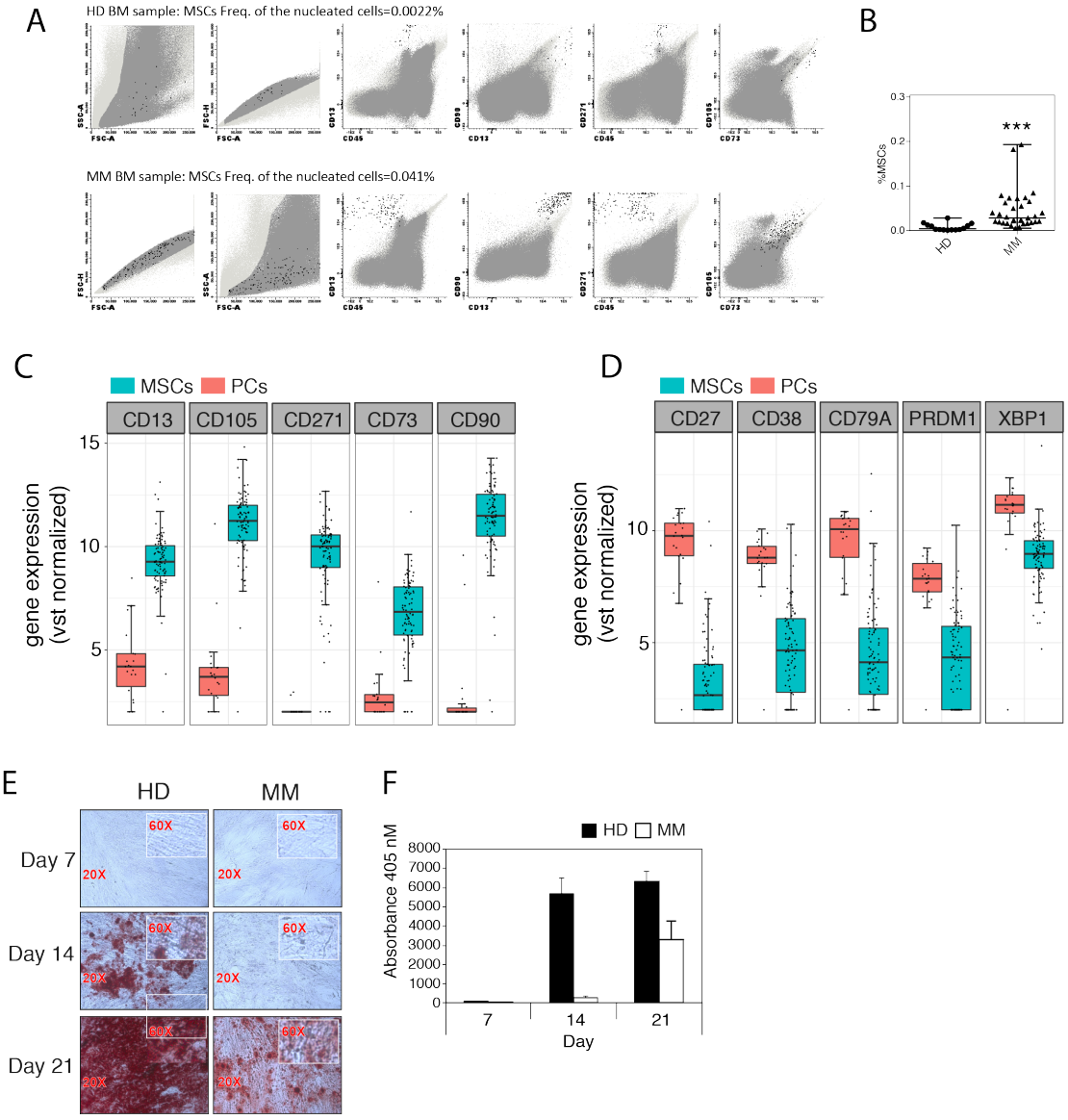
RNA-seq library construction

RNA-seq was performed in freshly isolated and *ex vivo* expanded MSC from HD and MM patients at diagnosis, MRD+ and MRD- stages. The protocol was adapted from MARS-seq protocol.[2] Briefly, samples were FACS sorted directly into 50µl of Lysis/Binding Buffer (Invitrogen). Poly-A RNA was selected with Dynabeads Oligo (dT) (Invitrogen) and reverse-transcribed with AffinityScript Multiple Temperature Reverse Transcriptase (Agilent) using oligo(dT) primers carrying a 7bp-index. Up to 8 samples with similar overall RNA content were pooled together and subjected to linear amplification by *in vitro* transcription using HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs). Amplified RNA was fragmented into 250-350bp with RNA Fragmentation Reagents (Invitrogen) and dephosphorylated with thermosensitive alkaline phosphatase (FastAP, Thermo). Partial Illumina adaptor sequences were ligated with T4 RNA Ligase 1 (New England Biolabs), followed by a second reverse transcription reaction. Full Illumina adaptor sequences were added with KAPA HiFi DNA Polymerase (Kapa Biosystems). Libraries were sequenced in an Illumina NextSeq 500 at a median sequence depth of 10 million reads per sample.

Bioinformatic analysis

Sequencing data quality was assessed with FastQC software and Illumina adapter sequences, polyA tails, and short reads (less than 20 bases) were trimmed using Cutadapt. Then, reads were aligned to the hg19 reference genome with STAR software[3] followed by gene expression quantification with public quant3p script (github.com/ctlab/quant3p) based on HTSeq package.[4] Downstream analyses were performed in R.[5] Minimal gene expression was established at 4cpm, and genes with lower levels of expression in more than 20% of samples from at least one sample

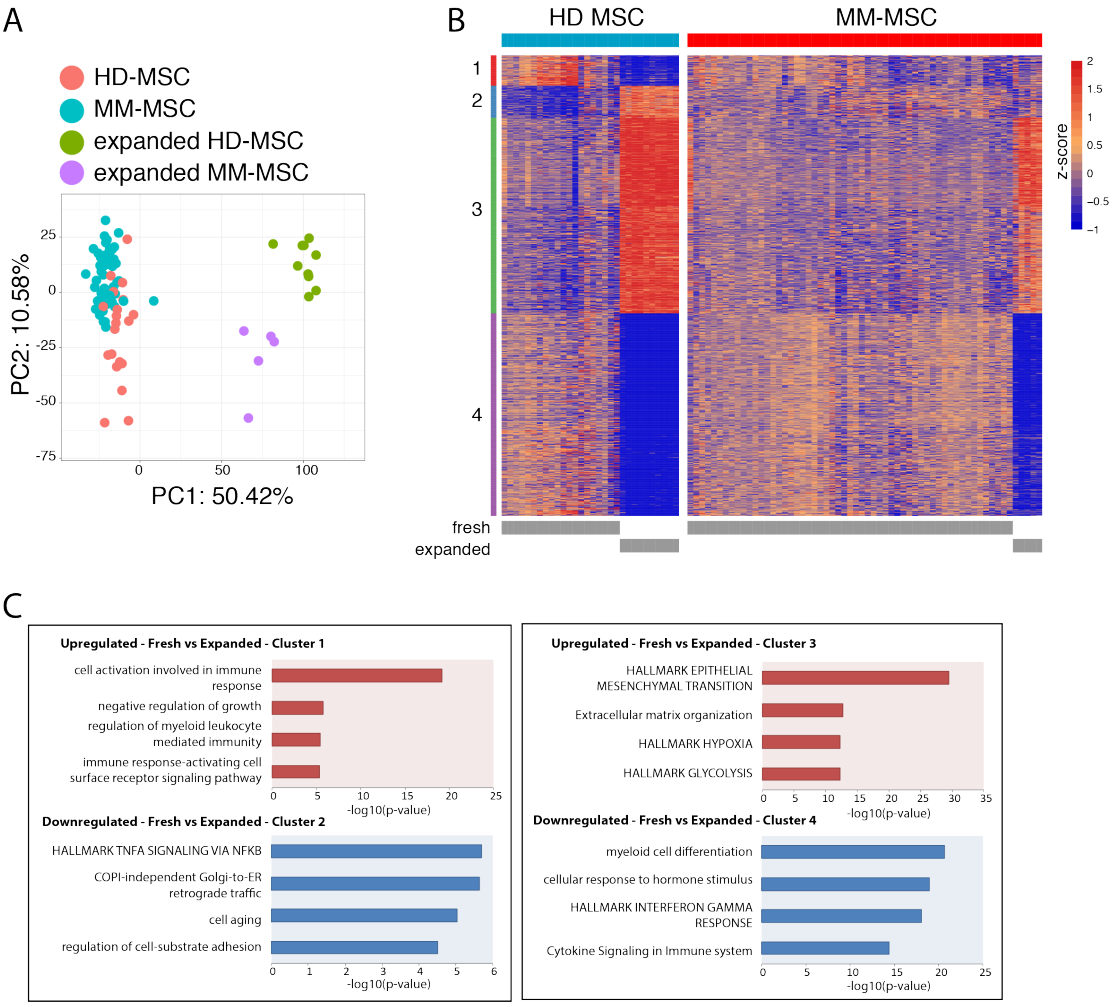
group were removed. Additionally, we filtered 20% of most variable genes per group for increased consistency. Differential gene expression analysis and variance-stabilizing transformation (vst) were performed with DESeq2.[6] Genes were considered adjusted p value (p-adj) < 0.05 and absolute log transformed fold-change ($|\log_2 FC|$) > 1, unless otherwise indicated. vst expression values were used for data visualization and unsupervised analysis. Batch effect associated with library preparation was removed by introducing "library" variable into the model formula (~library+condition) for DESeq2 analysis. Paired samples were analyzed with the model formula ~library+patient+condition. Data was processed using RemoveBatchEffect function from Limma analysis package[7] for data visualization purposes. Multiple R-packages were employed for hierarchical and kmeans clustering, volcano-plots, boxplots or barplots. Principal component analysis was performed with prcomp function and ggplot2 R-package.[8] Functional enrichment analysis was performed with Metascape[9] (http://metascape.org). Briefly, top 5 non-redundant biological functions (GO Biological Processes, KEGG Pathway, Hallmark Gene Sets and Canonical Pathways) were selected from first in-group features by p-value and enrichment level, -log10(p-value) of each term in each analyzed gene set was used for heatmap visualization.



Supplementary Figure 1. Isolation and molecular characterization of MSCs.

(A) Representative flow plots showing the gating strategy (CD45-/CD73+/CD105+/CD271+/CD13+/CD90+) and the frequency of MSCs in a HD and a MM sample. (B) Quantification of the frequency of MSCs in healthy donors and MM patients' samples. ***p-value<0.001. (C) and (D) Transcriptional validation of the mesenchymal identity, revealing differential expression in cell lineage markers in comparison with PC (Alameda et al., unpublished); (C) High Positive expression of

CD13, CD105, CD271, CD73 and CD90 in MSCs and (D) Low Levels of expression of CD27, CD38, CD79A, PRDM1 and XBP1 in MSCs. (E) Histologic analysis of *ex vivo*-expanded MSCs differentiated to osteoblast after 7, 14 and 21 days stained for mineralization (Alizarin Red) in HD and MM patients' derived samples. (F) Quantification of calcium deposits at days 7, 14 and 21 of MSCs osteoblast differentiation culture with Alizarin Red Solution staining.



Supplementary Figure 2. Transcriptional changes observed in MSCs after *ex vivo* expansion.

(A) PCA of transcriptional data from freshly isolated and ex vivo expanded MSC from both, MM and HD BM samples. Median absolute deviation (mad) was used as variability measure. (B) Semi-supervised kmeans clustering (k=4) of a total of 1556 differentially expressed genes (p-adj < 0.01 and $|\log_2 FC| > 1$) between freshly isolated or *in vitro*-expanded HD and MM-MSCs. Pairwise differential expression across groups of samples followed by k-mean clustering. Gene expression data was z-score standardized by row for visualization purposes. (C) Top selected biological functions enriched in each cluster (Supplemental Figure 2B). Left, functions enriched in clusters

of genes only altered in HD-MSCs by *in vitro* manipulation. Right, functions disturbed by *in vitro*-expansion independently of MSCs condition. Red, upregulated. Blue, downregulated.

Subject	Condition	Age
S062	MM	34
S010	MM	41
S028	MM	43
S033	MM	44
S009	MM	49
S024	MM	52
S088	MM	54
S048	MM	55
S008	MM	58
S037	MM	58
S025	MM	59
S029	MM	59
S040	MM	59
S058	MM	59
S030	MM	60
S057	MM	61
S002	MM	62
S067	MM	63
S006	MM	65
S065	MM	65
S055	MM	66
S061	MM	66
S063	MM	66
S109	MM	68
S047	MM	69
S068	MM	69
S070	MM	69
S073	MM	69
S007	MM	70
S072	MM	70
S084	MM	70
S066	MM	71
S005	MM	72
S041	MM	72
S049	MM	72
S078	MM	72
S036	MM	73
S045	MM	73
S064	MM	75
S069	MM	75
S076	MM	75
S081	MM	75
S046	MM	76
S085	MM	76
S077	MM	78
S038	MM	79
S071	MM	79
S080	MM	79
S060	MM	81
S042	MM	83
S004	MM	84
S054	MM	84
S074	MM	84
S075	MM	84
S043	MM	87
S050	MM	89
S022	Elderly HD	65
S011	Elderly HD	66
S015	Elderly HD	68
S108	Elderly HD	69
S107	Elderly HD	70
S020	Elderly HD	71
S012	Elderly HD	76
S014	Elderly HD	86
S098	Young HD	18
S094	Young HD	20
S096	Young HD	20
S097	Young HD	20
S105	Young HD	21
S089	Young HD	22
S090	Young HD	22
S092	Young HD	22
S093	Young HD	22
S100	Young HD	22
S101	Young HD	22
S106	Young HD	22
S102	Young HD	23

Supplementary Table 1. Subjects age distribution.

Sample	Condition	Time to follow-up (months)
MSC053	MRD+	8.1
MSC060	MRD+	8.5
MSC069	MRD+	7.6
MSC097	MRD+	13.1
MSC102	MRD+	17.4
MSC107	MRD+	3.6
MSC118	MRD+	9.2
MSC049	MRD+	6.4
MSC032	MRD-	7.2
MSC050	MRD-	12.8
MSC067	MRD-	6.3
MSC089	MRD-	22.1
MSC094	MRD-	13.6
MSC108	MRD-	13.0
MSC079	MRD-	12.9
MSC091	MRD-	3.2

Supplementary Table 2. Time to follow-up of MSC samples from MRD patients.

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