

In the second-year of the project we focalized our attention on: *i)* the characterization of bone marrow-derived mesenchymal cells obtained from SDS patients (SDS-MSC); *ii)* the interaction between SDS-MSC and neutrophils; *iii)* the capacity of SDS-MSC to support the hematopoiesis, as stated in the first report.

Phenotypical and functional characterization of MSC obtained from SDS patients

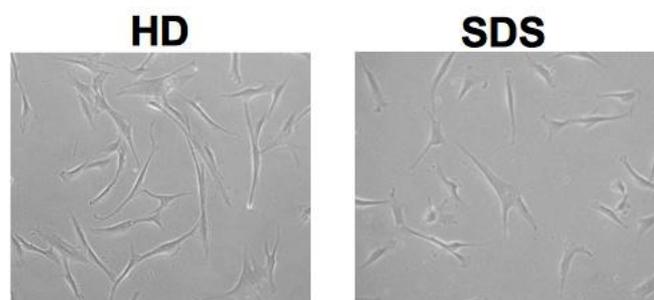
The mechanisms underlying the bone marrow failure in SDS patients is not fully understood. Dror and Freedman (Dror et al, *Blood*, 1999) reported a generalized marrow dysfunction in SDS patients with abnormal bone marrow stroma in terms of its ability to produce fat clusters and to support and maintain hematopoiesis.

Several studies demonstrated that MSCs represent the pivotal organizers for the generation, maintenance and plasticity of hematopoietic stem cell (HSC) niche thorough supporting the proliferation and differentiation of HSCs and their progenies (Bianco et al *Blood*, 2011). MSCs generate a number of stromal cells which have been shown to impact HSC behavior, including adipocytes pre-osteoblast, osteoblasts and chondrocytes. The hematopoietic microenvironment controls the formation of blood cells through the production and secretion of cytokines and extracellular matrix molecules. To our knowledge no study has examined the functional properties of MSCs obtained from patients with Shwachman-Diamond syndrome (SDS-MSCs). In this study we analyzed SDS-MSCs and we investigated their possible defects in supporting hematopoiesis (*paper in preparation*).

Results

Mesenchymal cells were obtained from bone marrow of 27 patients with SDS and were characterized for their proliferation capacity, antigen expression and differentiation ability along mesengenic lineages. Figure 1 showed that SDS-MSCs displayed a typical fibroblast-like morphology, similarly to HD-MSCs.

Figure 1: Morphology of SDS-MSC



MSC were subcultured when they reached 80-90% of confluence and cell growth was monitored by determination of population doubling (PD) time from passage 1 up to passage 5 of culture. Population doubling of MSCs obtained from HDs and SDS patients was comparable at each time-point considered (Students t-test>0,05). It is interesting to note, that the proliferation rate was reduced with passages in all MSCs tested. In particular, 2 days were necessary to double the cell number of both SDS-MSCs and HD-MSCs at P1; whereas PD was $4,3 \pm 1,4$ days for SDS-MSCs and $3,7 \pm 0,9$ days for HD-MSCs at P5. SDS-MSCs were also analyzed for their phenotype at P3. Flow cytometric analysis showed that SDS-MSCs, as well as HD-MSCs, were negative for hematopoietic (e.g., CD34, CD45 and MHC class II), lymphoid (e.g., CD19) and myeloid (e.g., CD11b and MHC class II) markers and positive for stroma lineage markers (e.g., CD73, CD90 and CD105). To further characterize SDS-MSCs, differentiation assays recommended by the International Society for Cellular Therapy (ISCT) were performed at P5. Adipocytic, osteoblastic and chondrocytic differentiation assays were performed in all cases. SDS-MSCs were able to differentiate into osteoblasts, as demonstrated by the histological detection of calcium deposition stained with Alzarin Red. The osteogenic differentiation was also confirmed by qRT-PCR, under osteogenic permissive condition at day 14 of culture. The expression of ALKP, OCN and OPN osteogenic genes has been observed both in SDS-MSCs and HD-MSCs. In addition, SDS-MSCs were able to differentiate into adipocytes, as revealed by the formation of lipid droplets stained with Oil Red O. Similarly to HD-MSCs, SDS- MSCs increased the expression of LEP, LPL and PPAR- γ . Within the first 24 hours of chondrogenic induction, the pellet becomes free-floating. During the induction of differentiation, sulfated proteoglycans accumulate in the extracellular matrix and multilayered cell pellet increases in size. To identify the development and accumulation of the cartilage matrix, proteoglycans were detected by safranin and fast green staining. No difference was observed in chondrogenic tissue formation between SDS-MSCs and HD-MSCs.

In order to evaluate the functional properties of SDS-MSCs, we analyzed their capacity to suppress lymphocyte proliferation, their ability to maintain the viability of CD34+ cells and their capacity to preserve neutrophils from apoptosis. The immunoregulatory activity of SDS-MSCs was evaluated by assessing their interaction with lymphocytes activated with mitogenic stimuli. PBMCs from healthy donors were stimulated with PHA in the presence or absence of various concentrations of irradiated MSCs. Figure 2 showed that SDS-MSCs were able to strongly inhibit lymphocyte proliferation in a dose-dependent manner. In particular, SDS-MSCs inhibited more than 50% of the lymphocytes proliferation (mean, 56%; range, 16-66%) at 1:32 ratio. This result was also observed in the presence of HD-MSCs.

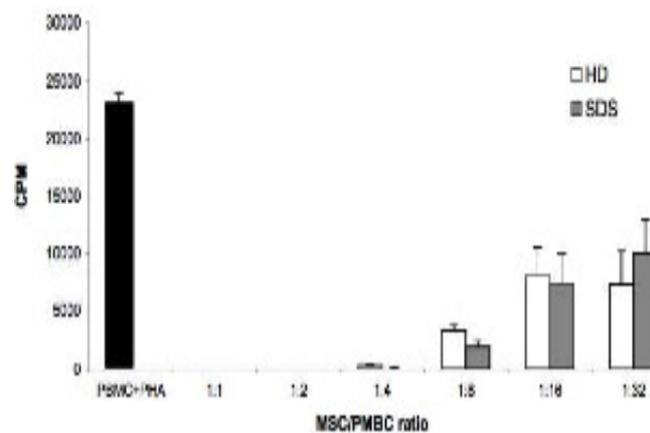


Figure 2: Lymphocytes proliferation in the presence or absence of SDS-MSCs.

Neutrophils are very sensitive cells and once released from BM they circulate in the PB and have a short half-life. We investigated the effects of MSCs on spontaneous neutrophil apoptosis. Neutrophils from healthy donors were cultured in the absence or presence of MSCs, both from patients and controls, for 72 hours. The majority of neutrophils cultured with medium alone died after few hours (data not shown). In contrast, MSCs were able to support the viability of neutrophils at different time points. At 24 hours about 13% (mean, 13,2%; range, 12,88-13,50%) of neutrophils were alive (double negative cells) while only necrotic and apoptotic neutrophils were detected after 72 hours. SDS-MSCs were able to sustain the neutrophils viability as the healthy donors counterpart.

CD34+ cells were co-cultured with MSCs from both patients and controls. When the 2 experimental conditions of CD34+ cells on mesenchymal cells were compared, numbers of total cells harvested during the 3 weeks were similar. The total number of cells increased at third week after an initial decline. After sorting more than 95% of cells expressed the CD34 marker. As expected, the percentage of CD34+ cells diminished during the co-culture. In particular, after 21 days the cells suspension still contained around 20% of CD34+ cells (data not shown). Importantly, SDS-MSCs as well as HD-MSC were able to preserve the stemness of these cells as CD34+ form hematopoietic colonies in methylcellulose after 3 weeks of co-culture. In both groups the CFU-E were not detected during culture. No statistical differences in the number of BFU-E and CFU-GM- after co-culture were observed between SDS-MSC and HD-MSC (Figure 3).

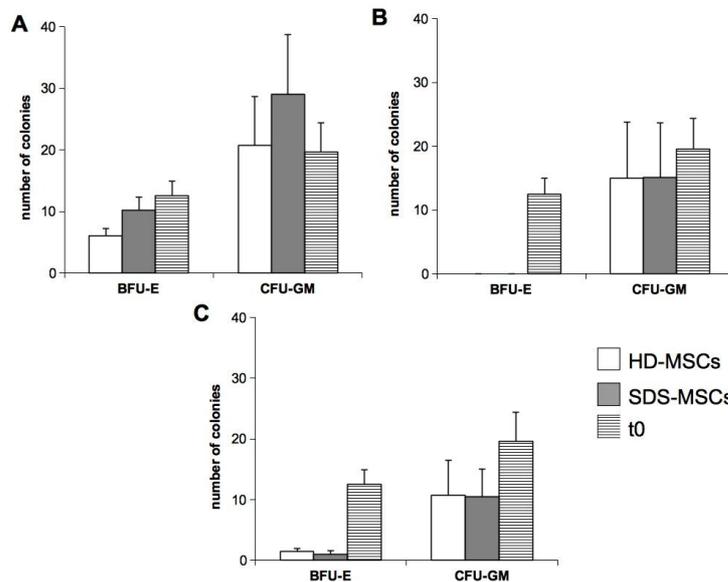


Figure 3: Detection of the BFU-E and CFU-GM colonies after 7 (A), 14 (B) 21 days of culture with MSC. CD34+ cells were seeded ($0,001 \times 10^6$) in wells containing methylcellulose. Colonies were scored as, BFU-E and CFU-GM using an inverted microscope after 21 days. Data represent the mean of 8 experiments performed \pm SE, Students t-test $> 0,05$.; BFU-E, erythroid burst-forming unit and CFU-GM, granulocyte-macrophage colony-forming unit.

Overall these results indicate that SDS-MSCs act in vitro just like their normal counterparts. Despite their pivotal role in the bone marrow niche, our data suggest that MSC themselves do not seem to be responsible for the hematological defects typical of SDS patients. Further in vivo studies mirroring the whole architecture of the hematopoietic stem cell niche are needed to comprehend the possible MSCs defects in SDS patients.