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Improving the Understanding of Shwachman-Diamond Syndrome

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"It is the time you have wasted for your rose
that makes your rose so important"
(Antoine De Saint-Exupéry)

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Chapter 1

Introduction

1.1 Shwachman-Diamond syndrome: general introduction

In 1964, Shwachman-Diamond Syndrome (SDS, OMIM 260400) was described as a disease in a cohort of patients with cystic fibrosis (CF). The syndrome was first characterized in England by Bodian in May of 1964[1] and then later the same year the disease was reported by Shwachman, Diamond and Oski[2]. Shwachman-Diamond syndrome is a rare autosomal recessive inherited disorder characterized by bone marrow dysfunction, exocrine pancreatic insufficiency and an increased risk of developing myelodysplastic syndrome (MDS) and/or acute myeloid leukemia (AML). In almost all affected patients, the disorder manifests a broad range of additional clinical features including skeletal abnormalities, neutropenia (recurrent infections, sometimes fatal, are common) and cognitive impairment. It can be

diagnosed in children of all ages or in adults and is extremely heterogeneous. Data show that SDS is the third leading inherited bone marrow failure syndrome after Diamond-Blackfan anemia and Fanconi anemia[3] and the most common cause of pancreatic insufficiency in children next to cystic fibrosis. The syndrome has an estimated incidence of 1 in 76000 (based on the observation that it is approximately $1/20^{th}$ as frequent as CF in North America[4]) with the heterozygote frequency of 1/138[5] and has no gender or ethnic predilection. In 2003, Boocock et al. showed that approximately 90% of patients have hypomorphic mutations in the Shwachman Bodian Diamond Syndrome gene ($SBDS$)[6] located on chromosome 7. The exact function of the SBDS protein remains unclear; however, recent studies in yeast and patient bone marrow cells show that *SBDS* gene is involved in RNA metabolism and ribosome biogenesis[7][8][9]. *SBDS* is an essential gene in embryogenesis and it is also implicated in cell division and cellular stress response[10][11].

1.2 Clinical presentation

In addition to case reports, several large cohort studies have summarized the clinical features associated with Shwachman syndrome.

1.2.1 Hematological abnormalities

Hematological abnormalities are present in all SDS patients. In particular, neutropenia is the most common hematological

deficiency. This defect is cyclical, persist throughout life and is the main cause of morbidity and mortality. Neutropenia, absolute neutrophil count (ANC)<1500 cells/μL, occurs in up to 95% of patients[12] and it is most frequently intermittent rather than persistent[13]. Hematological manifestations other than neutropenia include anemia, thrombocytopenia, raised fetal hemoglobin (HbF) levels and defects in the lymphoid lineage. Anemia is the second most common cytopenia in SDS, recorded in up to 80% of patients. Red blood cells are usually normochromic and normocytic but can also be macrocytic. Fetal hemoglobin was increased in 80% of patients[14]. Many patients (24% to 60%) develop a mild thrombocytopenia, defined by a platelet count$<150\times10^9$/L. Pancytopenia with progression to aplastic anemia has also been reported[15]. The development of aplastic anemia in patients with SDS suggests that the hematopoietic defect may reside at the level of an early hematopoietic stem cell. It has been demonstrated that patients with SDS had significantly lower number of $CD34^+$ cells on bone marrow aspirates. In addition, SDS $CD34^+$ cells showed markedly impaired colony production potential when plated in methylcellulose for clonogenic assays. The ability of marrow stromal cells from SDS patients to support normal $CD34^+$ was also diminished. In addition, patients also have a generalized marrow dysfunction with abnormal bone marrow stroma in terms of its ability to produce fat clusters and to support and maintain hematopoiesi[16]. The pivotal mechanism of bone marrow failure in SDS appears mediated by increased

apoptosis. This increased propensity for apoptosis is linked to high expression of the Fas antigen on marrow cells and to hyperactivation of the Fas signaling pathway[17][18].

Infection and immune abnormalities

Patients with SDS are particularly susceptible to recurrent viral, bacterial and fungal infections. Upper and lower respiratory tract infections and otits media are also frequently reported. Overwhelming sepsis is a well-recognized fatal complication of this disorder, particularly early in life. Neutropenia of variable degrees probably contributes to the susceptibility to infections. An additional mechanism possibly involves an inherited defect of neutrophil mobility, migration, and chemotaxis. Defects in lymphocyte mediated immunity have also been described. Several defects in B and T lymphocytes and natural killer (NK) cells were demonstrated[19]. B cells defects are comprised of low IgG production, low percentage of circulating B lymphocytes and decreased *in vitro* B cells proliferation. Also T and NK abnormality are comprised of low percentage of total circulating cells. Hematopoiesis deficiency is probably linked to defects in the bone marrow.

Risk of myelodysplastic syndrome and acute myeloid leukemia evolution

Similar to other marrow failure syndromes, patients with SDS have an increased risk for MDS and malignant transformation, in particular development of AML. The risk of leukemic and

dysplastic transformation increases with age varying from 14% to 30%[20][21]. Myelodysplastic syndromes are clonal hematologic disorders characterized clinically and morphologically by ineffective hematopoiesis. In general, myelodysplastic conditions are pre-leukemic disorders but not all cases of myelodysplasia evolve to AML[22]. Only if the percentage of leukemic blasts in the bone marrow is over 20% does the patient have leukemia, and not MDS. There is an increased frequency of clonal cytogenetic abnormalities in SDS, but estimates of the incidence of cytogenetic abnormalities in SDS patients are difficult to ascertain from a review of case reports. In a cohort study[23], six of 88 patients developed clonal cytogenetic abnormalities. Five of 12 patients had clonal abnormalities in another study[13]. A third longitudinal prospective study of 14 SDS patients with up to 5 years of follow-up found clonal marrow cytogenetic abnormalities consisting of i(7)(q10) or del(20)(q11) in four of 14 patients[24]. Abnormalities of chromosome 7, such as monosomy 7 or isochromosome 7q, are common. Other chromosome 7 abnormalities included monosomy 7, combined i(7q) and monosomy 7, and deletion or translocations involving 7q and accounted for 33% of the reported cytogenetic abnormalities in these patients. Deletion of long arm of chromosome 20 was the next most common chromosomal abnormality reported in 16% of cases. Although clonal cytogenetic abnormalities are typical in MDS and AML associated with SDS, the clinical significance of many of these cytogenetic abnormalities arising in the absence of morphologic MDS is unclear. There is some evi-

dence that i(7)(q10), resulting in duplication of 7q, is associated with a lower risk of MDS/AML[25] when compared with alteration commonly associated with MDS and rapid transformation to leukemia like monosomy of 7, deletion of 7q, derivative of 7, deletion of 5q, trisomy of 8 or complex abnormalities of 5q and 7q[26]. Alter reviewed 510 reported SDS cases, and no patient carrying the i(7)(q10) had developed MDS/AML, whereas patients with overt evolution into MDS/AML, other chromosome changes, including complex karyotypes, were observed[21]. In particular, Minelli et al.[27] studied eight patients with SDS carrying the i(7)(q10) who were heterozygotes for *SBDS* mutations. They demonstrated that all cases carried a double dose of the 258+2T>C and they suggested that, as the 258+2T>C mutation still allows the production of some amount of normal protein, this may contribute to the low incidence of MDS/AML in this subset of SDS patients. In a recent work, Pasquali[28] showed that detection of i(7)(q10) and del(20)(q12) abnormalities during the course of SDS is somehow a stochastic event and that the behavior of the abnormal clone was extremely variable. These results strongly suggest that the SDS karyotype instability is part of the natural history of SDS through a specific mutator effect, leading to the frequent acquisition of clonal chromosome anomalies in the bone marrow (BM), with changes of chromosomes 7 and 20 that may persist for a long time, or even regress, but may eventually promote MDS/AML with increasing age. It could well be that this evolution, or even the occurrence of i(7)(q10), is secondary to the acqui-

sition of other mutations in the BM, that are still unknown. Rujkijyanont and coworkers[29] demonstrated that SDS marrow mononuclear cells exhibit abnormal gene expression patterns. They used oligonucleotide microarray to identify gene expression patterns associated with leukemogenesis in marrow mononuclear cells of nine SDS patients without overt transformation compared to healthy controls. Among 154 known leukemia-related genes, several oncogenes were found to be up-regulated including *TAL1* and *MLL*, and several tumor suppressor genes were down-regulated including *DLEU1*, *RUNX1*, *FANCD2* and *DKC1*.

Recent data show that specific changes in mesenchymal cells (MSCs) of the hematopoietic microenvironment may be sufficient to initiate a complex phenotype of disordered homeostasis with similarities to myelodysplasia. Scadden[30] demonstrated the ability of this abnormality to result in the emergence of a clonal neoplasm in a cell type of clearly distinct lineage with distinct secondary genetic changes. The data indicate that individual, well-defined, mesenchymal microenvironment constituents can be primary enablers of neoplastic changes in a heterologous cell type. Shwachman syndrome has always been associated with risk of MDS/AML progression but recently, for the first time, a case of solid tumor in SDS patient has been reported. Singh et al.[31] describe a novel case of a breast cancer in a 30 years old SDS patient with a lack of family story for breast cancer and no mutations on *BRCA1* and *BRCA2* genes; the possibility of a solid tumor predisposition in Shwachman

patients requires further study.

1.2.2 Gastrointestinal features

One of the hallmarks of SDS is exocrine pancreatic dysfunction of varying severities. The pathophysiology of the pancreatic defect in Shwachman syndrome is due to the replacement of pancreatic acinar cells with fatty tissue[12][14][23]. Patients exhibit impaired enzyme production with low serum trypsinogen and low serum isoamylase levels[32]. The pancreatic deficiencies lead to malabsorption, steatorrhea, failure to thrive and low levels of fat-soluble vitamins A, D, E, and K. Pancreatic insufficiency is most significant between birth and two years of age and many patients require treatment with pancreatic enzyme replacement. The insufficiency spontaneously improves with increasing patient age but it is still unclear why this occurs[33]. Also hepatomegaly with elevated liver transaminases is often seen in young patients.

1.2.3 Skeletal abnormalities

Most or all patients with SDS have skeletal abnormalities[12][33]. Approximately half of patients have metaphyseal dysostosis, which most often involves the femoral head and is usually asymptomatic. One third to one half of children with SDS have rib-cage abnormalities, including short ribs with flared ends and a narrow rib cage. In a few cases, these abnormalities may lead to thoracic dystrophy and respiratory failure in the new-

born period. Other skeletal problems include delayed appearance of secondary ossification centers, osteopenia and vertebral collapse[34]. Osteoporosis and osteomalacia are often observed, most likely due to a lack of vitamin D and vitamin K[35]. However McLennan and colleagues observed no improvement in the bone abnormalties with intensive vitamin D therapy[36]. No phenotype-genotype correlation was observed[14] and the severity of the skeletal findings varied even in patients with identical *SBDS* mutations[35].

1.2.4 Additional features

Endocrine abnormalities in SDS include insulin-dependent diabetes, growth hormone deficiency, hypogonadotropic hypogonadism, and hypothyroidism. Cardiomyopathies have been noted in some cases. Psychomotor delay of varying severity, urinary tract anomalies, renal tubular acidosis, cleft palate and dental abnormalities also occur[37][38].

Multilineage cytopenias, hypoplasia of pancreatic acinar tissue and short stature are the predominant clinical manifestations of SDS. However, the mechanism explaining how loss of *SBDS* contributes to this phenotype is unknown. As the *SBDS* encodes a ribosomal associated protein, it is plausible that loss of *SBDS* leads to translational insufficiency. The consequences of ribosomal impairment is more prominent in tissues requiring a higher demand of protein synthesis. It is also interesting that whereas pancreatic dysfunction in SDS tends to normalize with age, skeletal and hematological changes are progressive.

1.3 Molecular pathogenesis

1.3.1 *SBDS* gene: structure, functions and mutations

In the 2003, Boocock and colleagues linked the cause of SDS with mutations on Shwachman-Bodian-Diamond syndrome gene (*SBDS*)[6]. Though the disease became labeled as SDS (for Shwachman and Diamond), Bodian's contribution has been recognized by the naming of the gene as the *SBDS* gene. Human *SBDS* gene encodes a member of a highly conserved protein family of unknown function with orthologues in diverse species including archaea, plants, and eukaryotes. *SBDS* gene has a 1.6 Kb transcript and encodes a predicted protein of 250 amino acids.

Structure

The human *SBDS* is located on chromosome 7 with the interval of 1.9 cM at 7q11, and it is composed of five exons spanning 7.9 kb. The paralogous is located 5.8 Mb distally and contains an unprocessed pseudogene copy of *SBDS* named *SBDSP*. The pseudogene transcript is 97% identical to *SBDS* and contains deletions and nucleotide changes that disrupt coding potential. Seventy-five percent of disease-associated mutations appeared to be the result of gene conversion that occurs when *SBDS* and its pseudogene recombine at meiosis. Shammas et al.[39] determined the structure of the A. *fulgidus SBDS* orthologue (*AfSBDS*). The AfSBDS structure has a three domain architec-

ture: an N-terminal domain with a mixed α/β-fold, a central three-helical bundle and a C-terminal ferredoxin-like domain. They showed that the N-terminal domain is the most frequent target for disease mutations.

Functions

The *SBDS* gene has been implicated in multiple biologic processes including ribosome biogenesis, stabilization of the mitotic spindle and cell motility. However the functional defect that causes the Shwachman-Diamond syndrome phenotype has not been clear. *SBDS* is an essential gene in embryogenesis and its ablation leads to early lethality at E6.5 in mice[40].

Studies in yeast and mammalian cells have demonstrated a role for *SBDS* in the ribosome maturation[9][41]. Premature joining of the ribosomal subunits is prevented by a protein called eukaryotic translation initiation factor 6 (eIF6) which binds the 60S ribosomial subunits. After nuclear export of 60S subunits, SBDS cooperates with elongation factor-like 1 (EFL1) to cause the release of eIF6 from the 60S to form the mature ribosome (Figure 1.1).

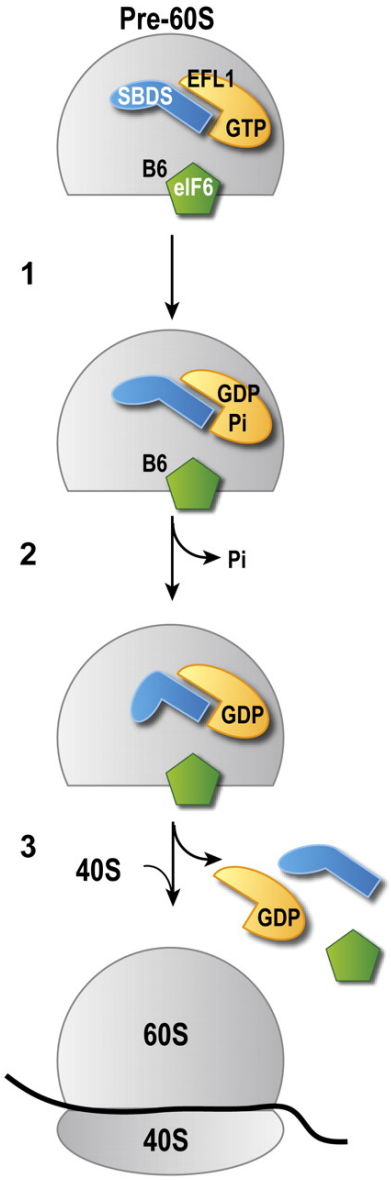

Figure 1.1: Model of eIF6 release by SBDS and EFL1. (1) SBDS stimulates 60S-dependent GTP hydrolysis by EFL1, generating EFL1.GDP.Pi. (2) Following release of inorganic Pi, EFL1 adopts its GDP-bound conformation and domain I of SBDS is rotated relative to domains II and III, directly or indirectly disrupting the intersubunit bridge B6. (3) Binding of eIF6 is destabilized, release of eIF6 is triggered and EFL1.GDP and SBDS dissociate from the ribosome. Release of eIF6 allows the formation of actively translating 80S ribosomes[9].

In addition, Austin and co-workers[42] also demonstrated that SBDS colocalized with the mitotic spindle, promoting its stability and chromosome segregation during the cell division process. The spindle stability defect in *SBDS* cells may explain the high frequency of chromosomal abnormalities observed in the bone marrows of patients.

A postulated role for *SBDS* in neutrophils has been suggested. Studies on SDS patient neutrophils show impaired mobility, migration and chemotaxis[19]. Orelio and Kuijpers[43] demonstrated that SDS neutrophils have chemotatic defects characterized by inabilities to orient toward a spatial chemoattractant gradient. Using the amoeba *Dictyostelium discoideum*, Wessels et al.[44] reinforced the hypothesis of *SBDS* role in chemotaxis. Little is known about how *SBDS* contributes to normal neutrophil development. Knockdown of *SBDS* in the murine myeloid 32Dcl3 cell line showed normal neutrophil maturation but reduced survival of granulocyte precursor cells, indicating that *SBDS* acts to maintain survival of immature cells of the neutrophil lineage[45].

The role of *SBDS* in skeletal formation was also demonstrated. Leung et al.[46] reported that murine *Sbds* is required for *in vitro* and *in vivo* osteoclastogenesis (OCG). Sbds-null murine monocytes form osteoclasts of reduced number and size because of impaired migration and fusion required for OCG. They concluded that murine *Sbds* is required for osteoclastogenesis and impaired osteoclast formation could disrupt bone homeostasis resulting in skeletal abnormalities seen in Shwachman patients.

Shwachman-Diamond syndrome is an inherited marrow failure disease and is characterized by cancer predisposition like other bone marrow failure syndromes, such as Diamond-Blackfan anemia, which are also thought to be ribosome disorders. It remains unclear how the ribosomal defects directly contribute to increased cancer risk. Mutations in *SBDS* plausibly predispose patients to cancer, probably by altering ribosome biogenesis and/or functions. The involvement of *SBDS* in myelodyplasia was also suggested by Raaijmakers[30]. In a recent paper they demonstrated that conditional deletion of *Dicer1* in osteoprogenitors showed reduce expression of murine *Sbds* and, in turn, deletion of *Sbds* in mouse osteoprogenitors induced bone marrow dysfunction with myelodysplasia.

Mutations

Most mutations in the *SBDS* gene arise from a mechanism of gene conversion between *SBDS* and its highly homologous pseudogene (*SBDSP*) located 5.8 Mb distally on the same chromosome[6]. The *SBDSP* transcript shows a 97% sequence homology with *SBDS* but also contains critical deletions and nucleotide changes that would render nonfunctional the hypothetical encoded protein (Figure 1.2).

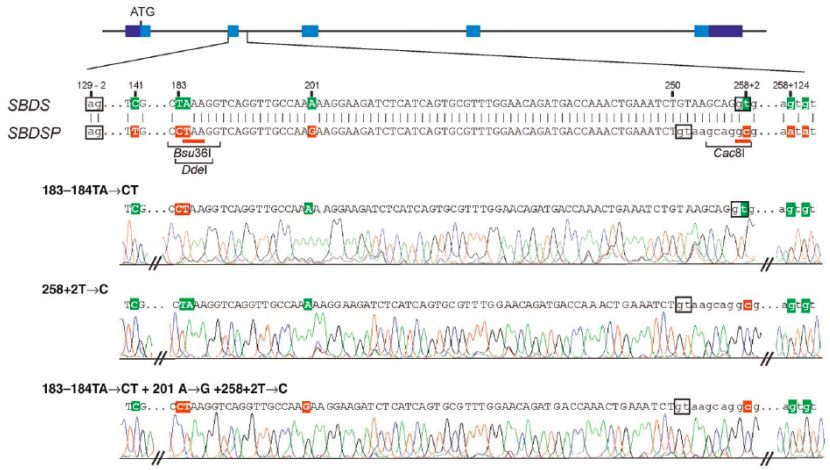

Figure 1.2: Map of *SBDS* and sequence alignment of the exon 2 region of *SBDS* and *SBDSP*[6].

Compound heterozygous mutations of the *SBDS* gene were detected in the 90% of patients with SDS. The 258+2T>C and 183-184>CT mutations are the most common[47][48].

The 258+2T>C mutation results in the disruption of the donor splice site of intron 2 and the use of a cryptic splice donor site at position 251-252. The resulting abnormally spliced mRNA results in a frameshift and the premature truncation of the 250 amino acid SBDS protein at amino acid 84.

The 183-184>CT mutation introduces a premature stop codon, resulting in the truncation of the SBDS protein at amino acid 62. Interestingly, no patients homozygous for this mutation were found; this could suggest that such genotype might be lethal during prenatal life. Other mutations are listed on (Figure 1.3).

Nucleotide change-position	Protein	Effect	Exon/ Intron	Number of chromosomes
c.95A>G	**p.Y32C**	missense mutation	1	1
c.101A>T	**p.N34I**	missense mutation	1	1
c.141C>T;c.183_184TA>CT	**p.K62X**	stop mutation	2	2
c.141C>T;c.183_184TA>CT;c.201A>G	**p.K62X**	stop mutation	2	4
c.201A>G;c.258+2T>C		splice mutation/ stop mutation	2 and int 2	5
c.258+2T>C		splice mutation/ stop mutation	int 2	11
c.141C>T;c.183_184TA>CT;c.201A>G; c.258+2T>C	**p.K62X**	stop mutation	2 and int 2	2
c.129-443A>G;c.129-433G>A;c.141C>T; c.183_184TA>CT;c.201A>G;c.258+2T>C	**p.K62X**	stop mutation	int 1-int 2	1
c.307_308delCA	**p.Q103fs**	frameshift/ stop mutation	3	1
c.624+1G>C		deduced splice mutation	int 4	1
c.635T>C	p.I212T	polymorphism	5	5
c.652C>T	**p.R218X**	stop mutation	5	1

Figure 1.3: Summary of mutations associated with SDS[48].

Data provide strong evidence that there is a subgroup of patients, about 10%, with clinical features of SDS who do not have *SBDS* mutations. Since the sequence analysis was limited to exons, splice junctions, and the immediate 5'- and 3'-untranslated regions, it is possible that mutations outside of the sequenced regions may have been missed. However, there are at least two other possibilities. It is possible that patients with normal *SBDS* alleles have a distinct clinical syndrome. Alternatively, SDS may be a genetically heterogeneous disorder. Mutation of

a gene or genes that disrupt a pathway shared by SBDS may result in disease with identical clinical features[49][50].

In 2009, Newman et al.[51] described a novel A>G substitution in exon 1 of the *SBDS* gene in a Fijian boy. This mutation resulted in a mutant serene codon at amino acid position 34, replacing the normal asparagine codon. The other allele carried the mutation 183-184>CT. Nicolis and co-workers[48] for the first time described the case of *de novo* mutation in patient with Shwachman syndrome. The affected subject resulted compound heterozygous: his mother was a carrier of the 258+2C>T mutation, but his father revealed only wild-type sequence. Since the paternity was confirmed, they suspected that a *de novo* gene conversion occurred.

Clinical variations are present even among the patient with identical genotype and further study will be required to explain the clinical.

1.4 Clinical management

1.4.1 Hematology

All patients with SDS should be monitored by a hematologist. Marrow evaluation with aspirate and biopsy including cytogenetics to assess for marrow cellularity, MDS, acute leukemia or other clonal disease is recommended on a yearly basis. Regular monitoring allows timely institution of therapy prior to the development of clinical complications. Neutropenia should be evaluated expeditiously with clinical examination, cultures of

any potential infectious sources including blood cultures and prompt institution of broad-spectrum antibiotics. For neutropenic patients (ANC of<500 cells/μL) with repeated infections or serious life-threatening infections, granulocyte colony-stimulation factor (G-CSF) administration may be considered. The role of G-CSF administration in promoting malignant myeloid transformation in bone marrow failure syndromes is controversial. The development of MDS and AML in SDS patients who are receiving G-CSF therapy has been reported. However, most SDS patients who develop myelodysplasia syndrome or leukemia had not received treatment with G-CSF[52].

1.4.2 Hematopoietic stem cell transplantation

The primary causes of death in SDS patients during infancy are related to malabsorption, infections and thoracic dystrophy. In older patients, the main causes of death are hemorrhage and infections due to associated hematological abnormalities such as marrow aplasia, neutropenia, MDS or acute leukemia. Hematopoietic stem cell transplantation (HSCT) is the treatment of choice for BM dysfunction associated with SDS. Being a rare disease, sources of few data are available on the results of conditioning regimes and HSCT, moreover most of the studied are from the case reports of a small number of patients[53]. Patients with SDS tend to have increased toxicity with intensive conditioning regimens and they seem to be especially suscep-

tible to cardiac toxicity[54]. Generally, the preparative regime include cyclophosphamide and busulfan or total body irradiation (TBI). Cyclophosphamide has a potent immunosuppressive activities and busulfan has a potent myeloablative effect. However, specific metabolites of cyclophosphamide are associated with an increased toxicity and mortality after conditioning, especially in the combination with busulfan. Indeed, the combination of cyclophosphamide and busulfan is prone to mediate endothelial damage, resulting in venous occlusive disease and pulmonary injury[55]. Causes of death in Shwachman patients included congestive heart failure and pulmonary hemorrhage. The special predisposition to this toxicity profile is poorly understood, but has been attributed to the cardiotoxicity mediated by cyclophosphamide[56]. Although earlier reports indicate that survival is fair, recent data indicate that transplantation of children with SDS using reduced-intensity conditioning is feasible and associated with modest mortality and morbidity[57][58]. Increased understanding of the genetic and biochemical basis for this disorder, and prospective careful data collection will hopefully allow optimization of therapy for this complex group of patients. Dror and Freedman[17] have demonstrated that BM mononuclear cells from patients with SDS show an increased propensity for apoptosis mediated by hyperactivation of the Fas-signaling pathway. It is possible that similar mechanisms are important in the increased susceptibility to organ toxicity with intensive conditioning regimens.

1.4.3 Gastroenterology

Patients with SDS should also be followed by a gastroenterologist for management of exocrine pancreatic insufficiency. Most patients require oral pancreatic enzyme supplementation. However, steatorrhea resolves spontaneously in roughly 50% of patients; therefore, assessment of continued need for pancreatic enzyme supplementation is indicated. Measurement of the fat soluble vitamins A, D, E, and K should occur with appropriate supplementation.

1.4.4 Skeletal

Measures to maximize bone density should be implemented, including adequate calcium and vitamin D intake. In addition, it is important to screen for and correct any underlying endocrine problems that may contribute to osteopenia, such as hypothyroidism or hypoparathyroidism.

1.5 Mesenchymal stem cells

There are two types of stem cells in the bone marrow, hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). Hematopoietic stem cells have been shown to produce blood cells and resident cells such as osteoclasts. Mesenchymal cells are thought to generate a number of non-hematopoietic cells including adipocytes, chondrocytes and osteoblasts and to support proliferation and differentiation of HSCs and their progenies. The hematopoietic microenvironment controls the for-

mation of blood cells through the production and secretion of cytokines and extracellular matrix molecules and the MSCs are the central component of hematopoietic microenvironment. Mesenchymal stem cells are a rare subset of stem cells of non-hematopoietic origin residing in the bone marrow where they closely interact with hematopoietic stem cells and support their growth and differentiation. In the BM mesenchymal cells represent 0.01-0.001% of total cells. In adult humans, steady-state hematopoiesis occurs in the bone marrow, which is situated within the bone cavity. Rather than being an inert structure, bone tissue undergoes a constant process of remodeling via a tight coupling between bone formation from osteoblasts (derived from mesenchymal stem cells) and bone resorption by osteoclasts (which are hematopoietic in origin)[59]. Osteoblasts play an active role as part of the regulating microenvironment or niche for HSCs and they are usually found in a layer along the endosteum at the interface between bone and marrow and periosteum, which comprise the internal and external surfaces of bone, respectively. The major functions of osteoblasts in bone remodeling are the secretion of unmineralized bone matrix proteins (collectively termed osteoid) and cells of the osteoblast lineage regulate osteoclast differentiation[59]. Hematopoietic microenvironment can regulate hematopoiesis by interacting directly with hematopoietic cells and/or by secreting regulatory molecules that influence, in a positive or negative manner, the growth of hematopoietic cells. Bone marrow plays also an important role in the homeostasis of neutrophils.

1.5.1 Role of MSCs in supporting Neutrophil survival

Neutrophils are an essential component of innate immune system and they differentiate from stem cells in the bone marrow by a process termed granulopoiesis. Neutrophils are generated at a rate of 1 to 2×10^{11} cells per day in a normal adult human under normal conditions[60]. The majority of neutrophils are reserved in the bone marrow. A large amount of neutrophils can be mobilized rapidly in response to infection and stress, which suggests that the bone marrow reserve is critical for host defense. CXCR4/CXCL12 signaling pathway plays a crucial role for maintaining neutrophils in bone marrow. It is reported that administration of G-CSF reduces the expression of CXCR4 on bone marrow neutrophils and the levels of bone marrow CXCL12. This explains why G-CSF administration mobilizes neutrophils from bone marrow to peripheral circulation. Once released from the bone marrow, neutrophils circulate in the peripheral blood and have a relatively short half-life (about 6-8 hours). Bone marrow serves also as an important site for neutrophil clearance under homeostatic conditions. Senescent neutrophils highly express CXCR4 and may home back to bone marrow via the CXCR4/CXCL12 chemokine axis. Once senescent neutrophils return to bone marrow, they are phagocytosed and destroyed by resident stromal macrophages, which in turn stimulates the production of G-CSF by bone marrow macrophages after uptake of apoptotic neutrophils. Sub-

sequently G-CSF acts as a positive feedback to promote granulopoiesis and regulate neutrophils release[61]. In particular, MSCs delay the apoptosis of bone marrow neutrophils through IL-6 production[62].

1.5.2 Role of MSCs in hematological disorders

In recent years, there has been an increasing interest on the biological characterization of MSCs in hematological malignancies since it has been demonstrated that in several hematopoietic disorders the marrow microenvironment is functionally abnormal and it may play an active role in inducing and/or sustaining hematopoietic disease. Whether MSCs alterations influence hematological disorders and how such alterations contribute to the progression of the disease remains controversial. In some disorders MSCs show alterations in the expression of some cell adhesion molecules and cytokines and have a reduce immunosuppressive efficiency[63]. Several studies have proposed that important quantitative and functional alterations occur in MSCs of patients with different hematological disorders[64][65]. Different studies suggest that MDS bone marrow derived MSCs contain MDS-associated cytogenetic abnormalities and others have contradicted these finding and suggested that these cells function normally. Lopez-Villar et al.[66] demonstrated by array-CGH and fluorescent in situ hybridization (FISH) analyses that MSCs from MDS patients display

genomic aberrations including -5q. In contrast, Menendez et al.[67] detected the *MLL-AF4* fusion gene in MSCs derived from MLL-AF4$^+$ leukemic patients, but not other rearrangements (*TEL-AML1*, *BCR-ABL*, *AML1-ETO*, *MLL-AF9*, *MLL-AF10* and *MLL-ENL*), suggesting that some cytogenetics alterations might arise in a prehematopoietic precursor and hit both hematopoietic and mesenchymal cells in the bone marrow. Raaijmakers et al.[30] showed that genetic alteration induced solely in osteoblast progenitors caused myelodysplasia with occasional transformation to myeloid leukemia. The investigators developed transgenic mice that delete the gene *Dicer* only in osteoblast progenitor cells, which express osterix. Although *Dicer*, which encodes a critical enzyme in the formation of microRNAs, was not deleted from the hematopoietic cells, these mice developed a form of myelodysplastic disease with ineffective hematopoiesis. Hematopoietic progenitor cells showed increased apoptosis and 2% percent of the mice developed leukemia. Of note, these leukemic cells expressed normal levels of *Dicer* proving that leukemia occurred in response to the microenvironment and not because of cell intrinsic deletion of *Dicer* within the myeloid cells. Investigators proved that the induction of MDS is stage-specific to osteoblast progenitor cells by *in vitro* co-culture of hematopoietic cells with *Dicer*-deficient osteoprogenitor cells and by *in vivo* deletion of *Dicer* specifically in more differentiated (osteocalcin positive) osteoblasts. To identify the mechanism by which *Dicer* deletion in osteoblast progenitors may cause MDS, the investigators performed gene

expression analysis. Differentially expressed genes and pathways included cytokines and stress response pathways, including significant down-regulation of the *Sbds* gene. Although the mechanism by which *Dicer* deletion is linked to *Sbds* expression is not clear, the investigators showed that knockdown of the *Sbds* gene in osteoblast progenitors reproduces MDS phenotype. Altered MSCs could generate conditions favorable to the development of preleukemic and subsequently leukemic states, being the primary cause of the development of a malignant myeloid disease.

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Chapter 2

Aims of the study

Shwachman-Diamond syndrome is a bone marrow failure disorder with a tendency to evolve into MDS/AML. The predisposition to cancer is not understood. The better comprehension of the pathogenesis of SDS could pave the way to new highly targeted strategies for the treatment of bone marrow failure and for the prevention of malignant evolution. More specifically, two lines of research focus on the improvement of the knowledge on SDS:

1. mesenchymal cells are crucial players in organizing the stem cell niche and defect in these cells severely impaired a correct hematopoiesis. Bone marrow MSCs obtained from SDS patients were analyzed for studying their potential defects in supporting hematopoiesis.

2. dissecting the risk factors and molecular events leading to malignant myeloid transformation might help develop strategies for prevention. We investigated the presence of

novel sub-microscopical lesions participating to the pathophysiology of the disease, either in the SDS phase or during the progression to MDS/AML. In addition, we investigated the potential role of bone marrow in promoting malignant transformation. The identification of signals from the microenvironment inducing transforming events might become targets for new therapeutic strategies.

Chapter 3

Characterization of mesenchymal stem cells in Shwachman-Diamond syndrome reveals an altered gene expression profile

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3.1 Rationale

Shwachman-Diamond syndrome is a rare autosomal recessive inherited disorder characterized by bone marrow dysfunction, exocrine pancreatic insufficiency and an increased risk of developing myelodysplastic syndrome (MDS) and/or acute myeloid leukemia (AML). In almost all affected patients, the disorder shows a broad range of additional clinical features including skeletal abnormalities and neutropenia. Data show that SDS is the third leading inherited bone marrow failure syndrome after Diamond-Blackfan anemia and Fanconi anemia[1]. Hematological abnormalities are present in all SDS patients. In particular, neutropenia is the most common hematological deficiency. Overwhelming sepsis is a well-recognized fatal complication of this disorder, particularly early in life. Hematological manifestations other than neutropenia include anemia, thrombocytopenia, raised fetal hemoglobin (HbF) levels and defects in the lymphoid lineage. The development of aplastic anemia in SDS patients suggests that the hematopoietic defect may rises at the level of an early hematopoietic stem cell or in the bone marrow stroma. The mechanisms underlying the BM failure in SDS patients and their predisposition to cancer is not fully understood. In 1999, Dror and Freedman[2] described stroma cells derived from patients. They reported a generalized marrow disfunction with abnormal bone marrow

stroma in terms of its ability to produce fat clusters and to support and maintain hematopoiesis. Similar to other marrow failure syndromes, patients with SDS have an increased risk for MDS and malignant transformation, in particular development of AML. The risk of leukemic and dysplastic transformation increases with age varying from 14% to 30%[3][4]. Scadden et al.[5] demonstrated that targeted deletion of miRNA processing endonuclease *Dicer1* in osteoprogenitors-mesenchymal cells (MSCs) could induce complex secondary changes in the organization of the hematopoietic lineages, including the development of independent genetic mutations and frank leukemia. This model candidates, for the first time, MSCs as a new actor in the multi-hit process of oncogenesis. Mesenchymal cells are thought to generate a number of non-hematopoietic cells including adipocytes, chondrocytes and osteoblasts and to support proliferation and differentiation of HSCs and their progenies. The hematopoietic microenvironment controls the formation of blood cells through the production and secretion of cytokines and extracellular matrix molecules and MSCs are the central component of hematopoietic microenvironment.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 and their potential role in promoting malignant transformation. We successfully obtained SDS-MSCs from 27 patients. These cells are similar to normal bone marrow derived MSCs

in morphology, growth capacity, antigen expression and mesengenic differentiation ability. In addition SDS-MSCs delayed neutrophils apoptosis probably through the production of IL-6. Moreover, we demonstrated that SDS-MSCs were comparable to MSCs obtained from healthy donors (HD-MSCs) in supporting the viability and clonogenic potential of $CD34^+$ cells. However, gene expression profile showed a specific gene signature for SDS-MSCs. The elucidation of affected molecular pathways in SDS-MSCs may identify potential therapeutic targets to prevent or treat marrow failure and leukemia evolution in SDS patients.

3.2 Methods

3.2.1 Patients

The bone marrow samples were material exceeding the diagnostic specimens collected from patients affected by Shwachman-Diamond syndrome. Twenty-seven patients diagnosed with SDS were included in this study. The median age of patients was 9 years (range from 1 to 27 years old) and all patients had mutations on the *SBDS* gene. Patients were diagnosed with SDS based on clinical criteria which include clear evidence for both hematological and exocrine pancreatic dysfunction. In all patients, the diagnosis was supplemented by positive *SBDS* gene mutation analysis. Clinical and genetic characteristics of the patients were summarized in Table 3.1. Bone marrow collection bags, which are normally discarded after the BM infu-

sion, provided the 25 controls. Informed, written consent was obtained in all cases.

3.2.2 Isolation and culture of mesenchymal stem cells

Mononuclear cells (MNCs) were isolated from BM using Ficoll-Paque PLUS (GE Healthcare) and seed at $0{,}16\times10^6$ cells/cm^2 in cultured medium at 37℃ with 5% CO_2. Expanded medium was Dulbecco's Modified Eagle's Medium (DMEM, Lonza) low glucose supplemented with 10% of fetal calf serum (FCS, Biosera), 1% of L-glutamine (Euroclone) and 1% of penicillin and streptomycin (Euroclone). After 24 hours the culture medium was replaced, after a wash with phosphate buffered saline (PBS, Euroclone), and non-adherent cells were removed; adherent cells were maintained with medium replacement twice a week. As the culture reached around 80-90% of confluence, cells were detached with 0,25% trypsin-EDTA (Euroclone) and seed at $0{,}03\times10^6$ cells/cm^2. Mesenchymal cells were used between passages 3 (P3) and 5 (P5). Mesenchymal cells growth was monitored by determining the number of population doubling (PD). The population doubling time was calculated at every passages with the formula $PD = t \cdot \log 2/(\log N_t - \log N_0)$[6].

Phenotypic characterization

The following monoclonal antibodies were used, according to the manufacturer's instructions, to characterize cultured cells

at P3 and demonstrated their non-hematopoietic origin: anti-CD11b (Biolegend), anti-CD14 (eBioscience), anti-CD19 (BD), anti-CD34 (IQ product), anti-CD45 (BD), anti-CD73 (BD), anti-CD90 (eBioscience), anti-CD105 (eBioscience), anti-HLA-ABC (BD) and anti-HLA-DR (BD). The stain was performed at 4℃ to diminish the possible nonspecific staining caused by temperature and in the dark to avoid fluorescent attenuation. BD-FACScalibur was used for standard acquisition/analysis and flowcytometry data were analyzed with CellQuest.

Multilineage differentiation

To assess the multilineage differentiation capacity of these cells, osteogenic, adipogenic and chondrogenic induction were performed *in vitro*. Expanded cells were seeded into dishes at a density of $0{,}02\times10^6$ cells/cm^2 in culture medium at passage 5. As the culture reached the confluence, the medium was switched to adipogenic or osteogenic induction medium and changed twice a week. Adipogenic medium contained DMEM high glucose (Euroclone) supplemented with 10% of FBS, 1 μM of dexamethasone (Sigma), 100 μM of indomethacin (Invitrogen), 500 μM of 3-isobutyl-1-methylxantine (IBMX, Sigma) and 10 μg/mL of insulin (Sigma). Osteogenic medium contained DMEM low glucose (Lonza) supplemented with 10% of FBS, 10 nM of dexamethasone (Sigma), 0,05 mM of ascorbic acid (Sigma) and 10 mM of β-glycerolphosphate (Sigma). Cells cultured in DMEM high or low glucose supplemented with 10% of FBS, 1% of L-glutamine and 1% of penicillin

and streptomycin were used as adipogenic or ostegenic negative control respectively. After 2 weeks, the differentation was verified by Oil Red O staining (Sigma) and Alizarin Red S (Sigma) for adipogenic and osteogenic differentiation severally. For chondrogenic differentiation, $0{,}3\times10^6$ cells were centrifuged in polypropylene tubes to form a pellet. The cells were maintained in DMEM high glucose supplemented with 1% of penicillin and streptomycin, 10 μM of dexamethasone (Sigma), 0,173 mM of ascorbic acid (Sigma), 1 mM of sodium pyruvate (Sigma), 10 μL/mL of insulin-transferrin-sodium selenite (ITS, BD biosciences), 10 μL/mL of non essential amminoacid (MEM, Gibco) and 10 μL/mL of TGFβ-3 (Sigma). Chondrogenic medium was changed twice a week. On day 21, the pellets were washed twice with PBS, fixed with a solution of PBS with 2% formaldehyde (Sigma) and sectioned. To identify the development and accumulation of cartilage matrix, proteoglycans and glycosaminoglycans were shown by safranin staining.

The expression of the following genes were examined for adipogenic and osteogenic differentiation after 14 days of induction: leptin (*LEP*), lipoprotein lipase (*LPL*), peroxisome proliferator-activated receptor gamma (*PPAR-*γ), alkaline phosphatase (*ALKP*), osteocalcin (*OCN*), osteopontin (*OPN*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as housekeeping gene. Total RNA was isolated using Trizol reagent (Invitrogen) and RNA samples were converted to cDNA using Superscript II enzyme (Invitrogen). All real-time PCR were performed on LightCycler 480 (Roche) using UPL. To

guarantee the reliability of the results, all samples were processed in triplicate. The Ct value of GAPDH was used as an endogenous reference for normalization and the values obtained were normalized versus the control and were expressed as fold changes.

3.2.3 Proliferation assay

To evaluating the MSC-mediated inhibition of mononuclear cell proliferation we used the [3H]-thymidine uptake method. Freshly healthy donor isolated mononuclear cells were plated in RPMI-1640 supplemented with 10% of FBS, 1% of L-glutamine and 1% of penicillin and streptomycin at $0{,}3\times10^6$ cells/well in U-bottom 96-well plates. Peripheral blood mononuclear cells (PBMCs) were plated either alone or in the presence of irradiated (35 Grey) MSCs at different MSC:PBMC ratios, from 1:1 to 1:32. The different ratios were obtained by serial dilution of MSCs. Mononuclear cells were treated with phytohemagglutinin (PHA, Irvine Scientific) 5 μg/mL prior to added to MSCs culture. After 48 hours of co-culture cells were pulsed for 16 hours with [3H]-thymidine at 1 μCi/well (Perkin Elmer) and then harvested. [3H]-thymidine incorporation was measured using Multipurpose Scintillation Counter (Beckman Coulter).

3.2.4 Neutrophils and MSCs co-culture

Neutrophils were isolated from peripheral blood of healthy donors under endotoxin-free conditions following the protocol described

by Boyum[7] allowing the isolation of pure and minimally activated cells. Briefly, leukocytes were separated from erythrocytes by 3% room temperature dextran (Sigma) sedimentation. Leucocytes were then centrifuged over cold Ficoll-Paque PLUS and the resulting neutrophil-enriched pellet was resuspended in RPMI-1640 (Euroclone) supplemented with 10% of FCS and 1% of L-glutamine and layered over a discontinuous gradient of 65% 285 Osm Percoll (GE Healthcare). Neutrophils were removed and washed with PBS. Cells were counted with Turk (Merck) and resuspended at 10^6 cells/mL in RPMI-1640 supplemented with 10% of FCS, 1% of L-glutamine and 1% of penicillin and streptomycin. Mesenchymal cells were seeded in 6-well plate in complete medium at $0{,}04\times10^6$ cells/cm^2. As the cells reached the confluence, neutrophil suspensions were incubated with MSCs. Neutrophils apoptosis was evaluated by GFP-Certified Apoptosis/Necrosis detection kit (Enzo Life Sciences) at different time points. Briefly, cells were washed with PBS^{++} and incubated with apoptosis detection and necrosis detection reagents for 15 minutes at room temperature according to the manufacturer's instructions. BD-FACScalibur was used for standard acquisition/analysis and flow cytometry data were analyzed with CellQuest. Supernatant from the co-culture were harvested and frozen until use. We used the supernatants to test the amount of IL-6 by ELISA (eBioscience), according to the manufacturer's instruction.

3.2.5 Co-culture of hematopoietic stem cells with mesenchymal cells layer

Mononuclear cells were obtained from healthy donors bone marrow collection bags. $CD34^+$ were purified using CD34 antibody conjugated magnetic beads according to the manufacturer's instructions (Miltenyi Biotec). To increase the $CD34^+$ purity, cells were passed through a second magnetic column. $CD34^+$ were suspended in RPMI containing 10% of FCS, 1% of L-glutamine and 1% of penicillin and streptomycin and plated at a density of $0{,}05\times10^6$ cells/cm^2 on a confluent irradiated (35 Grey) MSC layer in 48-well plate. After $CD34^+$ cells were layered over MSCs, hematopoiesis was assessed weekly. Every 7 day $CD34^+$ cells were counted and 1000 cells were resuspended in 80 μL of Iscoves Modified Dulbeccos Media (IMDM, Euroclone) and plated in methylcellulose-based medium (MethoCult, STEMCELL Technologies) supplemented with 1% of pen-strep and L-glutamine, 2 U of erythropoietin (EPO, Roche) and 100 μL of 5637 supernatant to quantify hematopoietic progenitor cells in the colony-forming cell (CFC) assay. Colonies were scored as CFU-E, BFU-E and CFU-GM using an inverted microscope after 21 days. With this method we assessed the ability of mesenchymal cells to support hematopoiesis for 3 weeks.

3.2.6 Western blot analysis

More than 1×10^6 of mesenchymal cells were collected at P5 by centrifugation and then washed with ice-cold PBS. Ice-cold

radioimmunoprecipitation assay buffer (1% NP40, 0,5% Na-deoxycholate, 0,1% sodium dodecyl sulfate, 5 M to 350 nM NaCl in PBS) containing fresh 0,25 nM of phenylmethane-sulfonylfluoride (PMSF, Sigma) and 100X protease inhibitor (Sigma). The suspension was transferred into a centrifuge tube and placed on ice for 30 minutes with occasional vortexing to ensure complete lysis of the cells. The cell suspension was cleared by centrifugation at 14000 g for 10 minutes at 4℃ and supernatants were stored at -80℃. Protein concentrations were determined using Bradford assay (Sigma). Fifty μg of protein were loaded per lane on a 10% sodium dodecyl sulfate-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane 0,45 μm pore size (Invitrogen) for 45 minutes at 45 V in Tris-glycine buffer. The membrane was blocked for 30 minutes in 5% nonfat dry milk in PBST at room temperature and then incubated in primary antibody overnight at 4℃. The anti-β-actin (Sigma) and the anti-SBDS (Santa Cruz) were incubated at 1:1000 in PBST/5% of milk. The membrane was then washed 3 times (10 minutes per wash) with PBST. The secondary antibodies were added at 1:20000 (anti-mouse-horseradish peroxidase; Sigma) a 1:5000 (anti-goat-horseradish peroxidase; Santa Cruz) dilution in PBST/5% of milk for 1 hour at room temperature. The membrane was washed 3 times (10 minutes per wash) in PBST and immunoreactive proteins were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

3.2.7 Karyotype and FISH analysis of MSCs

MSCs were cultured in medium supplemented with 10 μL/mL of colcemid (10 μg/mL, Sigma) for up to 6 hours. The cells were then washed with PBS and subsequently trypsinized and spun down. Pellet was resuspended carefully in hypotonic solution (KCl 0,075 M and sodium citrate 1%), rinsed and then fixed in methanol:acetic acid 3:1. Finally, the pellet was resuspended in appropriate volume of fixative solution and cells dropped onto glass slides. At least 20 metaphases were analyzed for each sample using QFQ banding technique, an epifluorescence microscope (Zeiss Imager Z1) and Ikaros software (v5.0, Metasystems). The karyotypes were described according to the International System for Human Cytogenetic Nomenclature (ISCN, 2009)[8]. Fluorescence in situ hybridization (FISH) was performed on MSCs using LSI D20S108 probe (Vysis) for the detection of deletion of long arm of chromosome 20 and D7S486/CEP7 FISH Probe Kit (Vysis) for the isochromosome 7 (Figure 3.1). Probes cut-offs were determined analyzing MSCs derived from healthy donors. At least 100 nuclei were analyzed for each case using Imager Z1 (Zeiss) epifluorescence microscope equipped with DAPI, FITC and TEXAS RED filters.

Figure 3.1: Illustration of the probes. In a normal cell the expected pattern when hybridized with the LSI D7S486/ CEP 7 probe is two orange and two green signal. In a hybridized abnormal cell containing the isochromosome 7, three orange and three green signal pattern will be observed. LSI D20S108 Single Color Probe hybridized to cells showing two orange signal pattern. In a hybridized abnormal cell containing the deletion of long arm of chromosome 20, one orange signal pattern will be observed.

3.2.8 RNA preparation and gene expression profiling

Total RNA was isolated using Trizol reagent (Invitrogen) from mesenchymal cells at P5 following the manufacturer's protocol. RNA quality and purity were assessed on the Agilent Bioanalyzer 2100 (Agilent Technologies) and RNA concentration was determined using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc). For microarray experiments, *in vitro* transcription, hybridization and biotin labeling were performed according to GeneChip 3IVT Express kit protocol (Affymetrix). Microarray data (.CEL files) were generated using default Affymetrix microarray analysis parameters of GeneChip Command Console Software (AGCC). Gene expression data were analyzed using R package (http://www.R-

project.org). Supervised analyses were performed using shrinkage test[9] and multiplicity corrections were used to control false discovery rate (FDR); probes with local FDR lower than 0,05 are considered significant. Genes with different expression pattern were validated on Light Cycler 480 (Roche) using the Universal Probe Library (UPL, Roche). Superscript II enzyme (Invitrogen) was used for cDNA synthesis and primers probes were selected according to the Software Probe Finder (Roche). Data were expressed using the comparative Ct method[10] and real time PCR experiments were performed in quadruplicates.

3.2.9 Statistical analysis

All values are expressed as mean ± SD or SE. Differences between groups were tested using Student's t test. P values $<$ 0,05 were considered significant. Figures show data from representative experiments.

3.3 Results

The following Table 3.1 summarizes patients' features.

Patient	Sex	Age at study (y)	ANC/μL	Infections	*SBDS* mutations	
UPN1	F	7	1100	NO	c.258+2T>C	c.183_184TA>CT
UPN2	F	3	1770	NO	c.258+2T>C	c.183_184TA>CT
UPN3	F	4	1820	NO	c.258+2T>C	c.183_184TA>CT
UPN4	F	3	1440	NO	c.258+2T>C	c.183_184TA>CT
UPN5	F	1	260	NO	c.258+2T>C	c.183_184TA>CT
UPN6	F	8	100	NO	c.258+2T>C	c.183_184TA>CT
UPN7	M	6	1600	URTI	c.258+2T>C	c.183_184TA>CT;c.258+2T>C
UPN8	F	1	260	NO	c.258+2T>C	c.183_184TA>CT;c.258+2T>C
UPN9	M	18	380	NO	c.258+2T>C	c.183_184TA>CT
UPN10	F	4	1100	NO	c.258+2T>C	c.183_184TA>CT
UPN11	M	4	2400	NO	c.258+2T>C	c.258+2T>C
UPN12	M	19	950	NO	c.258+2T>C	c.624+1G>C
UPN13	M	27	1120	NO	c.258+2T>C	c.183_184TA>CT
UPN14	F	9	1000	NO	c.258+2T>C	c.258+2T>C
UPN15	M	20	250	NO	c.258+2T>C	c.183_184TA>CT
UPN16	M	10	2350	NO	c.258+2T>C	c.258+2T>C
UPN17	F	6	1620	NO	c.258+2T>C	c.183_184TA>CT
UPN18	F	7	1760	n.a.	c.258+2T>C	c.183_184TA>CT
UPN19	M	3	2960	NO	c.258+2T>C	c.183_184TA>CT
UPN20	F	15	278	NO	c.258+2T>C	c.652>CT
UPN21	M	18	1500	NO	c.258+2T>C	c.183_184TA>CT
UPN22	M	11	310	n.a.	c.258+2T>C	c.183_184TA>CT
UPN23	M	13	800	n.a.	c.258+2T>C	c.183_184TA>CT;c.258+2T>C
UPN24	F	12	n.a.	NO	c.258+2T>C	c.352A>G
UPN25	M	3	820	n.a.	c.258+2T>C	c.183_184TA>CT
UPN26	F	7	n.a.	NO	c.258+2T>C	c.183_184TA>CT
UPN27	M	7	n.a.	n.a.	c.258+2T>C	c.183_184TA>CT;c.258+2T>C

Table 3.1: Clinical and genetic characteristics of the 27 patients enrolled in the study. UPN, unique patient number; F, female; M, male; URTI, upper respiratory tract infection; PB, peripheral blood; n.a., not available. All the mutations are described according to the mutation nomenclature (www.hgvs.org/mutnomen).

3.3.1 Evaluation of MSC-typical features in cells expanded from BM of SDS patients

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 3.2 showed that SDS-MSCs displayed a typical fibroblast-like morphology, similarly to HD-MSCs. Mesenchymal cells 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) (Figure 3.3). 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.

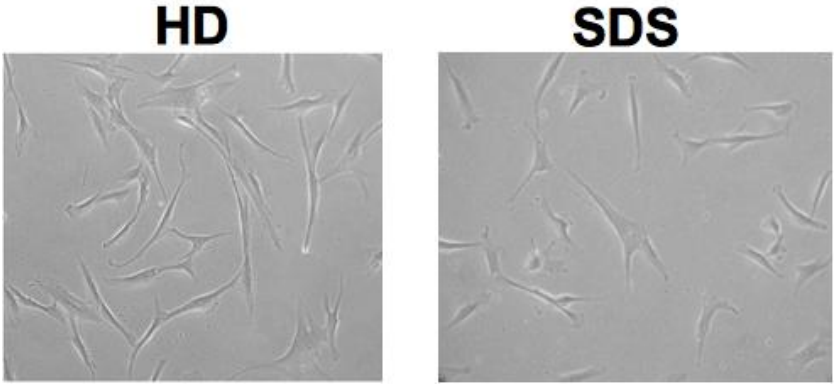

Figure 3.2: Isolation and expansion of SDS-MSCs. Mesenchymal cells were isolated from bone marrow of patients and controls and cultured in DMEM with 10% of FCS. Photos show fibroblast-like morphology of a MSCs layer from a normal donor and a SDS patient at P3.

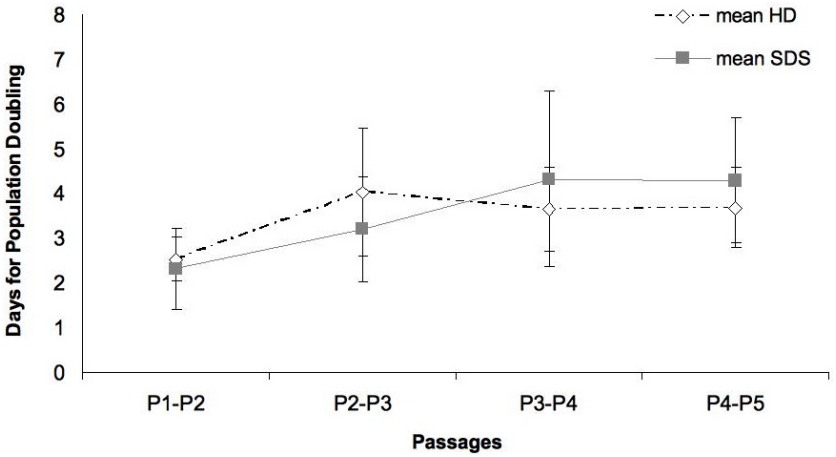

Figure 3.3: Growth characterization of SDS-MSCs. Population doubling (PD) of MSCs obtained from HDs and SDS patients was calculated. $PD = t \cdot \log 2/(\log N_t - \log N_0)$, where t represents culture time, N_0 and N_t represent cell number before and after seeding, respectively. Results were expressed as a mean ± SE.

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) (Figure 3.4).

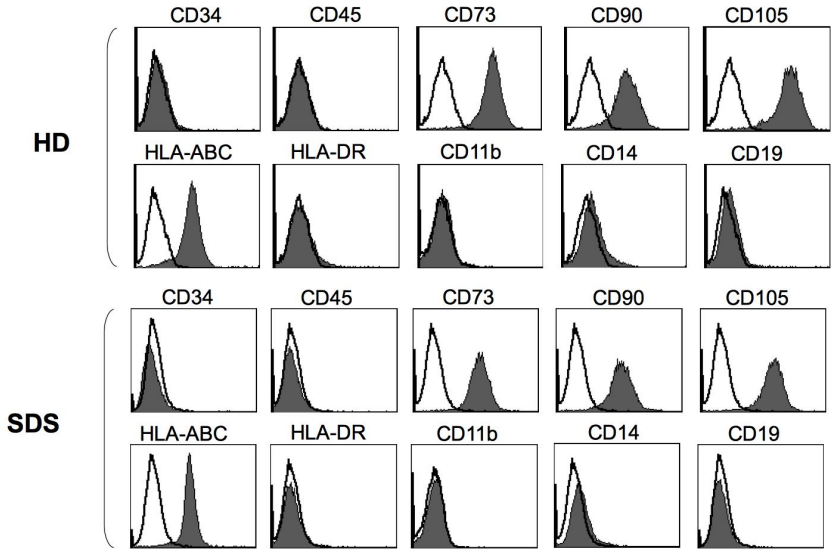

Figure 3.4: FACS analysis of MSC surface markers. Mesenchymal cells were stained with antibodies against surface specific markers (open profile); shaded profile represents unstained cells.

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 Alizarin Red (Figure 3.5, panel A). 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 (Figure 3.5, panel B).

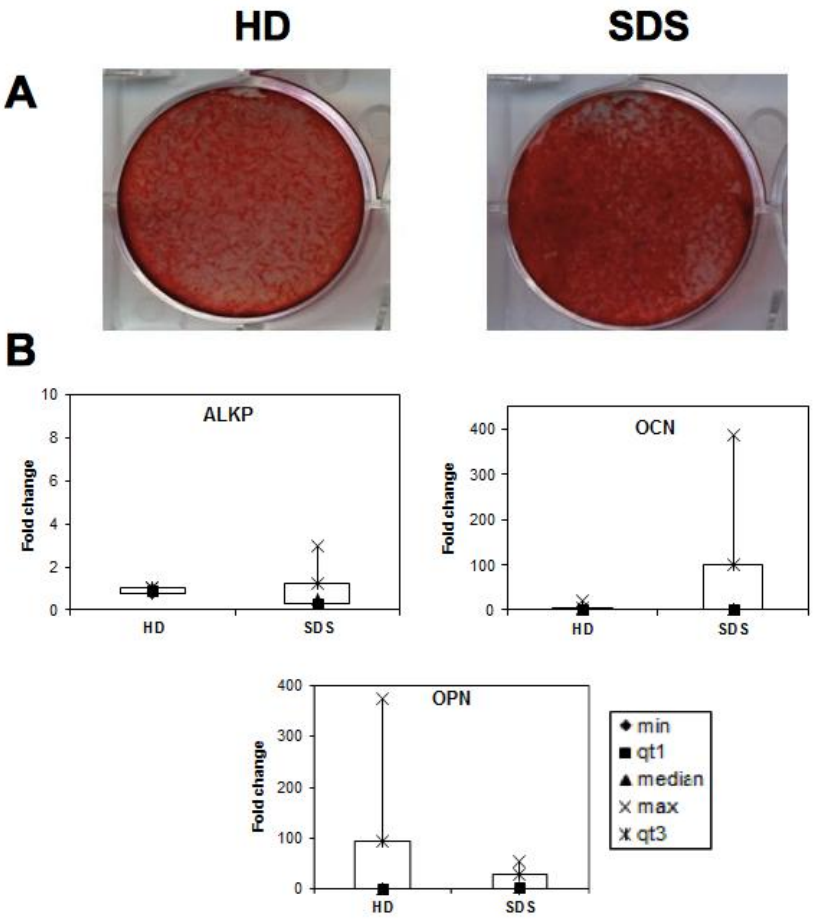

Figure 3.5: Osteogenic differentiation of MSCs. (A) After cells were treated with osteogenic medium for 14 days, calcium deposition in treated cells was revealed by Alizarin Red S staining. (B) Expression of genes up regulated during osteogenesis: *ALKP*, alkaline phosphatase; *OCN* osteocalcin and *OPN* osteopontin. The expression was measured in 4 patients and 4 controls using real-time PCR. The assay for each sample was performed in quadruplicate (Students t-test>0,05).

As shown in Figure 3.6 (panel A), 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 during the adipogenic differentiation process (Figure 3.6, panel B).

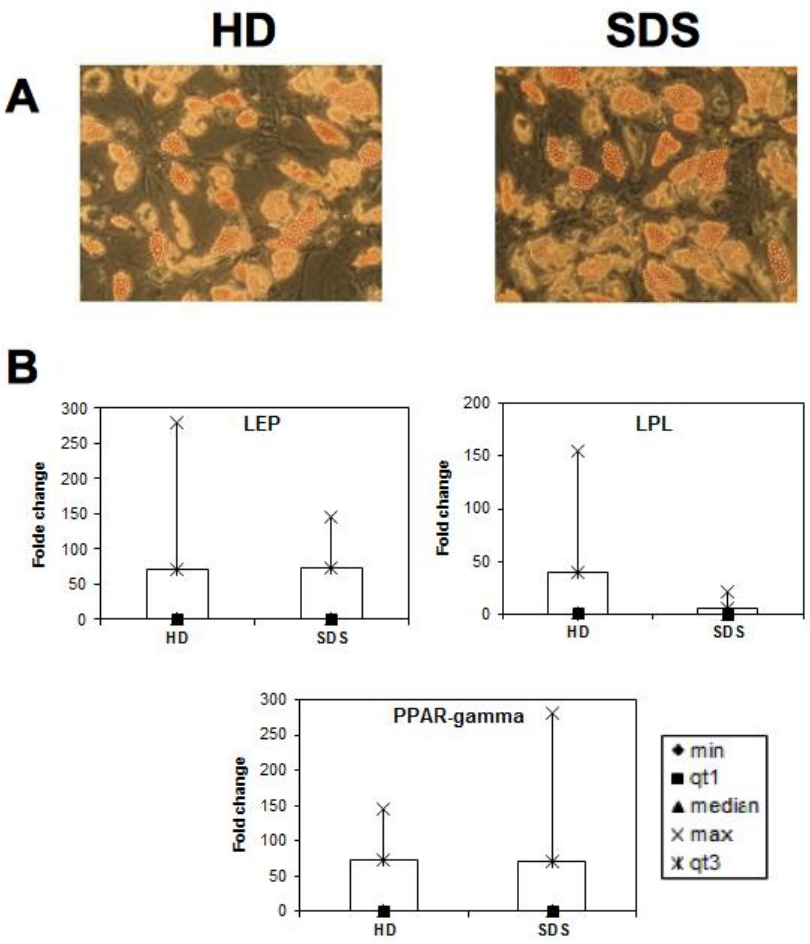

Figure 3.6: Adipogenic differentiation of MSCs. (A) After cells were treated with adipogenic medium for 14 days, lipid vacuoles were visualized by staining with Oil Red O. Magnification 20X. (B) Expression of genes up regulated during adipogenesis: *LEP*, leptin; *LPL*, lipoprotein lipase; *PPAR-γ*, peroxisome proliferator-activated receptor gamma; *ALKP*. The expression was measured in 4 patients and 4 controls using real-time PCR. The assay for each sample was performed in quadruplicate (Students t-test>0,05).

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. As shown in Figure 3.7 no difference was observed in chondrogenic tissue formation between SDS-MSCs and HD-MSCs.

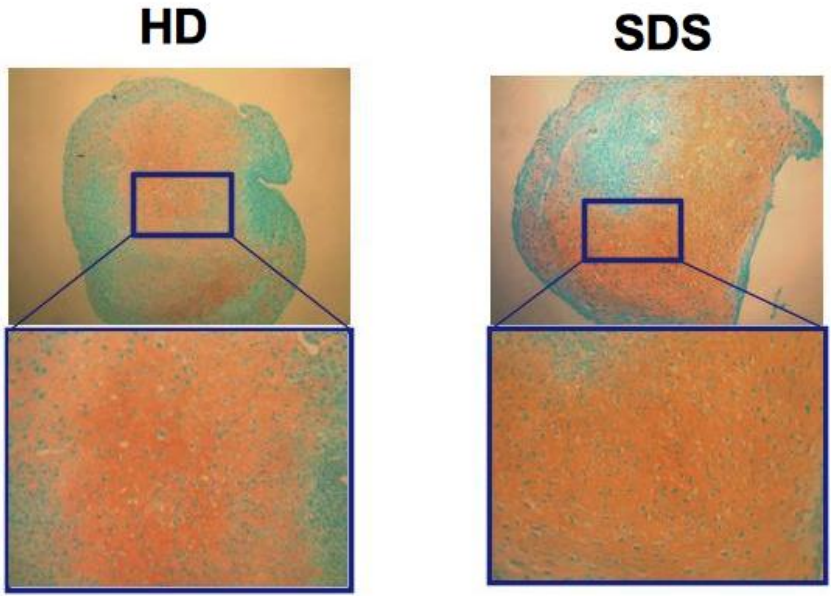

Figure 3.7: Chondrogenic differentiation. After a cell pellet was treated with chondrogenic medium for 21 days, sections of the cell pellet were stained with Safranin O that binds proteoglycans and glycosaminoglycans. Magnification 10X and 20X.

3.3.2 Functional characterization of SDS-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 immuneregulatory 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 3.8 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.

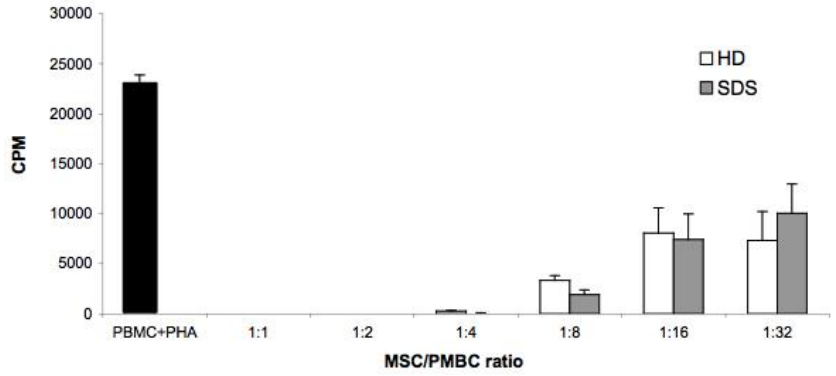

Figure 3.8: Lymphocytes proliferation in the presence or absence of SDS-MSCs. PBMCs ($0{,}3\times10^6$ cells/well) were stimulated with PHA and co-cultured with MSCs at different ratios in the 96-well plates. The PBMCs proliferation was measured 64 hours after co-culture by [3H]-thymidine incorporation. Bars represent mean ± SE; Students t-test>0,05. One experiment out of 10 performed. MSC, mesenchymal stem cells; PBMC, peripheral blood mononuclear cells; CPM, [3H]-thymidine incorporation; PHA, phytohemagglutinin.

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. Raffaghello et al.[11] demonstrated that inhibition of neutrophil apoptosis occurred at an MSC:neutrophil ratio ranging from 1:1 to 1:500 and disappeared at higher ra-

tios. Based on these results, all co-culture experiments were performed using an MSC:neutrophil ratio lower than 1:50. 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 (Figure 3.9). 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.

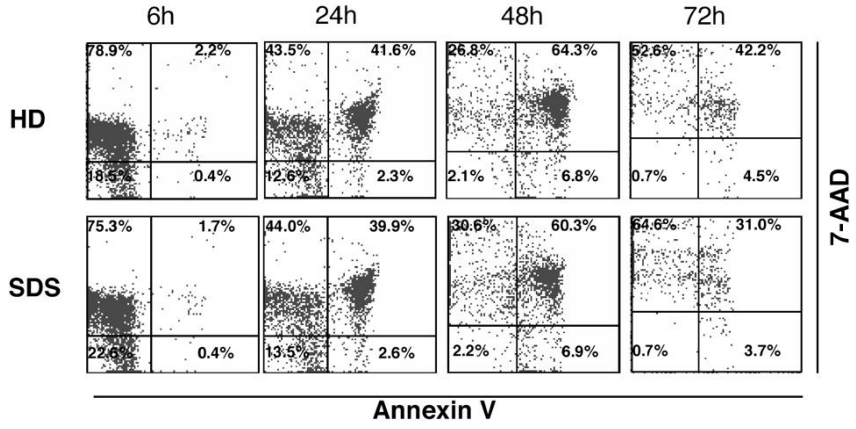

Figure 3.9: Evaluation of neutrophils apoptosis cultured with human mesenchymal stem cells. Neutrophils from healthy donors were cultured in the presence of MSCs for 72 hours. Apoptotic and necrotic neutrophils were assayed with Annexin V and 7-AAD double staining. One experiment out of 10 performed.

It has been demonstrated that interleukin-6 (IL-6) is constitutively produced by MSCs and inhibits neutrophil programmed cell death. We quantified IL-6 in supernatants of SDS-MSCs and neutrophils co-culture. As shown in Figure 3.10, SDS-MSCs and HD-MSCs are able to produce IL-6 in a time-dependent

manner. However, even if the IL-6 production was increased after 72 hours of culture with SDS-MSCs, there was no statistically significant difference between the two groups.

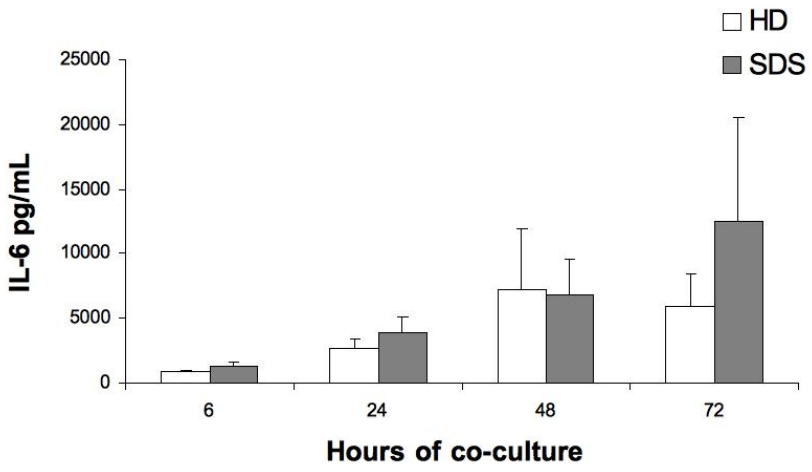

Figure 3.10: IL-6 secretion after neutrophils and SDS-MSCs co-culture. The quantification of IL-6 in co-culture supernatants was detected by ELISA at different time points. Experiments were performed in triplicate and data represent the mean ± SE, Students t-test>0,05. The experiment is representative of 8 performed.

$CD34^+$ 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 (Figure 3.11). The total number of cells increased at second week after an initial decline. After purification more than 95% of cells expressed the CD34 marker (Figure 3.12, panel A). As expected, the percentage of $CD34^+$ cells diminished during the co-culture. In particular, after 21 days the cells sunspention still contained around 20% of $CD34^+$ cells (Figure 3.12, panel B). Importantly, SDS-MSCs as well as HD-MSCs were able to preserve the

stemness of the 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 (data not show). No statistical differences in the number of BFU-E and CFU-GM after co-culture were observed between SDS-MSCs and HD-MSCs (Figure 3.13).

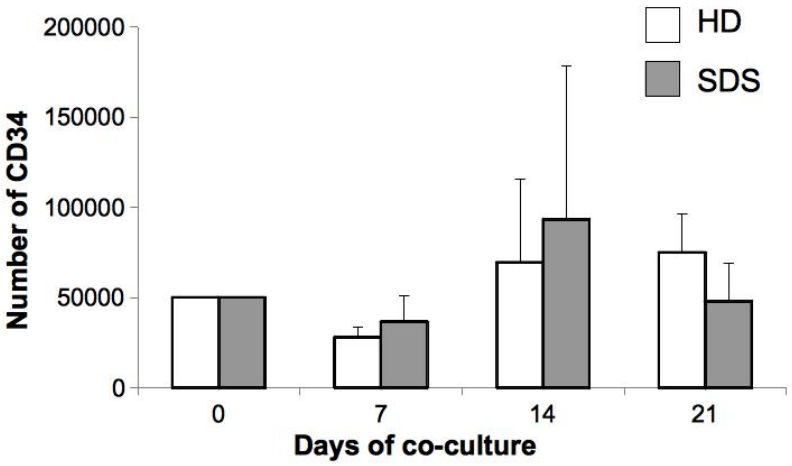

Figure 3.11: $CD34^+$ cells in co-culture with MSCs. Purified $CD34^+$ cells were counted and seeded ($0{,}05\times10^6$) in wells containing irradiated MSCs (day 0). Cells were harvested from individual wells after 7, 14 and 21 days and counted. Data represent the mean of 8 experiments performed ± SE, Students t-test>0,05.

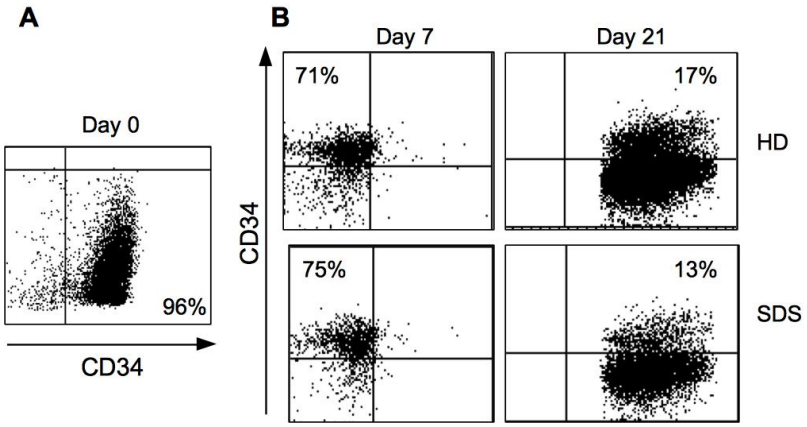

Figure 3.12: Percentage of CD34$^+$ after co-culture with HD-MSCs or SDS-MSCs. Representative experiment.

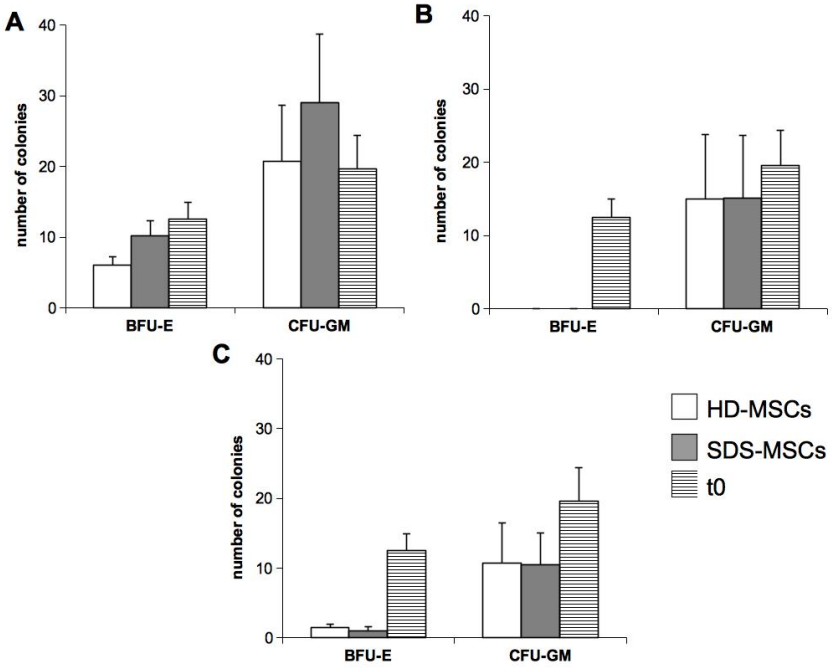

Figure 3.13: Number of colonies. CD34$^+$ cells were seeded ($0{,}001\times10^6$) in wells containing methylcellulose. Colonies were scored as BFU-E and CFU-GM using an inverted microscope after 21 days. (A) 1 week on MSC . (B) 2 weeks on MSCs. (C) 3 weeks on MSCs. Data represent the mean of 8 experiments performed $\pm$ SE, Students t-test>0,05. BFU-E, erythroid burst-forming unit and GM, granulocyte-macrophage colony-forming unit.

3.3.3 Analysis of SBDS protein in SDS-MSCs

It has been demonstrated that SBDS protein level is low in all hematopoietic cell lineages from SDS patients[12]. In order to assess the level of SBDS protein in SDS-MSCs, we analyzed protein lysates from 7 SDS-MSCs and from 8 HD-MSCs by western blot. As shown in Figure 3.14 (one representative experiment) a variable amount of protein of slighter more than 30 kDa was detected in HD-MSC lysates. Importantly, in line with previously reported studies on leukocytes[12] and with our results on *SBDS* transcript (data not show) SBDS protein was not discernible in SDS-MSCs.

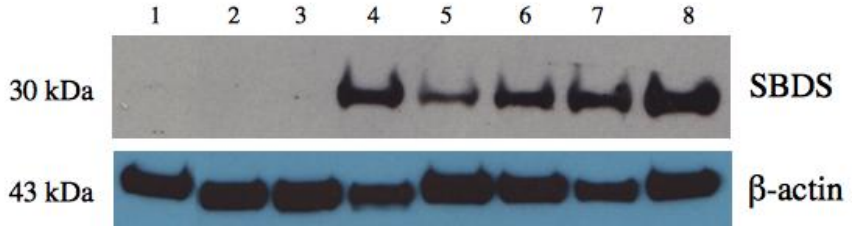

Figure 3.14: Detection of SBDS protein in SDS-MSCs by western blot. Proteins extracted from MSCs were analyzed by immunoblotting with the SBDS antiserum. β-actin protein level served as a loading control. SBDS protein is absent in SDS-MSCs. Shwachman patients are lanes 1,2 and 3; controls are lanes 4, 5, 6, 7 and 8. Molecular size markers are indicated on the left in kilodaltons.

3.3.4 Molecular genetics of SDS-MSCs

Karyotype and fluorescence in situ hybridization analysis of SDS-MSCs

Different studies demonstrated the presence of chromosome alterations in MSCs derived from MDS or leukemic patients[13]

[14]. Karyotype analysis was performed both in SDS-MSCs from 4 patients (UPN3, UPN6, UPN21 and UPN23) and 3 HD-MSCs. We also determined whether SDS-MSCs carried the same chromosomal aberrations observed in the BM of patients [UPN3, i(7)(q10); UPN6, i(7)(q10); UPN21, i(7)(q10); and UPN23, del(20)(q11)] by FISH. We demonstrated that SDS-MSCs did not show any chromosomal abnormalities previously observed in the BM of patients (Figure 3.15). FISH analysis of one representative experiment showed that SDS-MSCs displayed normal pattern of hybridization. Probe D7S486/CEP7 showed two orange and two green signals for D7S486 locus and chromosome 7 centromere (CEP7), respectively. Two orange signals were also observed for chromosome 20 specific probe (LSI D20S108). In accordance, cytogenetic analysis of SDS-MSCs did not show any chromosomal abnormalities observed in the BM patients (Figure 3.16).

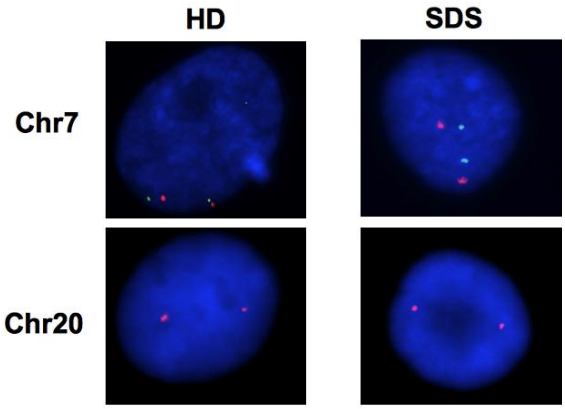

Figure 3.15: Fluorescence in situ hybridization analysis performed on SDS-MSCs. Isochromosome 7q and deletion of long arm of chromosome 20 were observed in the bone marrow population; using the D7S486/CEP7 and LSI D20S108 probes the above alterations were determined in SDS-MSCs (UPN6 and UPN23).

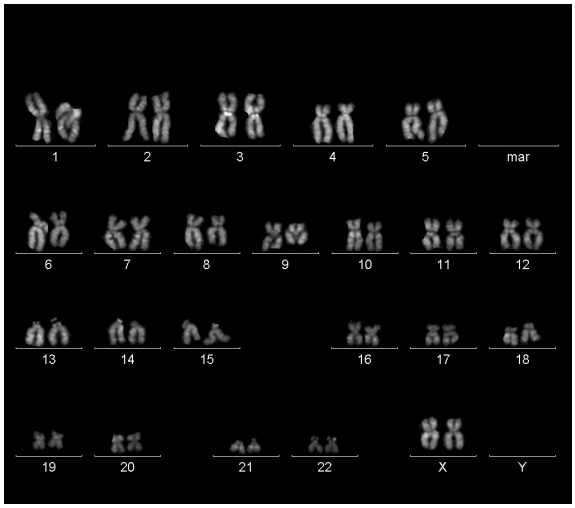

Figure 3.16: Representative normal karyotypes of SDS-MSCs (UPN6).

Gene expression profiling

We compared SDS-MSCs from 16 patients with those obtained from 11 healthy donor using HG U133 Plus 2.0 array. Unsupervised analysis was performed in order to see if patients and controls had a different expression pattern. The analysis revealed that patients and healthy donors did not cluster in a statistically significant manner (Figure 3.17). Supervised analysis identified 10 genes differently expressed between the two groups (Figure 3.18). Among these genes, we identified using pathway analysis mainly homeobox genes involved in the development process. To confirm the data, we analyzed the following genes by real-time PCR in MSCs from 14 patients and 14 healthy donors: *BCL11A* (B-cell CLL/lymphoma 11A), *FLJ30375* (hypothetical protein LOC440982), *HOXA2* (homeobox A2), *HOXA3* (homeobox A3), *HOXA5* (homeobox A5), *PCDH7* (protocadherin 7), *SBDS* (Shwachman Bodian Dia-

mond syndrome), *SHOX2* (short stature homeobox 2), *SIM1* (single-minded homolog 1) and *ZIC1* (Zic family member 1). Real-time PCR analysis confirmed the statistically significant down-regulation of *HOXA5* and *ZIC1* and the up-regulation of *BCL11A* and *SHOX2* (Figure 3.19). We observed a down-regulation of 42% of *HOXA5* and of 97% of *ZIC1*. We also observed an up-regulation of 6,2 times of *SHOX2* and of 5,8 times of *BCL11A*. Interestingly, *SBDS* gene transcript expression did not differ significantly between patients and controls and 4 patients had a gene expression profile close to controls (supervised analysis).

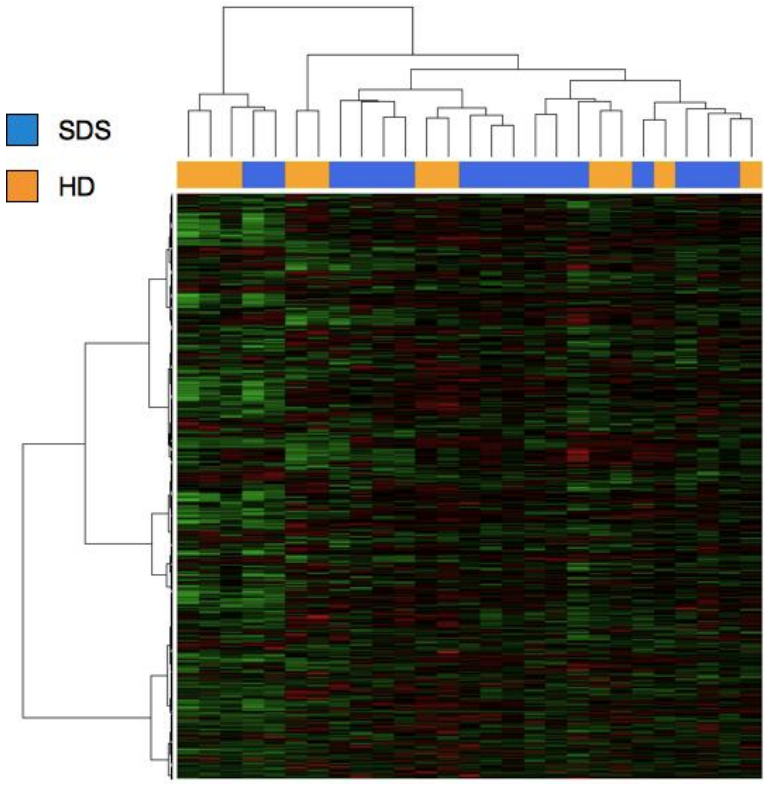

Figure 3.17: Unsupervised analysis.

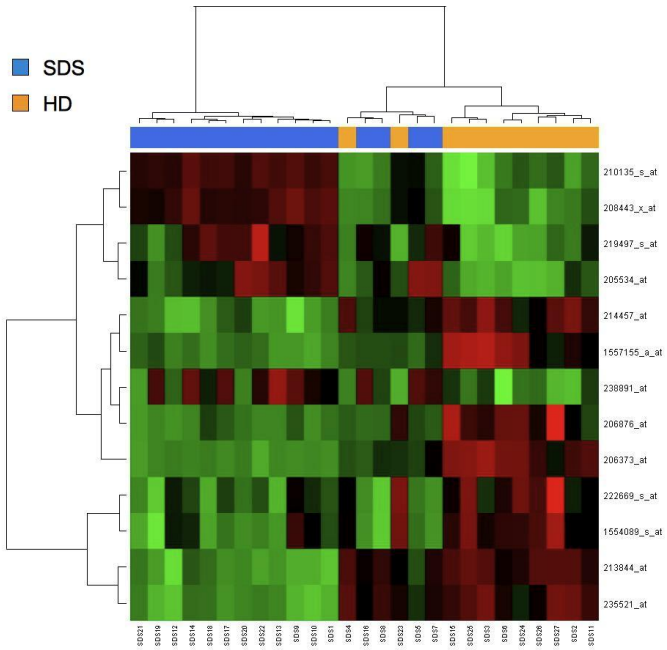

Figure 3.18: Heat map of gene expression profile of SDS-MSCs versus HD-MSCs. A set of 10 genes discriminates between the two groups. SDS (n=16), HD (n=11).

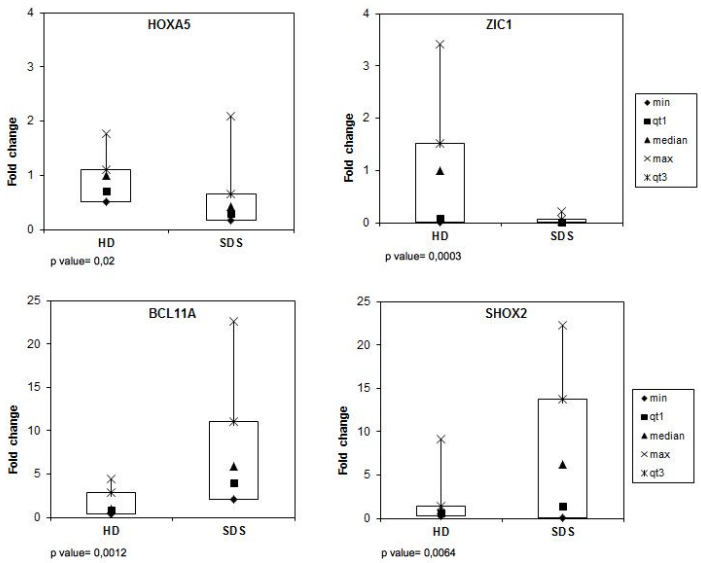

Figure 3.19: Real-time PCR analysis of the differentially expressed genes in MSCs. The differentially expressed genes were analyzed by real-time PCR using total RNAs obtained from 14 patients and 14 controls and GAPDH as internal control.

3.4 Discussion

Shwachman-Diamond syndrome is an autosomal recessive disorder characterized by hematological defects, exocrine pancreatic insufficiency and skeletal deformities. Patients may display a wide range of hematological abnormalities but the number of neutrophils in peripheral blood samples is reduced (neutropenia) in all individuals affected. Consistent with this neutrophil defect, SDS patients have an increased incidence of recurrent severe bacterial infections. Furthermore, individuals with this syndrome are cancer prone and have a risk of developing MDS and/or AML that increases with age. Additionally, the bone marrow of SDS patients shows reduced numbers of bone marrow precursors. Using long-term cultures of marrow stromal cells from SDS and unaffected subjects, Dror and Freedman[2] demonstrated that patients also have dysfunction of the bone marrow stroma: reduce ability to support and maintain hematopoiesis and a stem cell defect. Until now, the role of stromal cells or MSCs in the pathophysiology of the inefficient hematopoiesis observed in SDS is still unclear. In the present study we isolated and characterized MSCSs from bone marrow of 27 patients. MSCs should fit the functional and phenotypic criteria defined by the Mesenchymal and Tissue Stem Cell Committee of the ISCT[15]. We demonstrated that SDS-MSC were similar to normal HD-MSCs in terms of morphology and growth kinetics. Moreover, SDS-MSCs from patients were able to differentiate into adipocytes, osteoblast and chondrocytes

under appropriate induction conditions. Furthermore, flow cytometry data had illustrated that those cells presented several cell-surface antigens commonly found on HD-MSCs, such as CD73, CD90 and CD105. Finally, we demonstrated that SDS-MSCs strongly inhibit the proliferation of PHA-activated lymphocytes. The results here demonstrated that cells with characteristics of MSCs could be obtained successfully from bone marrow of SDS patients.

Following the MSCs characterization we tried to better understand the potential role of SDS-MSCs in the hematopoietic insufficiency observed in SDS patients. The basis for stromal impairment in SDS can be related to either reduced expression of hematopoietic cytokines, altered production of extracellular matrix, or abnormal expression of adhesion molecules necessary for the interaction between hematopoietic progenitors and stromal cells. We co-cultured SDS-MSCs with $CD34^+$ cells and, in our culture condition, we observed that SDS-MSCs were able to support the viability and the stemness potential of $CD34^+$ cells as well as HD-MSCs. The altered function of SDS stroma reported by Dror[2] has been obtained by *in vitro* experiments using bone marrow stroma as an integral unit of various cell types. Our results did not exclude the potential role of MSCs in determining hematopoietic insufficiency in SDS patients, but this effect could not be determined by *in vitro* co-culture experiments. We also analyzed the effect of SDS-MSCs on neutrophils and we demonstrated that, after co-culture, neutrophils were protected from apoptosis. As demonstrated by Raffaghello

et al.[14] for normal mesenchymal cells, SDS-MSCs produced IL-6, a cytokine involved in prevention of excessive or inappropriate neutrophils activation. Overall these results indicate that SDS-MSCs act *in vitro* just like their normal counterparts. 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.

Patients analyzed in our study were diagnosed with SDS based on clinical criteria and the diagnosis was supplemented by positive SBDS gene mutations. In agreement with previous reports, the 258+2T>C and 183-184>CT mutations were the most common in our cohort. Woloszynek et al.[16] detected the SBDS protein in samples from family members with normal *SBDS* alleles but not in patients. Full-length SBDS protein was not detected in leukocytes of SDS patients with the most common *SBDS* mutations, consistent with a loss-of-function mechanism. We demonstrated that, even though *SBDS* gene transcript expression did not differ significantly between patients and controls (data not show), SDS-MSCs did not express SBDS protein. The exact function of the SBDS protein remains unclear; however, recent studies in yeast and patient bone marrow cells show that *SBDS* gene is involved in RNA metabolism and ribosome biogenesis[17][18][19]. *SBDS* is an essential gene in embryogenesis and it also implicated in cell division and cellular stress response[20][21]. Further studies are needed to better comprehend the mechanisms compensating the absence of SBDS protein.

Similar to other marrow failure syndromes, SDS patients are

cancer prone and have a risk of developing myelodysplastic syndrome and/or leukemia. Raaijmakers et al.[22], using transgenic mice, showed that genetic alteration of cells in the bone marrow microenvironment can induce MDS with ineffective hematopoiesis and with occasional transformation to AML. Although it is generally agreed that the marrow microenvironment plays a role in the biology of hematological diseases, the mechanisms by which the stromal compartment promote the transformation is unclear. To study the potential implication of bone marrow mesenchymal cell in promote leukemia transformation in SDS patients we analyzed their molecular and genetic features. In spite of having chromosomal abnormalities in the bone marrow counterpart, we did not find any chromosomal alteration in SDS-MSCs. These results indicate that MSCs derived from SDS patients may be non-malignant cells. Probably, the most common chromosomal alterations [i(7)(q10) or del(20)(q11)] observed in SDS patients occurred in a more mature, already committed, progenitors which do not involve the mesenchymal compartment.

Gene expression profile analysis between patients and healthy donors was performed to see if the two groups were different at gene expression level. The heat map, derived from a supervised analysis, reveled differences in the signature pattern. We identified by expression profiling and confirmed by real-time PCR 4 genes differentially expressed: *HOXA5* is a DNA-binding transcription factor which may regulate gene expression, morphogenesis and differentiation, *ZIC1* is important during develop-

ment, *SHOX2* is thought to be responsible for idiopathic short stature and it is implicated in the short stature phenotype of Turner syndrome patients and *BCL11A* plays a crucial roles in lymphopoiesis and influence the progression of hematopoietic malignancies. These molecular differences may affect the functional property of mesenchymal cells but further studies are necessary to quantify the proteins expression and to identify the role of these genes in mesenchymal cells and if these changes may play a role in the initiation of myelodysplastic syndrome or leukemia.

We describe, for the first time at our knowledge, the isolation and expansion of MSCs from Shwachman patients. Our results showed that SDS-MSCs were similar to normal bone marrow derived MSCs in morphology, growth property, surface epitopes, and differentiation ability *in vitro*. Moreover, SDS-MSCs had normal karyotype and may be non-malignant. However, SDS-MSCs appeared to be different in gene expression profile. Our results may facilitate future studies on biological bases underlying Shwachman-Diamond syndrome. The identification of signals from the microenvironment inducing transforming events could pave the way to new highly targeted strategies for the prevention an treatment of malignant evolution.

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Chapter 4

SNP array analysis in Shwachman-Diamond syndrome for unraveling disease evolution

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4.1 Rationale

Shwachman-Diamond syndrome is a rare autosomal recessive disorder showing a wide variety of abnormalities and symptoms. It is mainly characterized by short stature, exocrine pancreatic insufficiency and bone marrow dysfunction with a tendency to evolve into myelodysplastic syndrome and leukemia. In 2001, the genetic defect of SDS was mapped to the centromeric region of chromosome 7 and in 2003 the defect was narrowed down to a single gene, which was named Shwachman-Bodian-Diamond Syndrome (*SBDS*)[1]. Mutations in the *SBDS* gene were identified in 90% of the SDS patients and this has provided the molecular basis for investigations into the underlying mechanisms defective in SDS. Clonal chromosome changes, mainly involving chromosomes 7 and 20, are often found in the bone marrow of SDS patients. The most frequent abnormalities are the isochromosome 7 [i(7)(q10)] and the deletion of the long arm of chromosome 20 [del(20)(q11)][2]. Similar to other marrow failure syndromes, patients with SDS have an increased risk for MDS and malignant transformation, in particular development of AML. The relationship between chromosome changes in BM cells and the risk of developing MDS and/or AML are still unknown. The risk of leukemic and dysplastic transformation increases with age varying from 14% to 30%[3][4]. In non-SDS patients the presence of clonal chromosome changes in BM is one of the criteria usually considered for MDS diagnosis[5], particularly in children[6], and is also relevant for

prognosis[7]. Determine risk factors and molecular events leading to malignant myeloid transformation might help developing strategies for prevention. With this aim, we investigated the presence of novel sub-microscopical lesions participating to the pathophysiology of the disease. We performed a genome-wide copy-number analysis of cells from patients' BM with clonal aberration [i(7)(q10) or del(20)(q11)]. The chromosomal abnormalities must be present in at least 50% of BM cells.

4.2 Methods

4.2.1 Patients

For this study 9 patients (mean, 13 years old; range, 3-32 years old) with Shwachman-Diamond syndrome have been enrolled. Patients were diagnosed with SDS based on clinical criteria which include clear evidence for both hematological and exocrine pancreatic dysfunction. In all patients, the diagnosis was supplemented by positive *SBDS* gene mutation analysis. Clinical, genetic and chromosomal characteristics of the patients are summarized in Table 4.1. Bone marrow from healthy controls (n=10) were used to determine if any lesions detected in SDS patients also might be present in controls. Informed, written consent was obtained in all cases.

4.2.2 DNA preparation, copy number and SNP analysis

The copy number alteration analysis was evaluated by Whole Genome Cytogenetic 2.7M array (Affymetrix) which allows us to perform genome-wide DNA copy number analysis at the highest available resolution, providing detection of DNA gains and losses, as well as copy number neutral loss of heterozygosity (LOH) on a single array. This new system contains 2.7 million markers across the genome, including 400000 single nucleotide polymorphisms (SNPs), known to be highly relevant for discovering and detecting chromosome aberrations. The median overall marker spacing of 735 base pairs provides the highest available coverage of the whole genome, allowing a 99.2% coverage for OMIM genes and 100% for cancer genes (marker spacing: 294 bp). Moreover, the 400000 SNPs to enable the detection of copy number neutral LOH, not visible with alternative techniques.

Briefly, for each sample, 100 ng of genomic DNA was amplified by whole-genome amplification reaction, purified by magnetic beads and fragmented to generate small (<300 bp) products which were labeled and loaded into a single array and hybridized over night. The chip was washed and stained in the Affymetrix GeneChip Fluidic Station 450 and scanned by Affymetrix GeneChip Scanner 7G. The results were analyzed by Chromosome Analysis Suite (ChAS) Software (Affymetrix). The segmentation file was generated by ChAS; amplifications,

duplications and deletions were identified by copy number state ($\geq$3 or $\leq$1), number of markers in the region $\geq$20 and size of the region $\geq$30 kb for losses and $\geq$50 kb for gains. The software is also able to detect loss of heterozygosity copy number neutral; segments considered as long continuous stretch of homozygosity (LCSH) have a copy number (CN)=2, size$\geq$2Mb and a number of markers $\geq$20. All abnormalities detected in patients samples and controls were considered constitutive copy number alterations (CNA). Abnormalities detected in at least two patients were considered recurrent. The SNP confirm the cytogenetic analysis.

4.3 Results

4.3.1 Cytogenetics and *SBDS* mutations

The following Table 4.1 summarizes *SBDS* mutations and chromosomal abnormalities observed in SDS patients. In agreement with previous reports, the 258+2T>C and 183-184>CT mutations were the most common in our cohort. One patient (UPN1) carried the 258+2T>C and 183-184>CT; 258+2T>C mutations. Another one (UPN9) carried the 258+2T>C and g.IVS1-71del83bp mutations. All patients had clonal abnormalities [i(7)(q10) or del(20)(q11)] in at least 50% of BM cells analyzed by FISH. Only one patient (UPN6) had a different chromosomal alteration, der(7)add(7).

Patient	**Sex**	***SBDS* mutations**		**Chromosomal alteration**
UPN1	M	c.258+2T>C	c.183_184TA>CT;c.258+2T>C	del(20)(q11)
UPN2	M	c.258+2T>C	c.183_184TA>CT	i(7)(q10)
UPN3	M	c.258+2T>C	c.183_184TA>CT	i(7)(q10)
UPN4	F	c.258+2T>C	c.183_184TA>CT	i(7)(q10)
UPN5	F	c.258+2T>C	c.183_184TA>CT	i(7)(q10)
UPN6	M	c.258+2T>C	c.183_184TA>CT	der(7)add(7)
UPN7	M	c.258+2T>C	c.183_184TA>CT	del(20)(q11)
UPN8	M	c.258+2T>C	c.183_184TA>CT	i(7)(q10)
UPN9	M	c.258+2T>C	g.IVS1-71del83bp	del(20)(q11)

Table 4.1: *SBDS* mutations and cytogenetics of 9 SDS patients enrolled in the study. UPN, unique patient number; F, female; M, male. All the mutations are described according to the mutation nomenclature (www.hgvs.org/mutnomen).

4.3.2 Copy number and SNP analyses

In all patients at least one aberration was detected (mean, 14.3; range, 4-26). The total number of lesions detected was 129 (25

gains, 51 losses and 53 LOH). Alterations found in at least 2 patients were described in Figure 4.1. A region of 10 kb on chromosome 1p13.2 with no genes was found deleted in one case (UPN2) and showed LOH in an additional patient (UPN4). A gain on chromosome 3q13.32 involving the *IGSF11* gene was found in two cases. This was the unique abnormality detected in UPN6. Loss of short arm of chromosome 7 and duplication of long arm of the same chromosome [iso(7)(q10)] was found in UPN2, UPN4 and UPN5 (also confirmed by FISH, data not show). On chromosome 15q11.2 a loss of 189 kb containing different genes (*OR4N4*, *OR4M2*, *OR4N3P*, *LOC727924*) was observed in UPN1 as well as UPN2. In addition, UPN1 and UPN9 showed a loss of 44 kb with no genes on chromosome 15q25.3. Loss of 12 kb gene-less region on chromosome 20q13.2.

Chromosome	Region	Start	End	Minimal common region (kb)	Genes	UPN6	UPN5	UPN4	UPN2	UPN1	UPN7	UPN9
1	p13.2	112693696	112704581	10	no genes							
3	q13.32				IGSF11							
7	p22.3-p11.2	1	58163069		many genes							
7	q11.2-q36.3	61683836	159138663		many genes							
15	q11.2	22315180	22504198	189	OR4N4, OR4M2, OR4N3P, LOC727924							
15	q25.3	87825834	87870180	44	no genes							
20	q11.22-q11.23	32237470	34434935	2197	many genes							
20	q13.2	54491979	54503809	12	no genes							

Figure 4.1: Recurrent alteration in SDS patients. In yellow LOH, in green losses and in red gains. Patients UPN2 and UPN6 showed a gain of two different regions of *IGSF11* gene (UPN2: 118726778-118821008; UPN6: 120216380-120304973).

The most common alteration was the deletion of long arm of chromosome 20 (3 out of 9 patients) and LOH on the same chromosome (2 out of 9 patients) (Table 4.2). UPN1 and UPN7 showed the loss of chromosome 20q11.21-q13.32 whereas UPN9 showed loss of chromosome 20q11.21-q11.23. A LOH was ob-

served in UPN5 and UPN2 (20q11.22-q11.23).

Patient	**Type**	**Region**	**Start**	**End**	**Size (Mb)**
UPN1	Loss	q11.21-q13.32	30332789	57187392	26.8
UPN7	Loss	q11.21-q13.32	31277101	57283279	26
UPN5	LOH	q11.22-q11.23	31974394	35554045	3.6
UPN2	LOH	q11.22-q11.23	32237470	34434935	2.2
UPN9	Loss	q11.21-q11.23	30743246	35928882	5.2

Table 4.2: List of aberrations on chromosome 20 in SDS patients. UPN, unique patient number; LOH, loss of heterozygosity.

The minimal common region among these 5 patients is a region of 2.2 Mb in size (bp 32237470-34434935) which includes many genes (Figure 4.2).

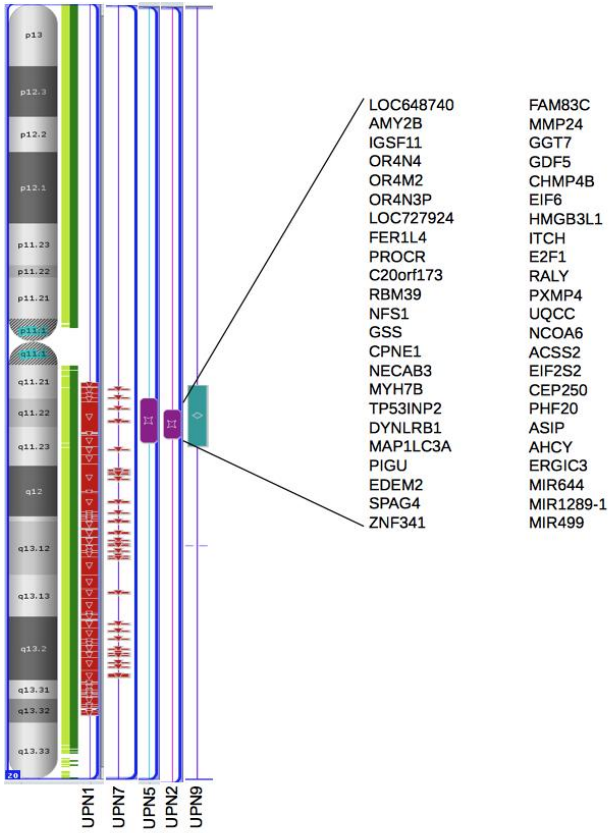

Figure 4.2: Minimal common region of LOH/deletion on chromosome 20. In red and green deletions, in purple LOH.

4.4 Discussion

Shwachman-Diamond syndrome is a rare autosomal recessive disorder showing a wide variety of abnormalities and symptoms and is caused by mutations in the highly conserved *SBDS* gene. It is mainly characterized by short stature, exocrine pancreatic insufficiency and bone marrow dysfunction with a tendency to evolve into myelodysplastic syndrome and leukemia. The estimated risk of developing acute leukemia in SDS patients is 18.8% at 20 years and 36.1% at 30 years[3]. The advanced age of leukemia diagnosis in SDS suggests that additional genetic events are necessary for leukemia progression in SDS. Chromosomal aberrations, mainly involving chromosomes 7 and 20, can be detected in the bone marrow of SDS patients. The most frequent abnormalities are isochromosome 7 [i(7)(q10)] and deletion of the long arm of chromosome 20 [del(20)(q11)][2]. To date, the relationship between these and other chromosomal changes in BM cells and the risk of MDS/AML is unknown. We had the opportunity to apply for the first time the Affymetrix Cytogenetics Whole-Genome 2.7M Array (Affymetrix) technology for a more sensitive analysis on SDS bone marrow cells. We hypothesized that with new precise methods, more cryptic karyotypic lesions participating to the pathophysiology of the disease can be uncovered. Traditional cytogenetics techniques, such as karyotyping and fluorescent in situ hybridization have been used to study chromosomal abnormalities for longtime; however, karyotyping only detects abnormalities at low resolu-

tions (larger than ∼5 Mb), and FISH is more focused and targeted. By contrast, due to the higher resolution of Cytogenetics Whole-Genome 2.7M Array as compared with cytogenetic analysis, smaller, previously cryptic deletions and duplications can be detected. A major advantage of this technology, over cytogenetic, is its ability to identify loss of heterozygosity (LOH) that occurs without concurrent changes in the gene copy number (CN). Since the array is a DNA-based technique that does not require live, dividing cells, it permits detection of aberrations in patients with non informative cytogenetic studies. We had applied this technique to study chromosomal lesions in bone marrow sample from 9 patients. We confirmed the cytogenetic analysis and we also found 129 lesions (25 gains, 51 losses and 53 LOH). Alterations were more frequent on chromosomes 1p13.2, 3q13.31, 7p22.3-p11.2, 7q11.2-q36.3, 15q11.2, 15q25.3, 20q11.22-q11.23 and 20q13.2. As far as newly identified aberrations were concerned, we focused on the lesions which recurred at higher frequency in our series, thus being candidate to retain a potential pathogenic significance. The most common alteration was the deletion/LOH of long arm of chromosome 20. A 2.2 Mb common region of loss on chromosome 20 between 20q11.21-q11.23 (bp 32237470-34434935) was determined. Following confirmation of the minimal common region of amplification, the involved genes in these regions were identified and we noticed the presence of *eIF6* gene. *eIF6* is located in the band q11.22 (bp 33330139-33336008). Finch et al.[8] showed that SBDS is coupled with the GTPase elongation factor-like 1

(EFL1) to cause the release of the ribosome anti-association factor eukaryotic initiation factor 6 (eIF6) protein from the nascent 60S ribosome subunit. Release of eIF6 allows the formation of actively translating 80S ribosome. Specific defects in the SBDS protein block the release of eIF6 from the 60S ribosomal subunit, causing an accumulation of the 60S subunit and an inadequate supply of mature ribosomes. *eIF6* over-expression has been observed in various tumors like colorectal neoplasms and head and neck carcinomas[9][10]. It is also down-regulated in cells committed to apoptosis. As demonstrated by Pressato et al.[11], there is a strong correlation between the deletion of chromosome 20 and the consequent loss of *eIF6* with a benign prognosis in Shwachman patients. These evidences lead us to postulate that mutation in *eIF6* gene can bypass loss of function mutations in *SBDS* gene, facilitate eIF6 eviction from pre-60S subunit allowing the ribosome maturation. Our investigation represents an application of SNP array for the detection of submicroscopical chromosomal defects in patients with Shwachman syndrome. In our cohort we found a consistent number of lesions and a recurrent alteration on chromosome 20, where *eIF6* resides. DNA sequencing is needed to verified if *eIF6* is mutated in bone marrow of Shwachman patients and *in vitro* and *in vivo* models are also necessary to test if mutations on *eIF6* gene can rescue its release from pre-60S subunit and the consequences of this unconventional mechanism. The chromosome 20 deletion or LOH was always associated to at least another recurrent lesion. It would be interesting to explore whether the associa-

tion of del(20)/*eIF6* deletion or LOH with other abnormalities (recurrent or not) can be related to the pathogenesis of the hematological disorder of SDS. In addition, the other recurrent abnormalities themselves or in association with non-recurrent lesion can be related to alternative pathogenetic mechanisms. Increase the number of patients could help to find a solid correlation between alterations describe in this work and MDS/AML evolution.

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Chapter 5

Summary, conclusion and future directions

Shwachman-Diamond Syndrome is a fascinating human autosomal recessive disorder characterised by hematological defects, exocrine pancreatic insufficiency and skeletal deformities. In the first part of our work we focused our attention on the biological question about the role of SDS-MSCs in the hematopoietic defects typical of this disease. SDS-MSCs do not appear different from HD-MSCs from a phenotypical and functional point of view. However, SDS-MSCs appeared to be different in gene expression profile. For the future, the genes resulted to be differentially expressed will be functionally tested to confirm their role in the pathogenesis of SDS. To date, no involvement of *SHOX2*, *ZIC1*, *HOXA5* and *BCL11A* has been reported in mesenchymal cells or in Shwachman syndrome. The complete understanding of the molecular characteristics of SDS-MSCs represents the driving element toward the understand the role

of mesenchymal cells in myelodysplastic and leukemic evolution in SDS.

In addition, in order to study whether SDS-MSC are able to recreate in vivo the hemalological alteration typical of SDS patients, we will test the formation of the Haematopoietic Progenitor Stem Cells (HPSC) niche after MSC-derived endochondral pellet transplantion into subcutaneous tissue of immunosuppressed mice. It is possible that the interactions between MSCs and stem cell niche are perturbed in SDS. Recently, it has been demonstrated that MSCs can create a niche able to sustain cancer evolution and progression. In recent years, the paradigm that tumor stromal components are just bystanders in the oncogenic process has changed. The prevailing view is now that the tumor microenvironment is a dynamic entity promoting leukemia evolution and invasion through mechanisms related to leukemic cell proliferation, immunosuppression and drug resistance, but the role of the mesenchymal component has not been fully elucidated. This study will offer the great opportunity of integrating clinical and preclinical data obtained from the analysis of MSCs isolated from SDS patients, allowing a deeper understanding of MSC biology and their role in leukemia evolution. We believe that, mirroring the whole architecture of the hematopoietic stem cell niche in vivo, we will comprehend the biological bases of leukemia evolution in SDS patients. The elucidation of tumor-promoting functions of MSCs may identify potential therapeutic targets to prevent or treat leukemia evolution in SDS patients.

The second part of the study focused on the detection of novel sub-microscopical chromosomal lesions participating in MDS and/or AML evolution. We analyzed 9 patients' bone marrow samples and we found a common region of alteration on chromosome 20 between 20q11.21-q11.23 by Cytogenetics Whole-Genome 2.7M Array. It would be interesting to explore whether the association of del(20)/*eIF6* deletion or LOH with other abnormalities (recurrent or not) can be related to the pathogenesis of the hematological disorder of SDS. The eIF6 protein is involved in the ribosome biogenesis together with SBDS. Specific defects in the SBDS protein block the release of eIF6 from the 60S ribosomal subunit, causing an accumulation of the 60S subunit and an inadequate supply of mature ribosomes. *eIF6* overexpression has been observed in various tumors like colorectal neoplasms and head and neck carcinomas[1][2]. It is also downregulated in cells committed to apoptosis. As demonstrated by Pressato et al.[3], there is a strong correlation between the deletion of chromosome 20 and the consequent loss of *eIF6* with a benign prognosis in Shwachman patients. These evidences lead us to postulate that mutation in *eIF6* gene can bypass loss of function mutations in *SBDS* gene, facilitate eIF6 eviction from pre-60S subunit allowing the ribosome maturation. In the future, we intend to increase the number of patients and to sequence the entire region of alteration or at least the *eIF6* gene. It will also be interesting to determine whether mutations on *eIF6* are involved in the pathophysiology of the disease and in its malignant evolution. This could potentially open the way

for testing specific drugs that modulate the affinity of the eIF6 on the 60S ribosome subunit interaction to treat Shwachman-Diamond syndrome.

By this work we tried to provide a better characterization of hematopoietic aspects in Shwachman-Diamond syndrome. Future work directed at understanding the molecular basis of the MDS/AML evolution and the role of mesenchymal cells should hopefully provide insight into the pathogenesis of this rare disease.

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