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SHWACHMAN-DIAMOND SYNDROME: FROM PATHOGENESIS TO DRUG TARGETING

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CHAPTER ONE

General introduction

1.1 Shwachman-Diamond Syndrome: a general introduction

Shwachman-Diamond Syndrome (SDS, OMIM 260400) is a rare autosomal recessive multi-organ disease, first described in 1964 by two different groups, which reported patients affected by pancreatic insufficiency and bone marrow hypoplasia[1,2]. The diagnosis of SDS is largely based on clinical phenotype: even if clinical presentation is not unique, exocrine pancreatic dysfunction and bone marrow failure are the principal features, associated with skeletal abnormalities, neutropenia (with recurrent infections), cardiac manifestations and cognitive impairment. Recent data show that the syndrome has an estimated incidence of 1 in 168000[3], with a ratio males to females 1,7:1[4], and no ethnic predilection. In 2003, Boocock *et al*. found in 89% of SDS patients recurring mutations in Shwachman Bodian Diamond Syndrome gene (*Sbds*)[5], mapping on chromosome 7 and involved in different aspects of cellular metabolism and survival.

1.2 Clinical presentation

Hematological features

All SDS patients present hematological abnormalities. The most frequent is neutropenia, affecting 88-100% of patients[6], defined as absolute neutrophil count (ANC)<1500 cells/μL. It can be either intermittent (in two thirds of patients) or persistent (in the other one third) and represents the major cause of morbidity and mortality. Other hematological symptoms are quite diffuse in SDS patients and include anemia, thrombocytopenia, raised fetal hemoglobin and defects in lymphoid lineage. Anemia occurs in 80% of patients, even if red blood cells are generally normocytic and normochromic, and this represents the second common manifestation of cytopenia in SDS. Fetal hemoglobin is generally elevated, reflecting a stressed hematopoiesis and difficulties in erythrocyte maturation. Some patients develop also mild thrombocytopenia (defined as platelets<$150*10^9$ cells/L). A general pancytopenia, sometimes including progression to aplastic anemia, has also been reported, with varying degrees of marrow hypoplasia, reduced numbers of bone marrow progenitors, such as granulocyte-monocyte colony-forming units (CFU-GM) and erythrocyte burst-forming units (BFU-E), and fat infiltration[7]. In fact, the percentage of bone marrow $CD34^+$ cells has been found significantly lower in SDS patients

compared to healthy controls and their clonogenic potential is significantly reduced when plated in methylcellulose for clonogenic assay. This suggests that hematopoietic defects observed in SDS patients may origin at a level of early hematopoietic stem cells. Moreover, it has been demonstrated that cells lacking SBDS are more prone to apoptosis, due to the high expression of Fas antigen on their surface and hyper-activation of the Fas-FasL pathway[8]. In addition, also bone marrow stroma cells present some generalized dysfunctions: their ability to support $CD34^+$ viability and maintain hematopoiesis is diminished and paucity of fat cell clusters was also reported[9].

Risk of myelodysplastic syndrome and other hematological diseases

In Shwachman-Diamond Syndrome there are no pathognomonic bone marrow features, but examination is required to monitor for marrow aplasia and for the emergence of clonal diseases. In fact, as for other bone marrow failure syndromes, patients with SDS are more prone to develop myelodysplastic syndrome and malignant transformations, in particular acute myeloid leukemia (AML). AML subtypes include AML-M0, AML-M1, AML-M4, AML-M5, and AML-M6[6]. AML-M6 is particularly common in SDS, occurring in about 30% of cases with classifiable leukemia[10]. Increasing with age, the risk of leukemia and dysplastic transformation varies from 14% up to

30%. In general, myelodysplastic conditions are pre-leukemic disorders, but fortunately not all cases evolve: only if the percentage of leukemic blasts in the bone marrow exceeds 20%, diagnosis of leukemia occurs. Different clonal cytogenetic abnormalities have been described in SDS. Abnormalities of chromosome 7, such as monosomy 7 or isochromosome 7q, accounted for 33% of the reported cytogenetic abnormalities in these patients. Interestingly, it has been reported that chromosome 7 aberrations may contribute to, but are not sufficient for, leukemic progression. Nevertheless, Dror *et al.* reviewed sixteen cases, and pointed out that chromosome 7 was involved in eleven of them, with an isochromosome for the long arm, i(7)(q10), in four. They concluded that the i(7)(q10) may be a fairly specific marker of myeloid malignant transformation in Shwachman-Diamond Syndrome[11]. In contrast, Alter and colleagues reviewed more than five hundred SDS cases and no patient with i(7)(q10) had developed MDS/AML. Other chromosome 7 abnormalities included monosomy 7, combined i(7q) and deletions or translocations involving 7q. Next most common chromosomal abnormality reported (16% of cases) was deletion of long arm of chromosome 20. Valli and coworkers reported a uniform loss of material of chromosome 20, often almost identical, in the region closer to the centromere, involving *EIF6* gene loss[12]. Data from different authors support the idea that SDS karyotype instability is part of the natural history of SDS, leading to frequent acquisition of clonal anomalies in the bone marrow. These chromosome anomalies may persist during time in patients bone marrow and eventually

promote MDS/AML development. On the other hand, different already unidentified mutations can occur in the bone marrow and lead to clonal evolution. Rujkijyanont *et al.* using a oligonucleotide microarray found multiple important genes significantly dysregulated in their expression in SDS marrow mononuclear cells[13]. Among 154 known leukemia-related genes, several oncogenes such as *TAL1* and *MLL* were found to be upregulated, while several tumor suppressor genes were downregulated, including *DLEU1*, *RUNX1*, *FANCD2* and *DKC1*. Kuijpers *et al.* reported also a patient compound heterozygous for two common mutations in exon 2 of the *Sbds* gene, that exhibited congenital aplastic anemia at early age[14]. Interestingly, Bezzerri *et al.* in a recent paper showed in both EBV-immortalized B cells and primary leukocytes obtained from SDS patients a constitutive hyper-activation of mTOR and STAT3 pathways. A basal enhanced activity of ERK1/2 seemed to be the driving force of this hyper-activation, and loss of SBDS expression was associated with this process. STAT3 and mTOR signalling pathway are both dysregulated in a variety of hematological disorders, including AML. Authors suggested that the basal hyper-phosphorylation of this two factors could partially explain the hematological defect observed in SDS patients, and provide molecular basis to understand the elevated risk of developing AML in SDS patients[15].

Immune dysfunctions

Patients with SDS are susceptible to recurrent bacterial, viral, and fungal infections, which affect in particular the upper respiratory tract and ears. Neutropenia but also defects in this lineage could represent contributing factors: in fact, it has been reported by Stepanovic *et al.* that PMNs from SDS patients were defective in the capacity of orienting and migrating in response to a chemoattractant[16]. Furthermore, Rothbaum and colleagues reported an aberrant surface distribution of Concanavalin A, reflecting a cytoskeletal defect in SDS neutrophils[17]. Neutrophils are not the only lineage affected in Shwachman-Diamond Syndrome. In fact, defects in lymphocyte mediated immunity are also reported: low percentage of T lymphocytes and Natural Killer cells have been demonstrated. In addition, also circulating B cells are diminished, showing low IgG production and decreased *in vitro* proliferation[7].

Gastrointestinal features

Exocrine pancreatic dysfunction is one of the typical features characterizing SDS patients. The acinar cells of the exocrine pancreas in these patients are severely depleted, and replaced with fatty tissue. Symptoms of pancreatic insufficiency, malabsorption and steatorrhea are present in 86% of patients but they spontaneously improve with increasing age in at least 50% of patients[4].

Tourlakis *et al*. demonstrated that disruption of *Sbds* in a mouse model was sufficient to recapitulate SDS phenotypes, with decreased pancreatic mass, fat infiltration and few zymogen granules[18]. Structural and functional abnormalities of liver, including hepatomegaly and elevated serum liver enzymes, are also observed.

Skeletal abnormalities

Skeletal abnormalities are frequently reported in SDS patients. Generally, children present rib-cage defects, with respiratory difficulties and thoracic dystrophy, metaphyseal dysostosis, osteoporosis and osteomalacia, most likely due to a lack of vitamin D and vitamin K[19] for malabsorption. No phenotype-genotype correlation was observed and severity of skeletal abnormalities varied even in patients with identical *Sbds* mutations.

Other features

Failure to thrive is common in SDS patients and it likely depends on multiple factors. Renal abnormalities, including urinary tract anomalies and renal tubular acidosis, and cardiac manifestations such as myocardial necrosis or fibrosis have also been described[6]. Lowered individual autonomy, intellectual disabilities, and reduced physical performances are also reported[20].

1.3 Molecular pathogenesis

Sbds gene and SBDS protein: structure and functions

Mutations in *Sbds* gene were found correlated with Shwachman-Diamond Syndrome in 2003, by Boocock and colleagues[5]. *Sbds* maps on chromosome 7 near the centromere, in the interval of 1.9 cM at 7q11, and encodes for a predicted protein of 28.8 kD. The gene is composed of five exons spanning 7.9 kb and gives rise to two additional mRNAs by alternative splicing, one that includes intron 1, and one that does not include exon 2[7]. *Sbds* has a pseudogene, called *Sbdsp*, located 5.8 Mb distally, characterized by a 97% identical transcript and containing deletions and nucleotide changes that disrupt coding potential. *Sbds* results to be highly conserved throughout evolution, with orthologues in different species, including Archaea, plants and eukaryotes, even if its functions are not been completely elucidated yet. The structure of the *A. fulgidus Sbds* orthologue AF0491 has been determined by Shammas and colleagues, representing a good paradigm for the SBDS protein family due to the high amino acid sequence conservation. They identified a three domain architecture, composed by an N-terminal domain with a mixed α/β-fold, a central three-helical bundle and a C-terminal ferrodoxin-like domain. Furthermore, they

demonstrated that the N-terminal domain is the most frequent target for disease mutations and that the most common are null mutations[21].

SBDS protein is widely expressed throughout human tissues, both in the cytoplasm and in the nucleus, but its prominent localization within sub-regions of the nucleus corresponds to the nucleolus, shuttling in and out depending on the cell cycle phase. One explanation of this localization is that SBDS could act in the nucleolus, which is a primary site of rRNA processing[22]. In fact, studies in yeast and mammalian cells demonstrated a role of SBDS in ribosome maturation[23,24]. Finch and colleagues demonstrated that SBDS cooperates with elongation factor-like 1 (EFL1) to directly catalyze eukaryotic translation initiation factor 6 (eIF6) release from pre-60S subunits[24]. In particular, eIF6 physically blocks the intersubunit bridge formation, preventing the premature joining of ribosomal subunits. SBDS catalyzes dissociation of eIF6 from nascent 60S ribosomes taking advantage of energy of EFL1 GTP hydrolysis and Pi. In this way SBDS proofreads the peptide-exit tunnel, having a key role in ribosome correct assembly and representing a final quality-control assessment of the integrity of the active sites of the 60S subunit.

Moreover, Zhang *et al.* demonstrated in a mouse model that the loss of the orthologous *Sbds* led to early embryonic mortality and the development of these *Sbds*$^{-/-}$ embryos arrested prior to embryonic day 6.5[25]. Thus SBDS assumes also a role in embryogenesis and early mammalian development.

SBDS in the Shwachman-Diamond Syndrome

Increased mitotic abnormalities were observed in SDS patients bone marrow stromal cells and lymphoblast cell lines by Austin and collaborators[26]. They demonstrated that SBDS co-localizes with the mitotic spindle, promoting its stability and chromosome segregation. Absence or defective SBDS could therefore give rise to spindle abnormalities, whose consequences cumulating over time could explain the high frequency of chromosomal abnormalities observed in SDS patients.

Even though SBDS was found not to be necessary for neutrophil maturation[27], in SDS patients these cells showed an impaired ability to migrate in response to chemoattractant. In particular, SBDS protein seems to be involved in regulating the F-actin cytoskeleton and its polymerization[28]. Nevertheless, in the murine myeloid cell line 32Dcl3, which has the potential to differentiate into mature neutrophils, SBDS acted to maintain survival of granulocyte precursor cells[27].

In addition to bone marrow dysfunctions, SDS patients present also skeletal dysplasia, generalized osteopenia and osteoporosis. Leung *et al.* demonstrated the role of SBDS in osteoclastogenesis: *Sbds*-null monocytes had a severe defect in forming osteoclasts *in vitro* and the gene itself appeared to have pleiotropic effects on the regulation of genes such as *RANK*, *NFATc1* and *Rac2*, without which subsequent osteoclast differentiation becomes blocked[29].

Furthermore, Raaijmakers and colleagues showed a reduction of *Sbds* expression in *Dicer1* deleted murine osteoprogenitors. This alteration gave rise to MDS features, with bone marrow dysfunctions and dysplasia, demonstrating in this way the involvement of *Sbds* in contributing to increase cancer risk[30].

Sbds mutations

Mutations in *Sbds* are accountable for the onset of Shwachman-Diamond Syndrome in 90% of patients. In particular, gene conversion due to recombination between *Sbds* and its pseudogene *Sbdsp* leads to introduction of critical deletions and nucleotide changes. In fact, the two genes share 97% of homology, but *Sbdsp* carries genomic sequence that disrupts coding potential. In patients carrying mutations in *Sbds*, 96% of these are found in exon 2. The 258+2T>C and 183-184TA>CT are the most recurrent. The first mutation results in the disruption of the donor splice site of intron 2, with a consequent 8-bp deletion causing a frameshift and a premature truncation of SBDS at amino acid 84. The 183-184TA>CT mutation is a dinucleotide alteration that introduces an in-frame codon stop (K62X), with the translation of a truncated protein at amino acid 62. Interestingly, no homozygous patient for the gene conversion associated with this second mutation was found[5,31,32], suggesting that such genotype could be lethal during prenatal life.

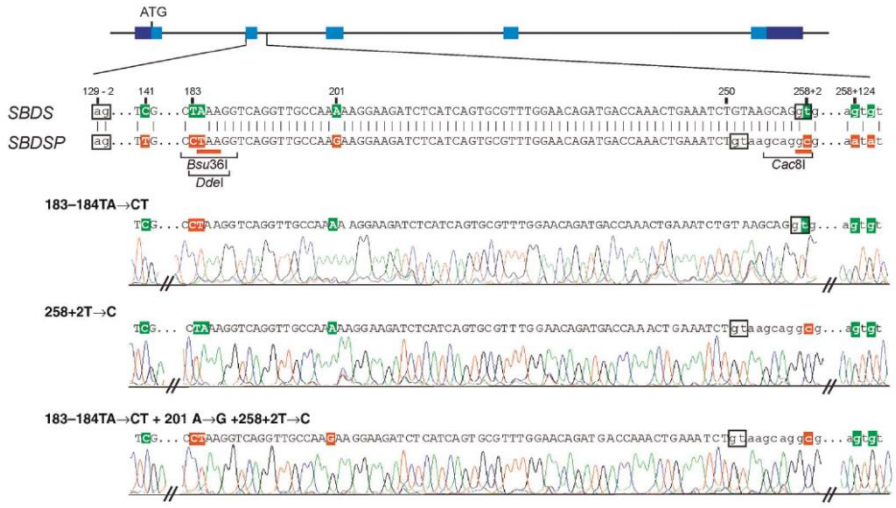

Figure 1.1: Map of *Sbds* and sequence alignment of the exon 2 region of *Sbds* and *Sbdsp*. Three converted alleles are shown[5]

Boocock and colleagues also identified alleles in affected individuals that contained an additional silent nucleotide change (201A>G) in the intervening segment[5]. Furthermore, different groups performed sequencing of alleles from SDS patients, listing a number of different alterations associated with the disease, leading to frameshift and missense changes that coded for a truncated protein[5,31].

Nucleotide sequence change	Amino-acid change
24C→A	N8K
96–97insA	N34fs15
119delG	S41fs17
131A→G	E44G
183–184TA→CT	K62X
183–184TA→CT + 258+2T>C	K62X
199A→G	K67E
258+2T→C	84Cfs3
258+1G→C	84Cfs3
260T→G	I87S
291–293delTAAinsAGTTCAAGTATC	D97–K98delinsEVQVS
377G→C	R126T
505C→T	R169C
635T→C	I212T

Table 1.1: Summary of mutations associated with SDS[5]

Interestingly, Nicolis *et al.* reported for the first time a patient with *de novo* gene conversion. In fact, the subject carried the genotype [c.183-184TA>CT;c.258+2C>T]+[c.258+2C>T]: his mother was a carrier of the 258+2C>T mutation, but his father revealed only wild-type sequence. Once confirmed paternity, they suspected that a *de novo* gene mutation occurred[31].

Regarding Italian Registry of Shwachman-Diamond Syndrome, here we reported the percentage of mutations present in this cohort of 131 patients, updated to March 2017 (Figure 1.2).

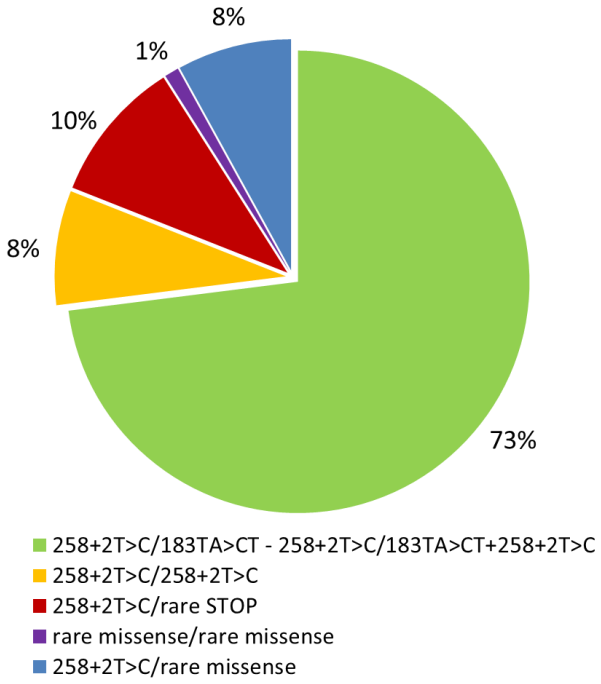

Figure 1.2: Percentage of mutations present in the Italian cohort of SDS patients

The mutation 183-184TA>CT is classified as a nonsense mutations. These kind of genetic disorders are responsible for different human diseases, such as Duchenne Muscular Dystrophy or cystic fibrosis. Therapeutic strategies to cure these pathologies are currently under investigation, and a particular approach will be further described in paragraph 1.6.

DNAJC21 in the Shwachman-Diamond Syndrome

About 10% of patients with clinical features of SDS constitute a subgroup that does not carry mutations in *Sbds* gene. Recently, Dhanraj and colleagues described four patients with diagnosis of SDS, negative for *Sbds* mutations, but carrying biallelic mutations in *DNAJC21*[33]. This gene encodes for an ubiquitous protein, involved as SBDS in the ribosome maturation, releasing factors from the pre60S subunit. In a recent paper[34] *DNAJC21* has been associated with a cancer-prone bone marrow failure syndrome, even if, in contrast with SDS, individuals who completely lack *DNAJC21* remain viable. Alterations described by Dhanraj in *DNAJC21* consisted in nonsense and missense mutations, and biallelic exon 5-6 deletion, while reduction in SBDS protein was not observed. Nevertheless, three additional SDS patients reported in this paper had mutation neither in *Sbds* nor in *DNAJC21*. Thus, it is likely that additional SDS-related genes remain to be identified[33].

SDS is a genetically heterogeneous disorder: clinical variations are described even among patients with identical genotype. On the other hand, mutations of different genes that share pathway with SBDS may result in disease with identical clinical features. For these reasons, further studies are needed to explain the phenotypic extent of SDS disease.

1.4 Clinical management

Due to the fact that currently there is no specific therapy for SDS patients, management of this disorder consists in different treatments based on clinical phenotype, as described below.

Hematology

Bone marrow aspiration and biopsy are essential for initial evaluation and should include assessment of cellularity, differential, iron stain and cytogenetics[10]. Patients with SDS should be monitored by a hematologist. Regular checks allow to supervise disease evolution prior to the development of clinical complications. Thrombocytopenia and anemia should require chronic transfusions, while mild to moderate bleeding episodes can be treated with local measures. Neutropenic patients presenting recurrent bacterial and/or fungal infections should be treated intermittently or chronically with granulocyte colony stimulating factor (G-CSF) in order to enhance neutrophil response.

In MDS and AML secondary to SDS, standard chemotherapy regimens are not indicated and due to a high risk of persistent aplasia an attempt should be made to provide Hematopoietic Stem Cell Transplantation (HSCT). In fact, apart from the supportive measures,

at present HSCT provides the only curative option for the hematological complications in SDS[7] because SDS-related leukemia presents a poor prognosis if treated with chemotherapy alone. In a multi-center study, Cesaro *et al.* reported twenty-six SDS patients undergoing HSCT and this therapy was found to be successful in more than half of them, with an overall survival comparable to that obtained with HSCT in children for acute leukemia[35]. In this study, most of the fatal toxicity was due to infections and organ failure, while Graft-*versus*-Host Disease accounted only for 35% of patients. Nevertheless, SDS patients have shown an increased toxicity with intensive conditioning regimens and they seemed to be more susceptible to cardiac toxicity. This augmented susceptibility has not been fully elucidated, but has been attributed to cyclophosphamide cardiotoxicity[36]. Furthermore, recent studies indicate that using reduced-intensity conditioning in SDS patients undergoing HSCT is associated with modest mortality[37]. For these reasons, further improvements might be achieved to adopt a less toxic condition regimen and better define the optimum time to transplant.

Gastroenterology

To manage the pancreatic insufficiency, patient with SDS should be followed by gastroenterologist. Once assessed steatorrhea, pancreatic enzyme replacement should be started. Blood levels of fat-soluble vitamins A, D, E and K should be measured every 6 to 12

months in young children, and supplementary therapy started if values are found low[10]. Furthermore, height and weight should be monitored at every clinical visit and patient should be eventually nursed by a dietitian.

Skeletal

Bone deformities may require orthopedic consultation and skeletal survey is recommended. Intake of vitamin D and calcium should be supplemented if necessary. Endocrine functionality should be screened, to exclude problems, such as hypothyroidism, that may contribute to osteopenia. In addition, dental problems are common in children with SDS and could be minimized with a regular dental care and appropriate advice from early age. Annual reviews are generally recommended.

1.5 Mesenchymal Stromal Cells

Bone marrow tissue is composed by two main populations: hematopoietic stem cells, able to produce blood cells, and cells constituting a specific microenvironment. The bone marrow microenvironment has a central role in supporting, maintaining, and regulating hematopoiesis by providing compartmentalization and production of soluble regulatory messages in ''stem-cell niches'' to which $CD34^+$ cells are anchored[9]. The main actor of this microenvironment is represented by Mesenchymal Stromal Cells (MSCs). MSCs were first isolated by Friedenstein and Petrakova in 1966 from rat marrow and currently this population is mainly isolated from bone marrow aspirates, where represents 0.001-0.01% of total population. *In vitro*, they proliferate as adherent cells and display a fibroblast-like morphology. MSCs are able to differentiate into mesodermal tissue and generate a number of non-hematopoietic cells, such as adipocytes, osteoblasts and chondrocytes. A specific antigen to identify MSCs has not been defined, but there is a panel of markers to which these cells result to be positive or negative. Mesenchymal Stromal Cells do not express hematopoietic markers such as CD45, CD34 and CD14, while are positive at variable levels for CD105, CD73, CD90.

Mesenchymal Stromal Cells in hematological disorders

In last years, the concept that Mesenchymal Stromal Cells are not barely an inert and supporting structure has arisen. In fact it has been demonstrated the involvement of these cells in support, drive and maintain several hematopoietic disorders, such as myelodysplasia and aplastic anemia. The exact role and contribution of MSCs in inducing malignancies are yet not fully understood. BM concentration of inflammatory cytokines, produced by both hematopoietic and stromal cells, have been found altered in numerous disorders, but also expression of adhesion molecules and protein by MSCs were changed. Alterations involved also changes in cytogenetic characteristics. Lopez-Villar *et al.* showed chromosomal imbalances in MDS MSCs, with genomic gain and loss, affecting genes involved in cell-cell adhesion and tumor development[38]. The striking evidence that genetic disruption in bone marrow niche can induce myelodysplastic syndrome was provided by Raaijmakers and collaborators. Using transgenic mice with *Dicer* deletion only in osterix-expressing osteoprogenitor cells, authors found that these mice developed a form of myelodysplastic disease with ineffective hematopoiesis. Survival, proliferation and differentiation of hematopoietic cells were affected, recapitulating key features of human MDS. These included hematopoietic progenitor cells increased apoptosis and development of leukemia. Of note, these changes were entirely microenvironment dependent because

hematopoietic and leukemic cells expressed normal levels of *Dicer*. Furthermore, *Dicer* deletion in osteoblast progenitors caused alterations in expressed genes and pathways, including significant downregulation of *Sbds* gene. The mechanism by which *Dicer* deletion is linked to *Sbds* is not clear, but investigators showed that knocking down *Sbds* gene in osteoblast progenitors recapitulated once again MDS phenotype[30]. Of note, Santamaría and colleagues quantifying *Sbds* in human MSCs from MDS patients found out a significantly reduction in expression of this gene by PCR analysis. Expression was affected also at protein level, but in this case only patients reporting 5q- syndrome showed reduction[39].

It is now clear that bone marrow microenvironment and, in particular, Mesenchymal Stromal Cells are able to influence and generate favorable conditions to development of hematological diseases, representing in this way a new potential therapeutic target in hematological management.

Mesenchymal Stromal Cells in SDS patients

In 1999, Dror and colleagues, using long term cultures from Shwachman-Diamond Syndrome patients bone marrow aspirates, identified defects in stromal cells, in terms of number, ability to form fat clusters and impaired cytokines production[9]. In contrast, recently Andrè *et al.* characterized deeply MSCs from twenty-seven SDS

patients. They demonstrated that SDS-MSCs are similar to HD-MSCs in terms of morphology, growth, differentiation ability and surface markers expression. Furthermore, they did not evidence any differences in the ability to support neutrophils growth, but found an increment in IL-6 production in supernatants[40]. Interestingly, unpublished data from the same group revealed differences in expression profile gene between healthy donor MSCs and sixteen SDS-MSCs. In particular *HOXA5*, a DNA-binding transcription factor which may regulate gene expression, morphogenesis and differentiation, and *ZIC1*, which has a role during development, were found downregulated in SDS-MSCs. On the other hand, *SHOX2*, implicated in the short stature phenotype of Turner syndrome patients, and *BCL11A*, which plays a crucial roles in lymphopoiesis and influences the progression of hematopoietic malignancies, were upregulated in SDS patients.

Once assessed these controversial data, the role of Mesenchymal Stroma cells in Shwachman-Diamond Syndrome remains still poorly investigated. It is absolutely relevant to find out a good model that could recreate the *in vivo* conditions and that can allow to mimic interactions that in bone marrow niche occur to better characterize the effective role of this cells in inducing and supporting hematological disorders.

Mesenchymal Stromal Cells as model

These cells have generated a great deal of interest because of their potential use in regenerative medicine and tissue engineering[41]. In fact, in stem cell therapy, undifferentiated cells are used to reach injured sites under the influence of focal signals and differentiate into cells of appropriate phenotype. Ponticiello *et al.* used a porous gelatin sponge to deliver MSCs in cartilage regeneration therapy, obtaining biocompatible cartilage-like extracellular matrix, able to give rise to both cartilage and bone cells[42]. *In vivo* heterotopic transplant of MSCs requires the use of mineralized, osteo-conductive scaffolds. Unfortunately, remnants of these materials are commonly found during analysis of cell populations after ossicles harvesting. Since these models have been used also to study bone marrow niche formation, scaffolds prevented the establishment of a complete normal architecture of bone and marrow. Recently, Serafini and coworkers were able to generate a human hematopoietic microenvironment niche transplanting free scaffold system MSCs based[43]. Unmineralized cartilage pellets were obtained from human bone marrow MSCs *in vitro* and transplanted subcutaneously in immunocompromised mice. Ossicles generated without any scaffolds completely recapitulated the architecture of bone marrow (cortical bone, medullar cavity) and were colonized by murine hematopoietic cells. Sacchetti *et al.* demonstrated that Mesenchymal Stromal Cells positive for CD146 were responsible for generation of heterotopic ossicles, had self-renewal potential, and associated with sinusoids

next to the endothelial layer[44]. Furthermore, Reinisch and colleagues demonstrated that only MSCs derived from bone marrow and no other sources (adipose tissue, umbilical cord, and skin) spontaneously formed a BM cavity when transplanted *in vivo* and exhibit a chondrogenic transcriptional program activated[45].

1.6 Stop codon mutation diseases

Different inherited diseases are caused by nonsense mutations. Depending on the disorder, nonsense mutations account for 5-70% of cases of genetic disorders, including cystic fibrosis, muscular dystrophy and several types of cancer[46]. For instance, some patients with cystic fibrosis carry stop codon mutations in *Cystic fibrosis transmembrane conductance regulator* gene, that lead to affected chloride ion channel functions, such as dysregulation of epithelial fluid transport in lung, pancreas and other organs. Otherwise, 7% of patients suffering from Duchenne Muscular Dystrophy account for non-sense mutations in the dystrophin gene.

Nonsense mutations are due to single-point mutations that inappropriately introduce in the mRNA coding sequence a UGA, UAA, or UAG triplet, transforming a codon coding for an amino acid into a stop codon. Consequently, the cellular machinery that translates mRNA into proteins reads the incorrect codon as a signal to terminate protein synthesis, causing premature cessation of translation due to a premature termination codon (PTCs) and formation of no more functional protein. Moreover, truncated proteins may also lead to damages to cellular processes and sometimes cause diseases. When PTCs are encountered by the elongating ribosome, mRNAs are subjected to an accelerated degradation process through a mechanism referred to as

nonsense-mediated decay (NMD)[47]. NMD is a conserved surveillance process able to minimize expression of defective genes and subsequently truncated and dysfunctional proteins. In fact, thanks to this mechanism, detection of a PTC during mRNA translation results in activation of a protein complex, in which the release factors eRF1 and eRF3 enter the ribosomal A site promoting dissociation of the two ribosomal subunits and mRNA decay. Discrimination between correct and wrong stop codons is based on the position of termination codon: in fact, if codon is further than about fifty nucleotides upstream of any exon-junction complexes, then the transcript is down regulated by NMD.

PTCs can be functionally overridden by a process called nonsense suppression. In fact, translation termination efficiency depends on competition between stop codon recognition and decoding of the stop codon by a near-cognate tRNA. This decoding leads to natural suppression, called readthrough, in which an amino acid is incorporated in place of the stop[48], translation termination is bypassed and synthesis of an extended protein occurs. The different stop codons are suppressed with variable efficiencies (in humans UGA≥UAG>UAA), and the level of suppression depends, in part, on the identity of the nucleotide immediately downstream from the stop codon (C>U>A>G)[48].

Therapies for nonsense mutations diseases

Nowadays, different agents are known to be able to induce ribosomal readthrough of nonsense mutation and lead to production of a functional protein. Gentamicin and G418 and other aminoglycosides antibiotics are small molecules able to partially restore synthesis of full-length proteins. They exert their antibiotic function, acting on bacterial protein synthesis, binding to specific ribosomal subunit. In a specular manner, these molecules act on mammalian protein synthesis, changing conformation of ribosomal A site. In this way, ribosome is no more able to discriminate against near-cognate tRNAs and synthesis continues. However these molecules show a wide range of efficiency, depending on the nature of the codon stop and the surrounding nucleotide context. For instance, Floquet *et al.* demonstrated that the presence of a cytosine in +4 position promotes higher gentamicin-induces readthrough, as well as a uracil residue immediately upstream from the stop codon is a major determinant for the response to this antibiotic[49]. The potential of this strategy was showed by Barton-Davis and coworkers, who first demonstrated in a *mdx* mouse model the restoration of dystrophin production (up to 10-20% compared to normal levels) after subcutaneous injection of gentamicin[50]. Encouraging results with this therapeutic strategy have been obtained sometimes in other genetic diseases, such as Duchenne/Becker Muscular Dystrophy, cystic fibrosis and hemophilia. For example, in three different clinical trials on cystic fibrosis patients, positive response to

gentamicin treatment was observed[51]. Aminoglycosides have minimal effects on normal translation termination, because the normal stop codons are surrounded by specific upstream and downstream sequences, whereas non sense mutation are not[51]. Nevertheless, one of the major drawbacks of aminoglycosides is their nephrotoxicity due to accumulation in tubular cells of the renal cortex and the irreversible ototoxicity. These huge side effects make these drugs unsuitable for continuous administration.

Ataluren

In 2007, Welch and co-workers performed two high-throughput screens to identify compounds that promoted UGA nonsense suppression. These analyses identified the new molecule PTC124 (3-[5-(2-fluorophenyl)-[1,2,4]oxadiazol-3-yl]-benzoic acid; $C_{15}H_9FN_2O_3$) as candidate for further studies. This compound is a 284.24 Da, achiral, 1,2,4-oxadiazole linked to fluorobenzene and benzoic acid rings, that has no structural similarity to aminoglycosides and is orally bioavailable when prepared in aqueous suspension[52].

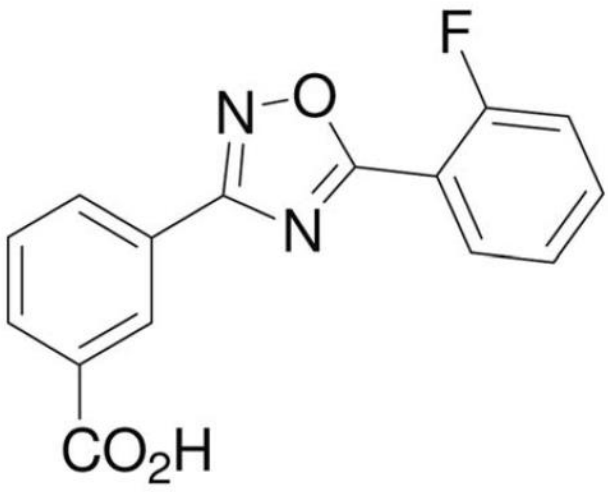

Figure 1.3: Structure of PTC124[53]

In this pioneering study, authors showed that PTC124 (now known as Ataluren or Translarna) promoted dose-dependent readthrough of all three nonsense codons in cell lines harboring LUC-190 nonsense alleles and that levels of suppression were inversely correlated with established termination efficiency[52]. Furthermore, PTC124 turned out to be a more potent suppressor than gentamicin, because exerted its role at lower concentrations compared to the antibiotic. Even in this case, surrounding amino acids influenced the efficiency of suppression: PTC124 was more active when a cytosine was located in the +1 position. The functional recovery was also demonstrated in *mdx* mice treated with PTC124 and in myotubes from primary cell

cultures derived from muscle biopsies of Duchenne patients. In both cases, the compound was able to restore the production of full-length dystrophin and no alteration on normal stop codon was observed. Safety of Ataluren was assessed in two phase I studies: the molecules had no serious adverse side effects and data supported initiation of phase II trials[53]. In recent years, phase III clinical trials for both Duchenne Muscular Dystrophy and cystic fibrosis have been initiated and Ataluren received its conditional first global approval on 31 July 2014 for the treatment of nonsense mutation in DMD[54].

Molecular mechanism responsible for Ataluren effects has not been yet completely elucidated. Roy *et al.* showed that enhancement of near-cognate tRNA insertion by Ataluren favors a subset of tRNAs, leading to incorporation of a precise amino acid at the PTC (Gln, Lys and Tyr at UAA and UAG codons, and Trp, Arg and Cys at the UGA codon). These results show that tRNA selection is probably due to base mispairing at codon positions 1 and 3, and not at the wobble one. For these reasons, authors postulated that Ataluren may target the ribosome; in fact the use of Tobramycin, which binds the A site, diminished the drug effects in a competitive manner[55].

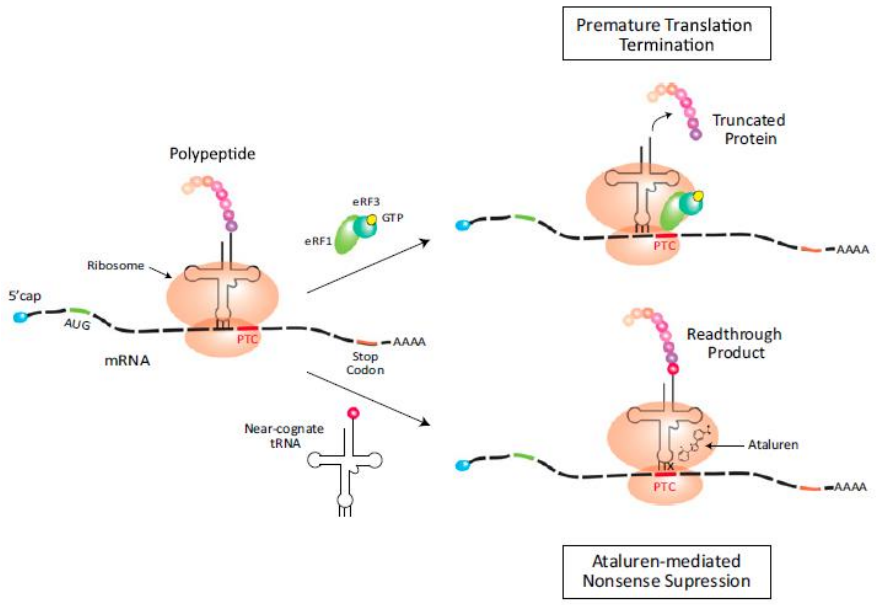

Figure 1.4: Schematic diagram illustrating Ataluren proposed mechanism of action[56]

The dinucleotide alteration 183-184TA>CT found in SDS patients is a nonsense mutation which introduces a premature termination codon resulting in the amino-acid change K62X. Notably, among the 131 SDS patients enrolled into the Italian SDS Registry, 73% of subjects carries the 183-184TA>CT nonsense mutation in one allele, similarly to the previous study based on a Canadian cohort of SDS patients[57]. Furthermore, about 20% of Italian SDS patients carrying 183-184TA>CT mutation developed MDS or leukemia, linking this

mutation to a sort of predisposition to develop hematological malignancies.

Given the fact that no therapies except bone marrow transplantation are now available to cure hematological disorders in these patients, considering the administration of compounds such as Ataluren could represent an intriguing strategy to partially restore SBDS production in this patients and attenuate disease symptoms, including enhanced risk of developing hematological diseases.

1.7 Scope of the thesis

Shwachman-Diamond Syndrome is a rare disease characterized by bone marrow failure and predisposition to develop myelodysplastic syndrome and leukemia. A better comprehension of the pathogenesis of this disease could unravel new therapeutic targets suitable to develop new strategies to counteract bone marrow failure and prevent malignant evolution.

In particular, in this work we focused our attention on two different lines of research:

1. to analyze defects of Mesenchymal Stromal Cells derived from patients in sustaining hematopoiesis and possibly driving bone marrow failure and malignant transformation, taking advantage of an *in vivo* model
2. to study the effects of Ataluren treatment on hematological and non-hematological cell derived from of SDS patients, and evaluate the possibility to consider this drug to cure hematopoietic disorders of these patients.

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CHAPTER TWO

Mesenchymal Stromal Cells derived from SDS patients fail to recreate a bone marrow niche in an in vivo model

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2.1 Introduction

Shwachman-Diamond Syndrome is a rare multi-organ disease characterized by bone marrow (BM) dysfunctions, exocrine pancreatic insufficiency and short stature. SDS is thought to be the third most common inherited bone marrow failure disorder after Fanconi anemia and Diamond-Blackfan anemia[1]. Diagnosis is mainly based on clinical phenotype, but must be confirmed on the bases of genetic analyses. In particular 80% of patients presents mutations on *Sbds* gene, which encodes for the SBDS protein, whose exact function is still unknown. Neutropenia and a generalized condition of cytopenia are typical features of these patients, along with affection of functional abilities, such as the capability of neutrophils to respond to chemotactic stimuli, and reduced numbers of bone marrow progenitors. Thus, these dysfunctions contribute to infections that frequently appear in young children. In addition, SDS patients have also an increased risk for bone marrow failure, such as myelodysplastic syndrome, and leukemia, in particular acute myeloid leukemia (AML), varying from 14 to 30% and increasing with age. The mechanisms responsible for BM failure in these patients and predisposition to develop hematological diseases are not fully understood. It is known from literature that bone marrow microenvironment has a fundamental role in supporting, maintaining, and regulating hematopoiesis. A pioneer study on

long-term bone marrow cultures (LTCs) derived from SDS patients demonstrated that in these cultures the production of fat cell clusters was markedly reduced and authors postulated that lack of this nutrient source might be a contributing factor for the reduced ability of stroma to support hematopoiesis[2]. Recently, Raaijmakers and colleagues identified a striking role of Mesenchymal Stromal Cells (MSCs) in driving hematological disorders. In fact, deleting the *Dicer1* gene in mesenchymal osteoprogenitors in an *in vivo* mouse model, they were able to recapitulate key features of human myelodysplastic syndrome (MDS). With this approach, they demonstrated that stage-specific perturbations in osteolineage cells could be responsible for the induction of hematological disorders and stressed the central role of stromal cells in tissue homeostasis[3]. Our group for the first time obtained and characterized MSCs derived from SDS patients comparing them to healthy donors. We found that these cells displayed morphology, growth kinetics and expression of surface markers comparable to healthy MSCs. SDS-MSCs were also able to support $CD34^+$ viability and their staminal potential and showed a normal karyotype[4]. Nevertheless, further unpublished data revealed that ten genes were differently expressed between SDS-MSCs and HD-MSCs. In particular, data from RT-PCR confirmed that *HOXA5* and *ZIC1*, which regulate gene expression and development respectively, were found downregulated, while *SHOX2*, implicated in the short stature phenotype of Turner syndrome patients, and *BCL11A*, which has a crucial roles in lymphopoiesis and influences the progression of hematopoietic malignancies, were

upregulated in SDS patients. Thus we postulated that these molecular differences may affect the functional properties of Mesenchymal Stromal Cells[5].

The aim of our study was to investigate if Mesenchymal Stromal Cells deriving from SDS patients were able to form a complete and functional hematopoietic niche taking advantage of an *in vivo* model that recapitulates bone marrow niche formation[6] and enable us to study in a more complex and complete scenario the behavior of these cells. Thanks to this model, we revealed that MSCs derived from sixteen patients were not able to form an *in vivo* bone marrow niche after two-month transplants. In particular, analyses at different time points of harvested pellets showed a differentiation arrest to mesengetic tissue, absence of vascularization and sometimes encapsulation in a fibrotic tissue. Evidences arising from our data revealed that even if SDS-MSCs show *in vitro* characteristics comparable to healthy donors, they display impaired functions once transplanted *in vivo*. Further studies will be necessary to better comprehend and unravel the molecular bases of bone marrow dysfunctions in SDS patients.

2.2 Methods

Patients

MSCs were obtained from thirty-six fresh or frozen bone marrow samples derived from diagnostic specimens collected from Shwachman-Diamond Syndrome patients. All patients were diagnosed as SDS based on clinical criteria and had mutations in *Sbds* gene, as screened at time of diagnosis. Table 1.1 shows the main genetic characteristics of all SDS patients enrolled in this study. MSCs from twelve healthy donors (HDs) were obtained from collection bags, discarded after BM infusion. Informed, written consent was obtained in all cases.

Isolation and culture

Mononuclear cells (MNCs) were isolated from BM aspirates using Ficoll-Paque PLUS (GE Healthcare, Waukesha, WI, USA) and seeded at 160000 cells per cm^2 in Dulbecco's Modified Eagle Medium low glucose (Lonza, Basel, Switzerland) supplemented with 10% of fetal bovine serum (Biosera, Ringmer, UK), 1% of L-glutamine (Euroclone) and 1% of penicillin and streptomycin (Euroclone) and cultured at 37°C with 5% CO_2. After 24h, the culture medium was replaced and nonadherent cells were removed washing with phosphate buffered saline (PBS, Euroclone). Adherent cells were maintained with

medium replacement twice a week, detached with 0.25% trypsin-EDTA (Euroclone) after reaching 70-80% confluence, and seeded at 1000 cells per cm^2. MSCs were used for our experiments between passages 3 (p3) and 5 (p5).

Multilineage differentiation and immunophenotype

To determine multilineage differentiation ability of these cells, *in vitro* osteogenic, adipogenic and chondrogenic induction were performed. MSCs at p4 were seeded into 6-well dishes at a density of 1000 cells/cm^2. Once reached confluence, the medium was substituted with adipogenic or osteogenic induction medium and changed twice a week. Adipogenic medium contained DMEM high glucose (Euroclone) supplemented with 10% of FBS, 1% of L-glutamine, 1% of penicillin and streptomycin, 1 µM of dexamethasone (Sigma-Aldrich St Louis, MO, USA), 100 µM of indomethacin (Invitrogen), 500 µM of 3-isobutyl-1-methylxantine (IBMX, Sigma) and 10 µg/mL of insulin (Sigma-Aldrich). Osteogenic medium was composed by DMEM low glucose supplemented with 10% of FBS, 1% of L-glutamine, 1% of penicillin and streptomycin, 10 nM of dexamethasone, 50 µM of ascorbic acid (Sigma-Aldrich) and 10 mM of β-glycerolphosphate (Sigma-Aldrich). MSCs cultured in DMEM high or low glucose supplemented with 10% of FBS, 1% of L-glutamine and 1% of penicillin and streptomycin were considered as negative control. After two weeks, differentiation was evaluated by Oil Red O staining (Sigma-Aldrich) and Alizarin Red S

(Sigma-Aldrich) for adipogenic and osteogenic differentiation respectively. To induce chondrogenic differentiation, 300000 cells at p3 were centrifuged in 15 mL polypropylene tubes to form a pellet. Cells were cultured in DMEM high glucose supplemented with 1% of L-glutamine, 1% of penicillin and streptomycin, 100 nM of dexamethasone, 50 µg/mL of ascorbic acid (Sigma-Aldrich), 1 mM of sodium pyruvate (Sigma-Aldrich), 10 µL/mL of insulin-transferrin-sodium selenite (ITS™ premix, BD biosciences, San Jose', CA, USA), 10 µL/mL of non-essential amino acid (MEM, Gibco) and 10 ng/mL of TGFβ1 (R&D Systems, Minneapolis, MN). Chondrogenic medium was changed twice a week. MSCs phenotype at p3 was assessed using these monoclonal antibodies, used according to the manufacturer's instructions: phycoerythrin PE-labeled anti-CD14 (eBioscience, San Diego, CA, USA); PE-labeled anti-CD34 (BD); PE-labeled anti-CD45 (BD); PE-labeled anti-CD73 (BD); PE-labeled anti-CD90 (eBioscience); PE-labeled anti-CD105 (eBioscience); fluorescein isothiocyanate-labeled anti-HLA-ABC (BD); PE-labeled anti-HLA-DR (BD) and PerCP-Cy5.5-labeled anti-CD146 (BD). Staining of at least 50000 cells was performed at 4° C for 30 minutes in the dark in presence of 1% of human serum to avoid aspecific binding. Samples were acquired by FACSCanto II (BD) and analyzed by FlowJo Software.

In vivo transplantation

All animal procedures were approved by Milano-Bicocca University and Italian Ministry of Health institutions. Cartilaginous pellets after 21 days of *in vitro* culture were transplanted subcutaneously into 8-15-week old anesthetized female SCID/beige mice (CB17.Cg-*Prkdc*^{scid} *Lyst*$^{bg-J}$/Crl, Charles River Laboratories) as close as possible to blood vessels. At different time points animals were sacrificed and ossicles harvested for histological and flow cytometry analyses.

Histology and flow cytometry

After 21 days of *in vitro* culture, before *in vivo* transplantation cartilaginous pellets were washed twice with PBS, fixed with a solution of PBS with 4% formaldehyde (Sigma-Aldrich) and accumulation of cartilage matrix, proteoglycans and glycosaminoglycans was evaluated by hematoxylin and eosin. Ossicles harvested at different time points were fixed in 4% formaldehyde in phosphate buffer, decalcified in 10% EDTA and processed for paraffin embedding. Sections were stained with hematoxylin and eosin. To analyze cell populations that colonized ossicles, after eight weeks HD ossicles were harvested and digested both mechanically and with 100 U/mL type II collagenase (Gibco, Grand Island, NY) in a PBS solution containing 3 mM $CaCl_2$ for 1 hour at 37° C. Cells obtained after centrifugation were stained firstly with a live/dead Viability stain (ThermoFisher Scientific) and then with different anti-mouse monoclonal antibodies: PE-Cy7-labeled

anti-CD45 (eBioscience); PerCP-Cy5.5-labeled anti-CD11b (eBioscience) and APC-labeled anti-Gr1 (eBioscience). Staining was performed at 4° C for 30 minutes in the dark in presence of 1% of human serum to avoid aspecific binding. Samples were acquired by FACSCanto II (BD) and analyzed by FlowJo. Bone marrow cells deriving from flushing of femur of the same mice were stained following the same protocol and used to compare percentage of population colonizing ossicles.

2.3 Results

Patient	Gender	Age at study (y)	*Sbds* mutations	
UPN1	M	27	624+1G>C	258+2T>C
UPN2	M	14	258+2T>C	258+2T>C
UPN3	F	4	183-184TA>CT	258+2T>C
UPN4	M	23	183-184TA>CT	258+2T>C
UPN5	F	9	258+2T>C	258+2T>C
UPN6	F	39	G63C	258+2T>C
UPN7	M	5	183-184TA>CT	258+2T>C
UPN8	M	9	183-184TA>CT	258+2T>C
UPN9	F	6	183-184TA>CT	258+2T>C
UPN10	M	11	183-184TA>CT	258+2T>C
UPN11	M	16	183-184TA>CT	258+2T>C
UPN12	M	6	183-184TA>CT+258+2T>C	258+2T>C
UPN13	M	9	183-184TA>CT	258+2T>C
UPN14	F	8	183-184TA>CT	258+2T>C
UPN15	M	38	183-184TA>CT	258+2T>C
UPN16	F	10	183-184TA>CT	258+2T>C
UPN17	F	12	183-184TA>CT	258+2T>C
UPN18	M	37	183-184TA>CT	258+2T>C
UPN19	F	9	258+2T>C	258+2T>C
UPN20	F	17	352A>G	258+2T>C
UPN21	M	18	183-184TA>CT+258+2T>C	258+2T>C
UPN22	F	20	183-184TA>CT	258+2T>C
UPN23	F	10	183-184TA>CT	258+2T>C
UPN24	F	4	183-184TA>CT	258+2T>C
UPN25	M	9	183-184TA>CT	258+2T>C
UPN26	M	4	258+2T>C	258+2T>C
UPN27	F	13	183-184TA>CT	258+2T>C
UPN28	F	5	183-184TA>CT	258+2T>C

UPN29	F	2	183-184TA>CT+258+2T>C	258+2T>C
UPN30	M	5	183-184TA>CT	258+2T>C
UPN31	M	15	183-184TA>CT	258+2T>C
UPN32	F	1	183-184TA>CT	258+2T>C
UPN33	M	7	183-184TA>CT	258+2T>C
UPN34	M	1	183-184TA>CT+258+2T>C	258+2T>C
UPN35	F	8	183-184TA>CT	258+2T>C
UPN36	M	2	nd	nd

Table 2.1: Features of thirty-six patients enrolled in the study. UPN, unique patient number; F, female; M, male. In Italic patients that were tested for *in vivo* studies. All mutations are described according to the mutation nomenclature (www.hgvs.org/mutnomen)

Characterization of new Mesenchymal Stromal Cells isolated from SDS patients

Mesenchymal Stromal Cells were obtained from the bone marrow of 17 newly collected patients. We succeeded in significantly increasing our patients cohort and a total number of thirty-six patients were enrolled in our study. All SDS-MSCs showed a typical fibroblast-like morphology, similarly to HD-MSCs and were tested for their antigen expression and their ability to differentiate into different lineages.

Phenotype of SDS-MSCs was analyzed at p3 by flow cytometric analyses. As for HD-MSCs, Mesenchymal Cells derived from patients were negative for hematopoietic markers (CD45, CD34 and MHC class II), while showed a strong positivity for stroma lineage markers, such as CD105 (Endoglin), CD73 (5'-nucleotidase) and CD90 (Thy-1) (Figure 2.1).

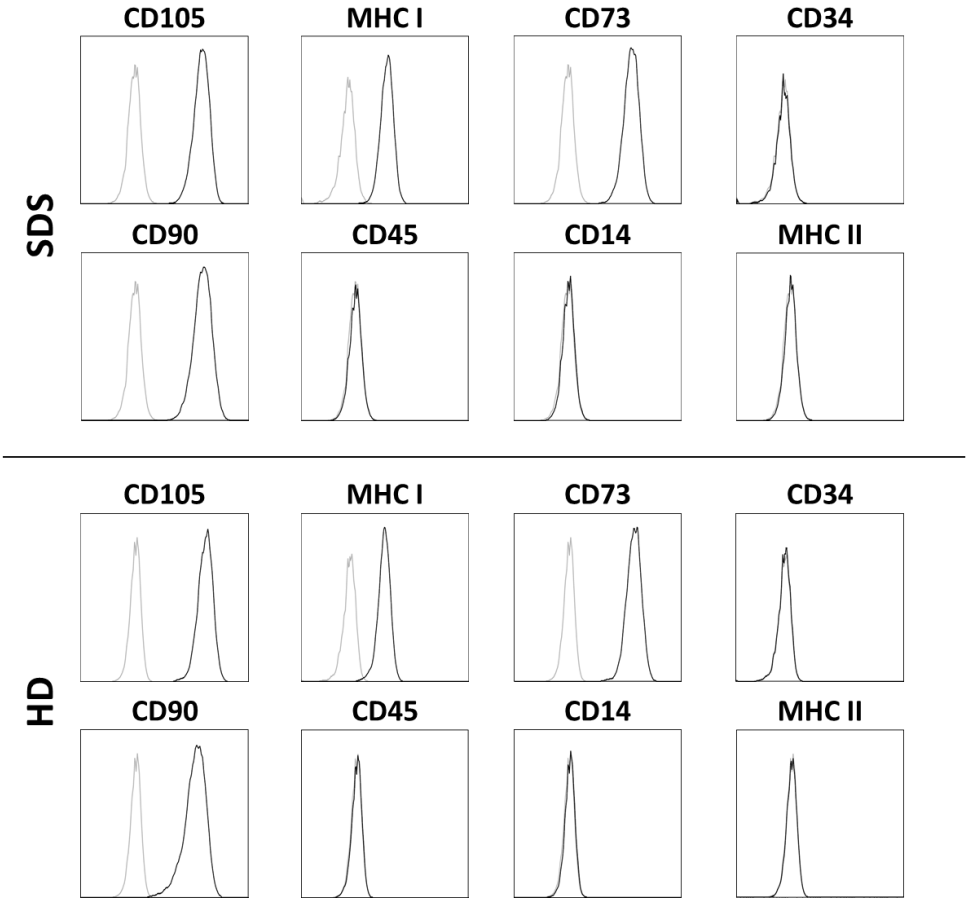


Figure 2.1: Analyses of SDS-MSCs marker surface. MSCs derived from SDS patients express typical markers of Mesenchymal Cells and present no differences compared to healthy donors one. Black profiles represent positive cells, while grey profiles are unstained cells

To further characterize new SDS-MSCs the ability to differentiate into different mesengetic lineages was assessed at p3. We performed differentiation assays in all new patients cases. All MSCs obtained from patients were able to differentiate into adipocytes, after induction of differentiation with specific medium, as revealed by the formation of lipid droplets stained with Oil Red O. SDS-MSCs also differentiated into osteoblasts as well as HD-MSCs and calcium deposition was verified by Alizarin Red staining (Figure 2.2).

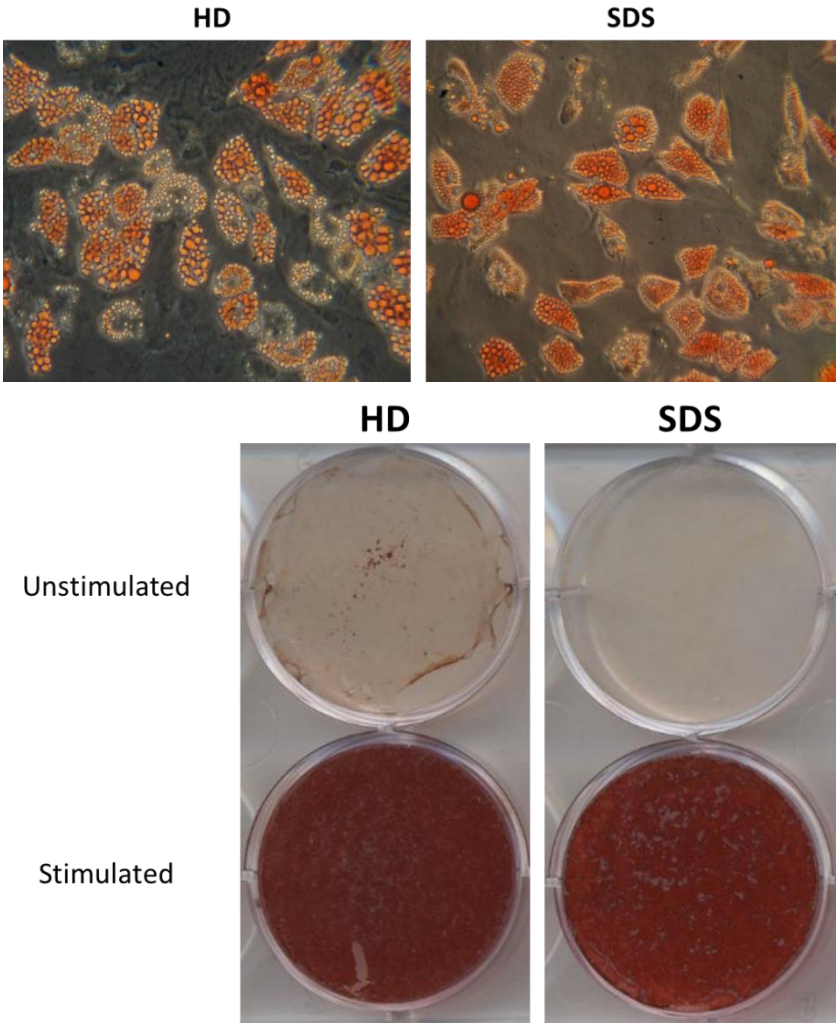


Figure 2.2: Upper panel. Adipogenic differentiation in SDS-MSCs. After 14 days of induction with differentiation medium, MSCs derived both from patients and healthy donors were stained with Oil Red O to visualize lipid droplets. Magnification 20X. **Lower panel.** Osteogenic differentiation of MSCs. Mesenchymal Cells derived from patients and donors were stimulated for 21 days with osteogenic medium. At the end of the culture, calcium deposition was visualized by Alizarin Red staining

Chondrogenic differentiation in SDS Mesenchymal Stromal Cells is comparable to HD

We also assessed the ability of SDS-MSCs to differentiate into chondrocytes and create semi-cartilaginous pellets. We induced chondrogenic differentiation for 21 days with medium supplemented with TGFβ1. After this period, the presence of cartilaginous matrix was assessed by hematoxylin and eosin staining. Histological analyses showed no difference in terms of chondrogenic tissue formation between SDS-MSCs and HD-MSCs as shown in a representative experiment in Figure 2.3, upper panel. In particular, we successfully obtained differentiation in 8 out of 12 healthy donors (68%) compared to 16 out of 26 SDS patients (61.5%) (Figure 2.3, lower panel).

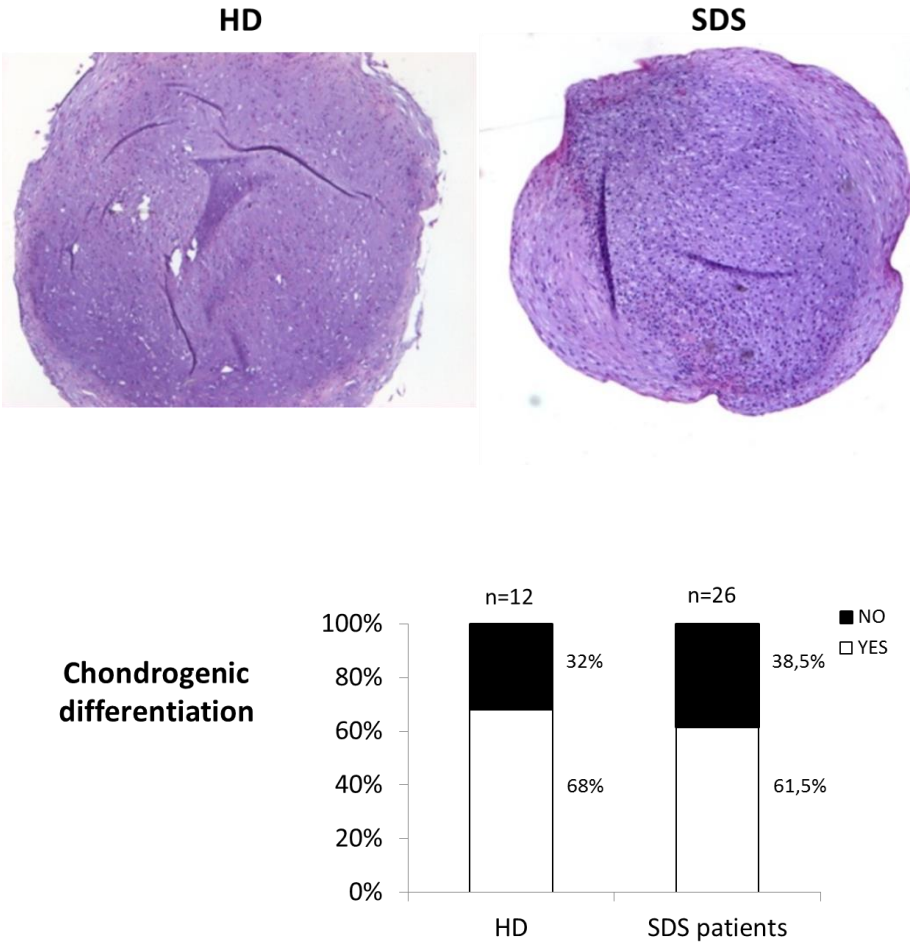


Figure 2.3: Chondrogenic differentiation of MSCs. Induction of differentiation was performed for 21 days with a specific medium. After this period, pellets derived from SDS and healthy MSCs were stained with hematoxylin and eosin to reveal cartilaginous matrix formation. Magnification 4X. The lower graph shows percentage of success of chondrogenic differentiation in all HD and SDS-MSCs tested

SDS-MSCs are not able to recreate an *in vivo* bone marrow niche

To investigate SDS-MSCs ability to support hematopoiesis, we took advantage of an *in vivo* model that could recapitulate bone marrow microenvironment[6]. After inducing *in vitro* chondrogenic differentiation by culturing MSCs at p3 in presence of TGFβ1 for three weeks, we obtained semi-cartilaginous pellets (SCPs). Only pellets that presented a correct grade of chondrogenic differentiation *in vitro*, revealed by histological analyses, were transplanted subcutaneously in immunocompromised mice (HD n=8; SDS patients n=16). The transplantation was performed in anesthetized 8-15-week old female SCID/beige mice and SCPs were placed as close as possible to blood vessels to facilitate vascularization and hematopoietic cells colonization. Eight weeks after implants, mice were sacrificed, ossicles harvested and analyzed by histological analyses to bone marrow establishment or digested for FACS analyses (Figure 2.4).

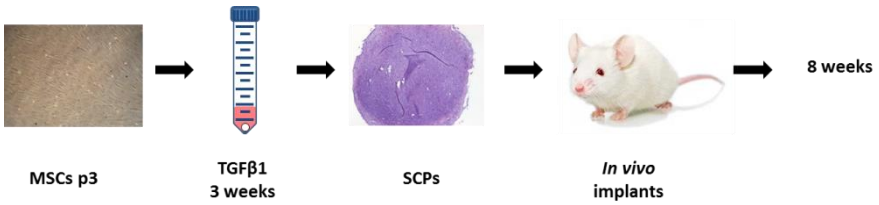


Figure 2.4: Schematic representation of the experimental plan

We found that bone remodeling and formation of marrow occurred only in SCPs derived from healthy donors, while SDS-SCPs were not able to recreate bone marrow niche *in vivo*.

In particular, ossicles derived from six different HD and harvested after 8 weeks *in vivo* macroscopically displayed a red color and a hard consistency. Microscopic analyses after histological staining revealed a complete heterotopic ossicles formation. Bone remodeling and formation of marrow occurred: structures including bone with intratrabecular spaces, murine marrow cells, marrow sinusoids and adipocytes lacuna were present in harvested ossicles, perfectly recapitulating bone marrow microenvironment (Figure 2.5).

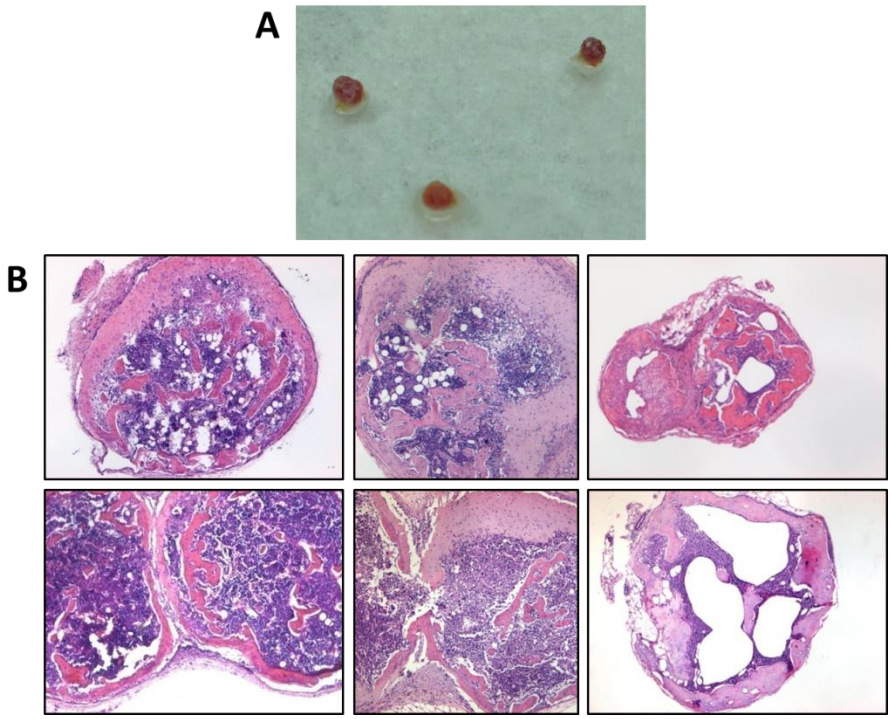


Figure 2.5: *In vivo* formation of HD ossicles. **A.** Macroscopic appearance of HD ossicles harvested after 8 weeks. **B.** Hematoxylin and eosin analyses of heterotopic ossicles derived from six different healthy donors. Images show a complete formation of bone marrow cavity, resembling normal bone architecture. Magnification 4X

Furthermore, FACS analyses of digested ossicles revealed that murine hematopoietic cells of different lineages were able to colonize HD-SCPs. In particular, we found cells positive for the hematopoietic marker CD45 and subpopulations that showed positivity for the pan-macrophage marker CD11b and cells expressing also Gr1, that can be classified as monocytes, neutrophils or eosinophils. Expression of these markers was compared to populations present in a SCID/beige bone marrow and were found comparable (Figure 2.6).

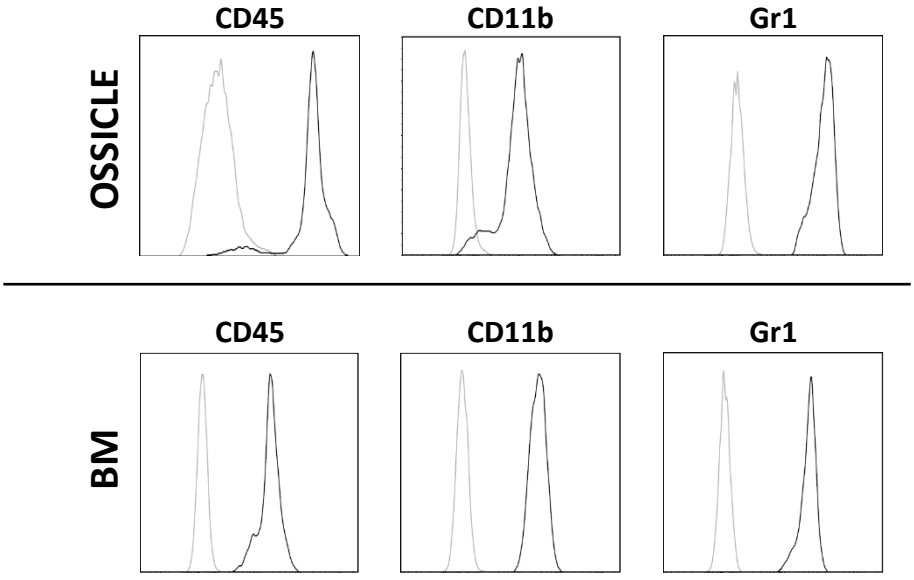


Figure 2.6: Expression of murine hematopoietic markers in HD heterotopic ossicles after eight weeks. Exemplificative flow cytometry of SCID/beige bone marrow is shown. Black profiles represent positive cells, while grey profiles are unstained cells

Regarding SDS-SCPs, we implanted a total number of 130 pellets obtained from 16 different patients. Surprisingly, at the end of the *in vivo* experimental procedure, we harvested only 18 pellets (13.8%) and none of them presented vascularization or bone marrow niche formation (Figure 2.7).

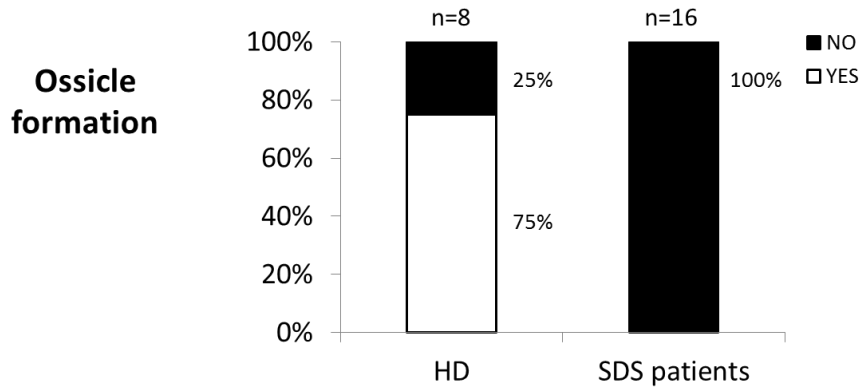


Figure 2.7: Percentage of success in ossicles formation in all HD and SDS-SCPs tested. Data revealed that only pellets derived from healthy donors were able to recreate a bone marrow niche *in vivo* after transplantation

In particular, histological analyses on collected samples revealed that harvested pellets were principally composed by undifferentiated mesengetic tissue or that the differentiative process that occurred to HD-SCPs in these cases remained at chondrogenic phase. In detail, in two pellets out of eighteen were found inflammatory elements, such as infiltrating macrophages (Figure 2.8, Panel A), while six pellets were composed by mesengetic undifferentiated tissue (Figure 2.8, Panel B). Regarding the other ten pellets, they were all constituted by chondrogenic tissue (Figure 2.8, Panel C and D) and three of them were also surrounded by a fibrotic capsule (Figure 2.8, Panel D). Concerning the remaining 86% of cases, SDS-SCPs were resorbed during the *in vivo* period, so we were not able to found them at harvesting time.

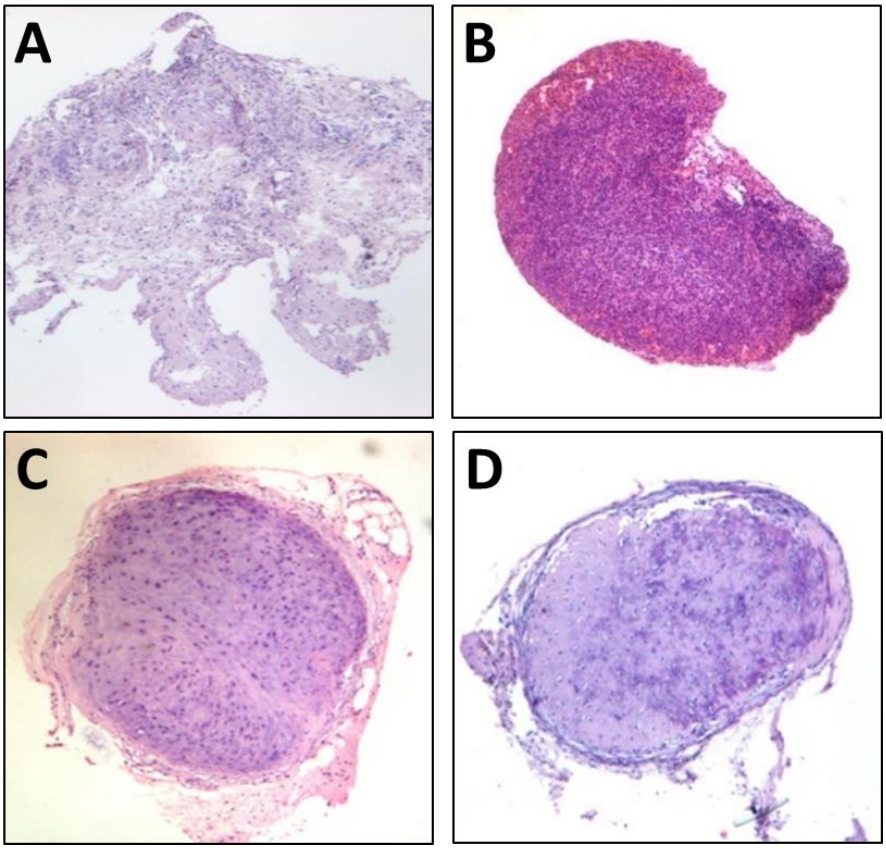


Figure 2.8: SDS patients are not able to form complete ossicles *in vivo*. Histology of SDS-pellets harvested after 8 weeks shows absence of vascularization and bone marrow structures. Moreover, differentiative process seems to be stopped at mesengetic (**B**) or chondrogenic phase (**C** and **D**) and sometimes inflammatory elements (**A**) or fibrotic capsule (**D**) are found

To further investigate the mechanisms underlying the inability of SDS derived pellets to form *in vivo* ossicles, we harvested ossicles at different time points (2 and 4 weeks) and perform histological analyses. We found that after 2 weeks, harvested SDS ossicles did not show any relevant differences in comparison to HD ones. In both cases chondrogenic pellets were found, characterized by total composition of endochondral cartilaginous matrix. At 4 weeks resorption of cartilage started in HD sample, with an initial deposition of osteogenic matrix, while in SDS ossicles the process seemed to be less evident and more slow. At the final time point, we successfully obtained bone marrow niche formation only in HD ossicles. One out of three SDS samples was not found, whereas in the other two cases the ossicles collected showed no vascularization or bone marrow niche formation. In accordance with previous described results, we found chondrogenic and osteogenic tissue without any evidence of progression in ossicles formation (Figure 2.9).

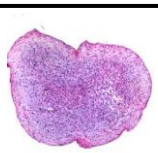
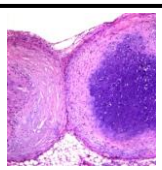
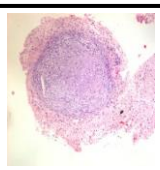
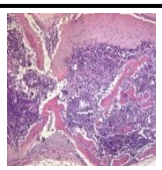
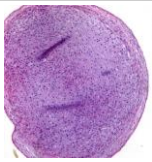
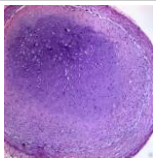
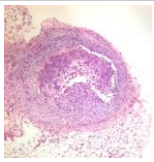
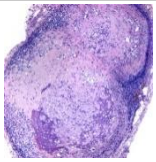
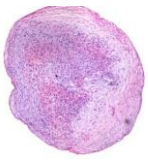
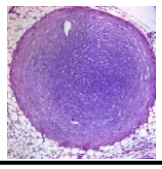
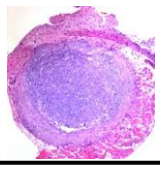
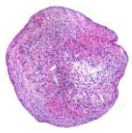
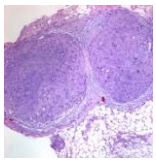
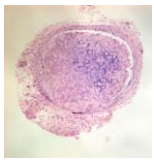
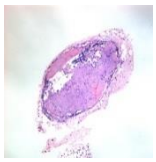
	Chondrogenic	2 weeks	4 weeks	8 weeks
HD				
UPN 26				
UPN 14				not found
UPN 20				

Figure 2.9: Histological analyses of ossicles derived from HD and SDS patients harvested at different time points. At two weeks after implantation, both HD and SDS pellets show a diffuse cartilage matrix, which starts to be resorbed in HD at four weeks to form a complete marrow niche at eight weeks. The process seems to be blocked in a cartilaginous/osteogenic phase in SDS patients, which on the other hand cannot form ossicles *in vivo*

Analyses of CD146 expression in MSCs

Recently, Sacchetti *et al.* demonstrated that, following transplantation, only MSCs that retained CD146 expression dynamically associate with developing sinusoids and generate heterotopic adventitial reticular cells[7] responsible for vascularization process. To assess if SDS-MSCs were defective in CD146 expression we performed FACS analyses, investigating the positivity for this surface antigen. We demonstrated that *in vitro* expression of this marker was identical between healthy donors and patients (Figure 2.10).

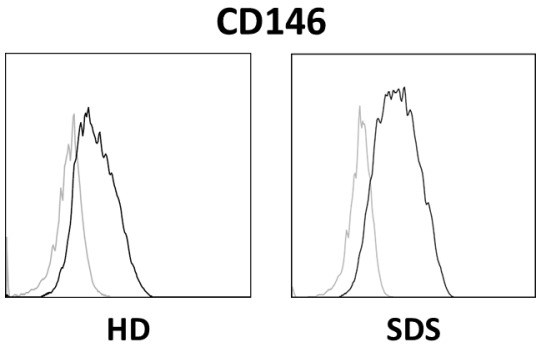


Figure 2.10: Expression of CD146 on MSCs surface does not differ between healthy donors and Shwachman-Diamond patients. Black profiles represent positive cells, while grey profiles are unstained cells. A representative experiment is shown

2.4 Discussion

Shwachman-Diamond Syndrome is a rare autosomal recessive disease, characterized by bone marrow failure, severe neutropenia, which represents the main cause of infections during early stages of life, exocrine pancreatic insufficiency and skeletal defects. Furthermore, patients are prone to develop cancer and present a high risk for leukemia (AML) and myelodysplastic syndromes (MDS). In addition, also number of bone marrow precursors is reduced and their viability can be affected due to dysfunctions of bone marrow stroma. Indeed, as demonstrated by Dror and Freedman using long-term cultures of SDS bone marrow samples, patients showed a reduced ability to support hematopoiesis and defects in cytokines production[2]. Nowadays, the role of Mesenchymal Stromal Cells in bone marrow niche, their ability to regulate and orchestrate hematopoiesis and also their influence in driving hematological diseases has been assessed, but their role in the pathophysiology of hematological defects in Shwachman-Diamond Syndrome has not been elucidated yet. Unpublished data from our group revealed that SDS-MSCs show a different gene expression profile. Supervised analyses, in fact, showed differences in their signature pattern and this was confirmed by real-time PCR. In particular, four genes were differentially expressed: *HOXA5* and *ZIC1* were downregulated, while *SHOX2* and *BCL11A* were found upregulated. We supposed that these

molecular differences could alter the functional properties of MSCs, even if no differences in phenotype, ability to differentiate and support $CD34^+$ viability were observed in *in vitro* culture[4]. To deeply investigate possible SDS-MSCs defects we took advantage of an *in vivo* model that could mirror the whole architecture of the hematopoietic stem cell niche. First of all, we increased our patients cohort and successfully obtained new MSCs for a total number of thirty-six patients. Regarding new patients, we isolated and characterized MSCs to confirm data previously published. As expected, all MSCs displayed a fibroblast-like shape, were able to differentiate into adipocytes, osteoblasts and chondrocytes under appropriate induction conditions and presented the same surface antigens commonly found on HD-MSCs, such as CD73, CD90 and CD105 but did not express hematopoietic markers. Thus, we confirmed that *in vitro* SDS-MSCs do not show differences if compared to HD-MSCs.

Similar to other marrow failure syndromes, SDS patients have an augmented risk of developing myelodysplastic syndrome and/or leukemia. Raaijmakers *et al.* showed in a mouse model that specific genetic alterations of osteoprogenitor lineage in bone marrow microenvironment can induce MDS with ineffective hematopoiesis[3]. Although it is known that marrow microenvironment can play a role in sustaining and promoting hematological diseases, the mechanisms by which the stromal compartment promotes transformation remain still unclear. Following SDS-MSCs characterization *in vitro* we tried to

better understand the role of these cells in driving and sustaining hematopoietic insufficiency observed in SDS patients. For this purpose, we generated semi-cartilaginous pellets deriving both from HD and SDS MSCs. We succeeded in obtaining correct cartilage matrix formation in similar percentage in healthy donors and SDS patients (68% *vs* 61.5% respectively). The remaining percentage of failure in inducing chondrogenic differentiation is linked to the great heterogeneity of *in vitro* cultured MSCs with respect to their differentiation potential. Although investigations in established MSCs cultures show them to be multipotent, with a tri-lineage differentiation potential, only a minority of cells seems to be effectively multipotent, with most of them having bi- or only uni-lineage differentiation capacity. Thus, it is hypothesized that the heterogeneous MSCs population contains only a small pool of immature cells with tri-lineage multipotency[8].

We then transplanted subcutaneously in immunocompromised mice SCPs that showed a good grade of chondrogenic differentiation (assessed by histological analyses) and allowed them to form a complete bone marrow niche within eight weeks. At the end of this period we collected ossicles and we found that only SCPs derived from healthy donors recreated an *in vivo* niche, that completely recapitulated the bone architecture, with bone trabeculae, murine hematopoietic cells and adipocytes. On the contrary, only 13.8% of total SDS-SCPs implanted were found and none of them presented bone marrow formation. Collected samples after *in vivo* transplants were constituted by chondrogenic and osteogenic tissue, sometimes

surrounded by a fibrotic capsule or within inflammatory elements. No vascularization or murine hematopoietic cell invasion was observed. Then we analyzed harvested ossicles at previous time point, to identify which step could be critical for niche formation in SDS-SCPs. We found that at two weeks after implants no evident differences between healthy donors and patients were revealed: in both cases, collected ossicles were composed by extended cartilaginous matrix. At the second time point (4 weeks) we observed in HD samples resorption of cartilage, with an initial bone deposition; on the contrary in SDS ossicles the process seemed to be less evident and more slow. At 8 weeks, we successfully obtained bone marrow niche formation exclusively in HD ossicles, while the few SDS samples that we could found were characterized by chondrogenic and osteogenic tissue without any evidence of progression in ossicle formation and most of them were completely resorbed. It is evident from our data that some critical events can or cannot occur to SDS ossicles in a critical lapse of time during *in vivo* experiment.

The exact function of SBDS protein remains still unclear and we need to clarify the reason why its lack in Mesenchymal Stromal Cells in SDS patients can lead to a failure in bone marrow niche establishment. It has been demonstrated by Serafini *et al.* that one critical step for bone marrow niche formation in ossicles is the replacement of unmineralized cartilage by stromal tissue which subsequently became colonized by blood vessels, which starts to occur at three weeks post transplantation[6]. Recent studies have

demonstrated that bone marrow Mesenchymal Stromal Cells that express on their surface the CD146 have a commitment to a vascular smooth muscle cell lineage[9]. Furthermore, Sacchetti *et al.* demonstrated in an *in vivo* model of hematopoietic microenvironment that, following MSCs transplantation a subset of these cells retains CD146 expression, dynamically associates with developing sinusoids and generates heterotopic adventitial reticular cells[7]. Expression of this surface marker was also tested in our SDS-MSCs by FACS analyses: no differences in CD146 expression was found between healthy donors and patients in mesenchymal cells deriving from *in vitro* culture. It could be interesting to investigate by histochemical analyses at our critical *in vivo* time points if changes in this marker expression occur, eventually compromising vascularization of pellets. Moreover, it has been reported the presence of high levels of Angiotensin-1 (Ang-1) in the heterotopic ossicles, localized close to adventitial reticular cells once hematopoiesis was established. Ang-1 is a key component of the HSC niche in postnatal murine bone and could be responsible, in association with CD146, for the vascularization of SCPs once implanted *in vivo*. Nonetheless, as well as Ang-1, other soluble factors, such as HIF-1α and VEGF, play a role in vascularization. Leung *et al.* showed that in plasma samples derived from SDS patients VEGF levels were comparable to healthy donors. Moreover, also VEGF secretion measured in supernatants derived from SDS long term culture did not show any consistent differences[10]. Nevertheless, as for the *in vitro* characteristics of MSCs, it is possible that to unravel

faint differences we need to recreate an *in vivo* model, where cells are influenced by surrounding microenvironment. In fact, undergoing complex mechanisms such as chondrogenic matrix resorption and bone marrow establishment, may highlight lack of functions that are not required in *in vitro* models. Thus, exploring these molecules expression in our ossicles could be helpful to better elucidate mechanisms undergoing during transplantation.

As for vascularization, another critical point for heterotopic ossicles formation is the deposition of bone matrix and formation of cortical bone. SDS patients had markedly reduced bone mineral density, reduced trabecular bone volume, reduced numbers of osteoclasts and osteoblasts, and reduced amount of osteoid, suggesting a primary defect in bone metabolism[11]. Our data suggest that also in *in vivo* model, the bone marrow process seems to be blocked in a cartilaginous/osteogenic phase, probably suggesting defects also in this mechanism.

In this study we successfully increased our Shwachman-Diamond Syndrome patients cohort and obtained fully characterized Mesenchymal Stromal Cells. Furthermore, we tried to establish, for the first time at our knowledge, a model of an *in vivo* hematopoietic niche deriving from SDS-MSCs. Our data revealed a defect in ossicles formation in SDS patients, that could not recreate a complete bone marrow microenvironment after heterotopic transplantation. Further studies are needed to better comprehend the mechanisms underlying these failure. The identification of altered molecular

pathways in SDS patients could reveal a new explanations for hematological and non-hematological defects and represent new appealing therapeutic target for this disease.

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CHAPTER THREE

Ataluren: a new potential treatment to correct SDS hematological defects?

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3.1 Introduction

Shwachman-Diamond Syndrome (SDS) is a rare autosomal recessive disorder caused by mutations in the Shwachman Bodian Diamond Syndrome gene (*Sbds*) which encodes for the homonymous SBDS protein. SDS is mainly characterized by multiple-organ involvement, including short stature and bone malformation, pancreas insufficiency, and hematological disorders with severe neutropenia. Neutropenia is the main responsible for frequent and severe infections, often recurring in early age and representing one of the major causes of death in these patients. SDS patients have also a tendency to develop myelodysplastic syndrome (MDS) and leukemia (AML) and risk varies from 14% up to 30%, increasing with age. SBDS is a protein widely expressed throughout human tissues and is thought to be a multitasking protein, with roles in ribosome maturation, stabilization of mitotic spindle, osteoclastogenesis and cell proliferation. Recently, loss of SBDS expression in LCLs derived from SDS patients has also been associated with increased basal phosphorylation of the mammalian Target of Rapamycin (mTOR)[1]. Interestingly, dysregulation of mTOR pathway has been associated to leukemia[2] and this could partially represent a reason for the increased risk of developing AML in SDS patients.

Mutations in *Sbds* gene are present in 90% of SDS patients and 96% of these are found in exon 2. The 258+2T>C and 183-184TA>CT

are the most recurrent. The first mutation results in the disruption of the donor splice site of intron 2, while the 183-184TA>CT mutation is a dinucleotide alteration that introduces premature termination codon (PTCs), with the translation of a truncated protein at amino acid 62. Interestingly, no homozygous patient for this mutation was found[3-5], suggesting that this genotype could be lethal during prenatal life. Notably, 73% of 131 SDS patients enrolled into the Italian SDS Registry carries the 183-184TA>CT nonsense mutation and about 20% of them developed MDS or leukemia. This percentage of malignant transformation is doubled if compared to SDS patients recorded in the French Severe Chronic Neutropenia Registry[6]. Interestingly, data from Italian SDS registry revealed that carrying the 183-184TA>CT mutation seems to be the crucial condition to developed MDS/AML, because none of the patients homozygous for 258+2T>C evolved in malignant transformation.

PTCs can be functionally overridden, a phenomenon called nonsense suppression, which allows the misreading of PTCs by near-cognate tRNA. This decoding leads to natural suppression, called readthrough, in which an amino acid is incorporated in place of the stop[7], translation termination is bypassed and synthesis of an extended protein occurs. Small molecules, such as aminoglycosides, can promote the readthrough of PTCs and lead to production of a functional protein. Thanks to this therapy, encouraging results have been obtained in genetic diseases, such as Duchenne/Becker Muscular Dystrophy, cystic fibrosis[8] and hemophilia. Unfortunately,

due to their well-known side effects (nephrotoxicity and irreversible ototoxicity) a therapy based on aminoglycosides continuous administration results to be inappropriate. In 2007, Welch and co-workers identified a new promising molecule, PTC124 (3-5-2-fluorophenyl)-[1,2,4]oxadiazol-3-yl]-benzoic acid; $C_{15}H_9FN_2O_3$), an achiral, 284.24 Da compound that has no structural similarity to aminoglycosides and is orally bioavailable when prepared in aqueous suspension[9]. PTC124 (now known as Ataluren or Translarna) is able to promote dose-dependent readthrough of all three nonsense stop codons and exerts its role at lower concentrations compared to aminoglycosides. Ataluren molecular mechanism has not been yet completely elucidated. It was shown by Roy *et al.* that Ataluren may target the ribosome, enhancing near-cognate tRNA insertion, leading to incorporation of a precise amino acid at the PTC[10] and allowing translation of a full-length protein. Currently, Ataluren has been approved for the treatment of nonsense mutation in Duchenne Muscular Dystrophy[11] and has been testing in phase III clinical trial for the treatment of cystic fibrosis[12].

Considering that nowadays no specific therapies are available to cure hematological disorders in Shwachman-Diamond Syndrome patients or prevent malignant transformations, the aim of our study was to investigate the effect of Ataluren on patients carrying the 183-184TA>TC mutation. We examined the effects of Ataluren treatment in hematological and non-hematological cells, derived from SDS patients. In addition, we analyzed SBDS protein restoration

and, finally, we investigated also the functional effect of this drug taking advantage of clonogenic assays.

3.2 Methods

Patients

Mononuclear cells (MNCs) and Mesenchymal Stromal Cells (MSCs) were obtained from fresh peripheral blood and bone marrow samples derived from diagnostic specimens collected from Shwachman-Diamond Syndrome patients. All patients included in this study were diagnosed as SDS based on clinical criteria and carried the 183-184TA>CT codon stop mutation, screened at time of diagnosis. Table 3.1 shows the characteristics of all SDS patients enrolled in this study. Informed, written consent was obtained in all cases.

LCLs stimulation

LCLs were obtained from five patients carrying the 183-184TA>CT codon stop mutation as previously described[1]. For our experiments, $2*10^6$ LCLs were incubated in RPMI-1640 (Gibco) medium containing 0.5% FBS (Lonza) for 24 hours, in order to synchronize cell growth. Cells were subsequently incubated in the absence or in the presence of Ataluren 5-10 μM in RPMI-1640 supplemented with 10% FBS. Cells were then lysed to obtain proteins and perform western blot analyses.

Isolation of Mononuclear cells (MNCs), stimulation and colony assay

MNCs were isolated from BM aspirates using Ficoll-Paque PLUS (GE Healthcare, Waukesha, WI, USA). For stimulation experiments, $2*10^6$ per condition were seeded in a 48-well plate in RPMI-Advanced (Life Technologies) supplemented with 10% FBS (Biosera, Ringmer, UK), 1% of L-glutamine (Euroclone) and 1% of penicillin and streptomycin (Euroclone) and cultured at 37° C with 5% CO_2 for 72 hours. 2% Phytohemagglutinin (PHA, IrvineScientific) was added to stimulate proliferation and Ataluren 5 µM was added as well at seeding time. At the end of experiment, both adherent and non-adherent cells were collected and protein extracted. For colony assay, MNCs were cultured in 6-well plates at a density of 10^5/mL in methylcellulose medium (Methocult H4230, Stem Cell, Vancouver, BC, Canada) in the presence of 10% conditioned medium from the 5637 tumor cell line and 2 U/mL erythropoietin. At seeding time, Ataluren was added or not (untreated condition) in the culturing medium at different concentrations (as indicated). After 14-21-28 days of incubation spontaneous growth of CFU-GM and BFU-E has been determined.

Isolation and culture of Mesenchymal Stromal Cells

After isolation, MNCs were seeded at 160000 cells per cm^2 in Dulbecco's Modified Eagle Medium low glucose (Lonza, Basel, Switzerland) supplemented with 10% of fetal bovine serum (Biosera), 1% of L-glutamine and 1% of penicillin and streptomycin at 37° C with 5% CO_2. After 24h, the culture medium was replaced and

non-adherent cells were removed by washing with phosphate buffered saline (PBS, Euroclone). Adherent cells were maintained with medium replacement twice a week, detached with 0.25% trypsin-EDTA (Euroclone) after reaching 70-80% confluence, and seeded at 1000 cells per cm^2. For our experiments, Mesenchymal Stromal Cells (MSCs) at p3 were seeded in a 6-well plate at 1000 cells per cm^2; once reached the sub-confluence, the culturing medium was replaced every day with fresh medium containing vehicle or Ataluren at final concentration of 2.5 or 5 µM. Cells were harvested at different time points. MSCs cultured in medium containing vehicle (DMSO 0.5 µL/mL) were considered as negative control.

Western blot analyses

At different time points of treatment, cells (LCLs, colonies and MSCs) were trypsinized, or simply collected by centrifugation and then washed with ice-cold PBS. Pellets were lysed using ice-cold Ripa buffer (1% NP40, 0.5% Nadeoxycholate, 0.1% sodium dodecyl sulfate, 350 nM NaCl in PBS) containing fresh 0.25 mM of phenylmethanesulfonylfluoride (PMSF, Sigma) and a cocktail of protease inhibitors (Sigma). To ensure complete cells lysis, suspension was frozen and thawed and then centrifuged at 14000 g for 10 minutes at 4° C and supernatants collected and stored at -20° C. Protein concentrations were determined using Pierce BCA protein assay (Thermofisher). Ten µg of protein were loaded per lane on a 4-20% sodium dodecyl sulfate polyacrylamide gel (Biorad). Proteins

were transferred to a PolyVinylidene DiFluoride membrane 0.2 μm pore size (Biorad) for 1 hour at 50 V in Tris-glycine buffer. The membrane was then blocked for 1 hour in 10% Bovine Serum Album (BSA) in PBS-Tween at room temperature. After blocking, membrane was incubated in primary antibody anti-SBDS (Abcam) at 1:5000 in overnight at 4° C. The secondary antibody was added at 1:5000 (anti-rabbit horseradish peroxidase; Biorad) in 10% BSA/PBS-Tween. The anti-β-actin (Sigma) was added at 1:6000 in 5% non-fat dry milk/PBS-Tween and incubated for 1 hour at room temperature; anti-mouse horseradish peroxidase antibody (Sigma) was used at 1:20000 in 5% milk/PBS-Tween and incubated for 1 hour at room temperature. Membrane was washed three times (10 minutes per wash) with PBS-Tween. Immunoreactive proteins were detected using Clarity^{TM} Western ECL Substrate (Biorad). Densitometry was performed by Image Studio Lite Ver 5.2.

3.3 Results

Patient	Gender	Age at study (y)	*Sbds* MUTATIONS	
UPN1	M	38	183-184TA>CT	258+2T>C
UPN2	M	10	183-184TA>CT	258+2T>C
UPN3	F	6	183-184TA>CT	258+2T>C
UPN4	M	10	183-184TA>CT	258+2T>C
UPN5	F	14	183-184TA>CT	258+2T>C
UPN6	F	5	183-184TA>CT	258+2T>C
UPN7	M	6	183-184TA>CT	258+2T>C
UPN8	M	16	183-184TA>CT	258+2T>C
UPN9	M	2	183-184TA>CT+258+2T>C	258+2T>C

Table 3.1: Patients enrolled in this study and genetic characteristics. UPN, unique patient number; F, female; M, male.

Ataluren restores SBDS in LCLs derived from patients

In order to investigate in hematological cells the read-through capability of Ataluren, we treated LCLs obtained from five SDS patients carrying the nonsense mutation 183-184TA>CT with 5-10 µM Ataluren for 24 hours. Results indicated that 5 µM Ataluren is sufficient to strongly restore full-length SBDS protein expression compared to untreated cells, as observed by western blot analyses (Figure 3.1).

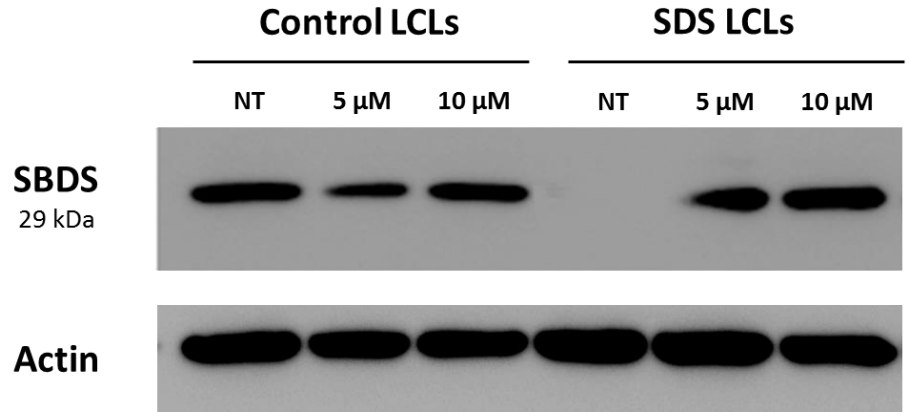


Figure 3.1: Ataluren is able to restore SBDS full length production in SDS-LCLs after 24h of treatment. Lymphoblastoid cell lines obtained from healthy donors (Control) or SDS patients carrying 183-184TA>TC mutation were treated with 5-10 µM Ataluren for 24 hours and cells lysates were analyzed by western blot. A representative experiment is shown

Ataluren restores SBDS in PHA-stimulated MNCs derived from patients

LCLs are immortalized cells obtained by infection with Epstein Barr Virus of B cells from peripheral blood. Even if they could be considered in close resemblance with lymphocytes, we decided to test Ataluren effects on primary hematopoietic cells derived from SDS patients. Mononuclear cells were isolated from bone marrow of two patients carrying the 183-184TA>TC mutation and stimulated in presence or absence of 2% PHA with Ataluren 5 µM for 72 hours. Results indicate that PHA is *per se* able to stimulate SBDS production, but in combination with Ataluren restoration of full-length SBDS production is strongly augmented (Figure 3.2).

Indeed, in UPN6 western blot analyses showed that the condition Ataluren 5 µM + PHA is able to strongly restore SBDS production, that resulted to be six times more expressed compared to control alone. In the second patient, UPN3, also Ataluren 5 µM by itself is able to restore SBDS production with a fold increase of 4, even if in combination with PHA triggers a massive full-length protein production compared to control (fold change 24). In both cases, despite PHA stimulation is able to induce a basal upregulation of the protein, the presence of Ataluren 5 µM robustly induces SBDS restoration.

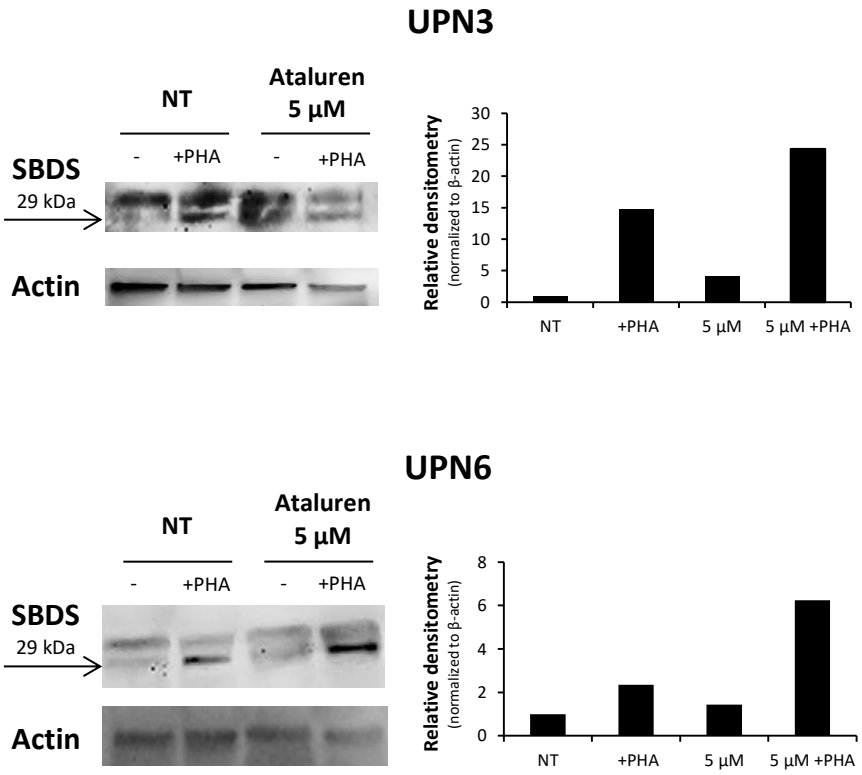
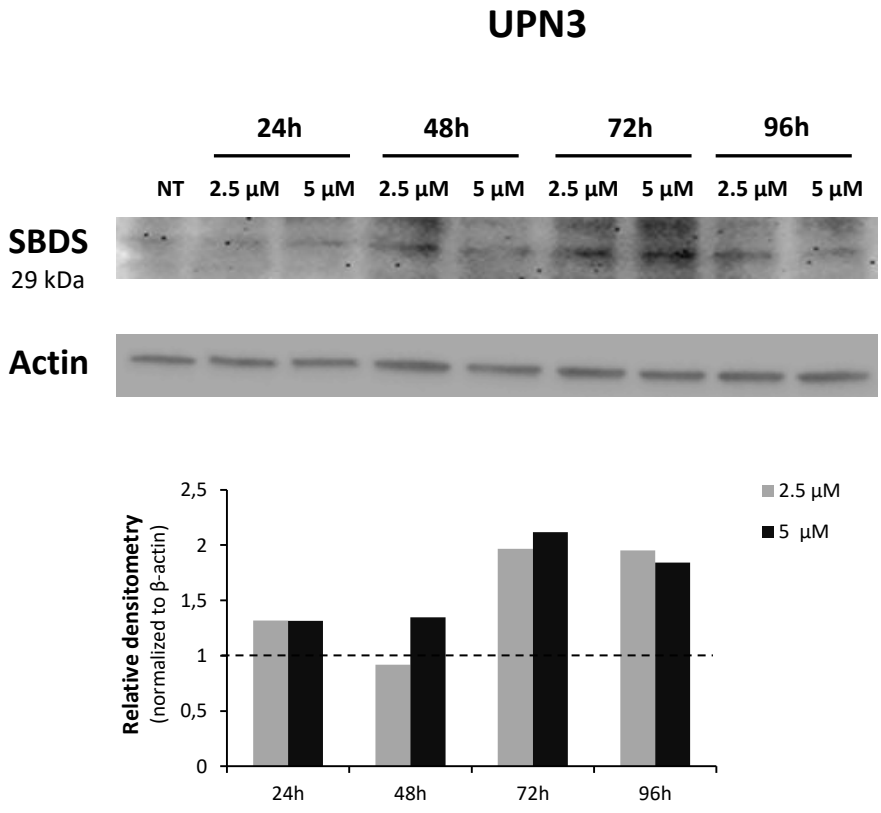


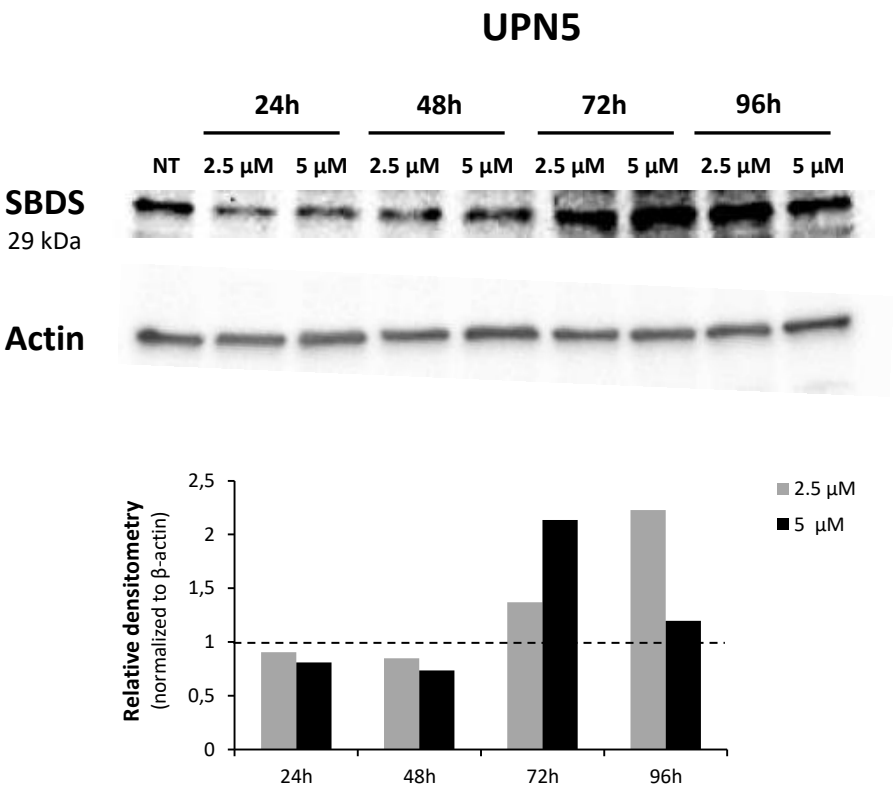
Figure 3.2: Ataluren restores SBDS production in patients MNCs. Western blot analyses of cell lysates show that Ataluren is able to strongly induce SBDS production after 72h of treatment. On right panels, densitometry analyses of bands. The untreated condition, normalized for β-actin, is considered as reference and set to 1

SBDS restoration in Mesenchymal Stromal Cells treated with Ataluren

In bone marrow compartment are present also cells that do not belong to hematologic lineage, such as Mesenchymal Stromal Cells. Since MSCs have a crucial role in supporting hematopoiesis in bone marrow, we also analyzed if Ataluren could restore SBDS protein in these cells. We treated SDS-MSCs from three different patients with 2.5 or 5 μM Ataluren and replacing it fresh every day in the culture medium. Cells were then lysed at different time points, proteins obtained and western blot analyses were performed. In all patients we found that Ataluren was able to duplicate SBDS full length production in Mesenchymal Stromal Cells. In particular, both concentrations of Ataluren exerted similar effects, at the same time points in all patients, upregulating SBDS production after 72 hours of treatment. In particular, we found that in all cases, both concentrations were ineffective in restoring SBDS production for the first 48 hours. Interestingly, the upregulation was observed after three days of treatment and was constantly maintained in the 96 hours-time point (Figure 3.3). In particular, regarding UPN3 an increase in fold change of 2 was obtained at the 72 hours time point for both Ataluren concentrations. In UPN5, the maximum level of upregulation was obtained with Ataluren 5 μM after 72 hours of treatment, when protein levels reached a fold change of 2.1 compared to control. Comparable upregulation level was obtained in the same patient at successive time point with Ataluren 2.5 μM. For

UPN6 upregulation presented a fold change of 1.8 following Ataluren 5 µM administration at 72 hours and remained stable for the next time point.




UPN5
24h
48h
72h
96h
NT
2.5 µM
5 µM
2.5 µM
5 µM
2.5 µM
5 µM
2.5 µM
5 µM
SBDS
29 kDa
Actin
Relative densitometry
(normalized to β-actin)
2,5
2
1,5
1
0,5
0
24h
48h
72h
96h
2.5 µM
5 µM

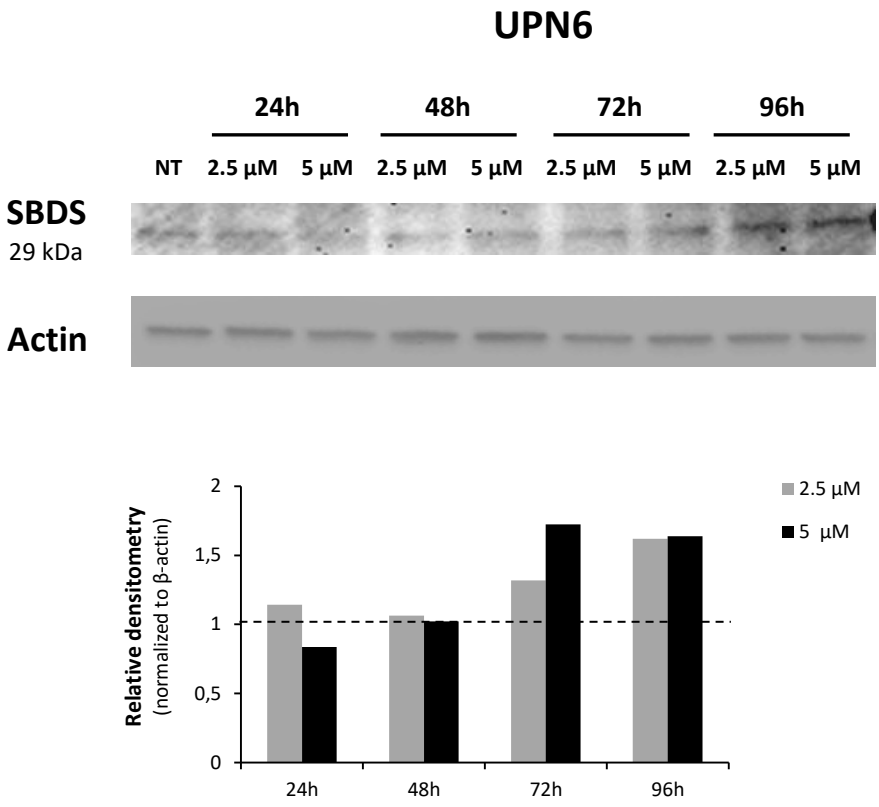


Figure 3.3: Ataluren restores SBDS production in SDS-MSCs. Western blot analyses of cell lysates from three different patients show that Ataluren is able to induce SBDS production after 72h of treatment. Densitometry analyses are shown. Red lines indicate protein expression in untreated cells and was set as 1

Analyses of functional effects of Ataluren in clonogenic assay

Once assessed the efficacy of Ataluren in restoring SBDS protein in different cell lineages, we analyzed the functional activity of restored SBDS. We treated with Ataluren MNCs derived from bone marrow aspirates and studied their clonogenic potential. SDS patients present always a reduced number of colonies, since lack of SBDS expression prejudices myeloid commitment in hematopoietic progenitors. We hypothesized that restoration of SBDS in hematopoietic precursors could improve their clonogenic ability. We started to test Ataluren in one patient carrying 183-184TA>CT mutation, performing clonogenic assay using the drug at 5 and 10 μM and evaluating colony formation at day 14 and 28 (Figure 3.4). At day 14 a toxic effect of 10 μM Ataluren was evident: the number of CFU-GM in absence of drug were 11, completely comparable to Ataluren 5 μM (10), while at the higher concentration colonies were less than half of control (4). Surprisingly we noted a strong effect on number of colonies formed with Ataluren 5 μM at day 28: in absence of drug CFU-GM were 15 while in presence of 5 μM Ataluren this patient showed an increase in CFU-GM, which doubled to 28. On the other hand, 10 μM Ataluren continued to show its toxic effect (4 CFU-GM). Interestingly, only CFU-GM were modulated by the drug, while no effect was observed on BFU-E colonies (data not shown).

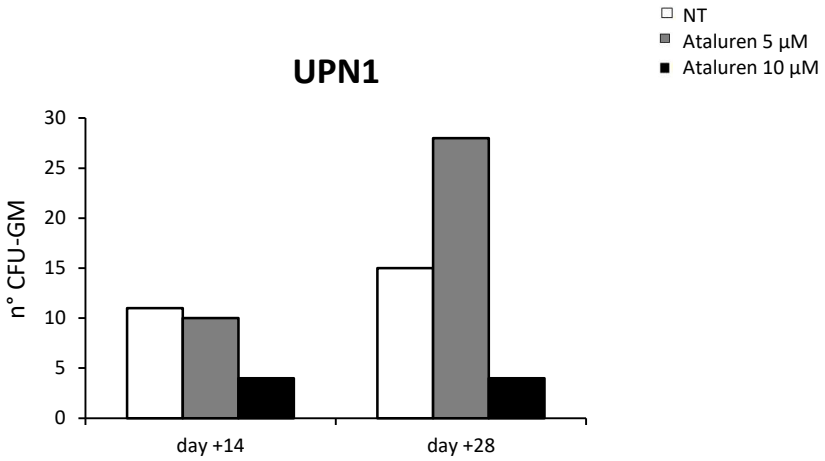


Figure 3.4: Ataluren can induce higher growth rate in CFU-GM colonies. For the first experiment, number of colonies was evaluated at day 14 and 28. Data, expressed as number of CFU-GM, indicate that in UPN1 5 µM Ataluren is able to duplicate number of colonies after 28 days of treatment, while 10 µM Ataluren results to be toxic at both time points

In following experiments, we decided to add a further intermediate monitoring time point (21 days) and lower Ataluren dose, testing 2.5 and 5 µM. We obtained MNCs from eight SDS-patients, and performed clonogenic assays with these new conditions. Data showed a significantly higher spontaneous growth rate of CFU-GM in three treated samples (37.5%) and none effect in five patients. Consistently, we observed that only CFU-GM were modulated by the drug, while BFU-E did not show any alterations in their number (data not shown). In patients in which drug exerted its effect, the augmented colony formation was striking, doubling

number of CFU-GM compared to untreated condition. In particular, at day 14 Ataluren showed already positive results on CFU-GM counts in all three patients. In detail, in UPN3 number of colonies in untreated condition was 8, while doubled in presence of the drug (16 and 15 for 2.5 and 5 μM respectively). At the next time points colonies growth augmented in treated conditions, reaching 18 for Ataluren 2.5 μM and 20 for Ataluren 5 μM, while no variation were observed in untreated condition (Figure 3.5 panel A).

Both UPN7 and UPN8 showed a striking effect in presence of 2.5 μM Ataluren. In particular, for UPN7 basal number of CFU-GM at day 14 was 50, while in presence of Ataluren colonies were 82 and 40 for 2.5 μM and 5 μM condition, respectively. Number of colonies counted had a tendency to diminish at both successive time points in all conditions, while the remarkable effect of Ataluren 2.5 μM remained evident (Figure 3.5 panel B).

In accordance with previous results, in UPN8 we observed a strong augment of CFU-GM with 2.5 μM compared to untreated condition, but Ataluren showed a quite toxic effect at 5 μM. In fact, at day 14 basal colony count was 6, while was more than doubled in presence of Ataluren 2.5 μM (15 CFU-GM); this ratio was maintained for the other two time points, with little variation in numbers: at day 21 we counted 6 colonies in untreated condition, 13 for 2.5 μM and 2 for 5 μM, while at day 28 CFU-GM were the same for 5 μM and 8 or 14 for basal and 2.5 μM condition respectively (Figure 3.5 panel C).

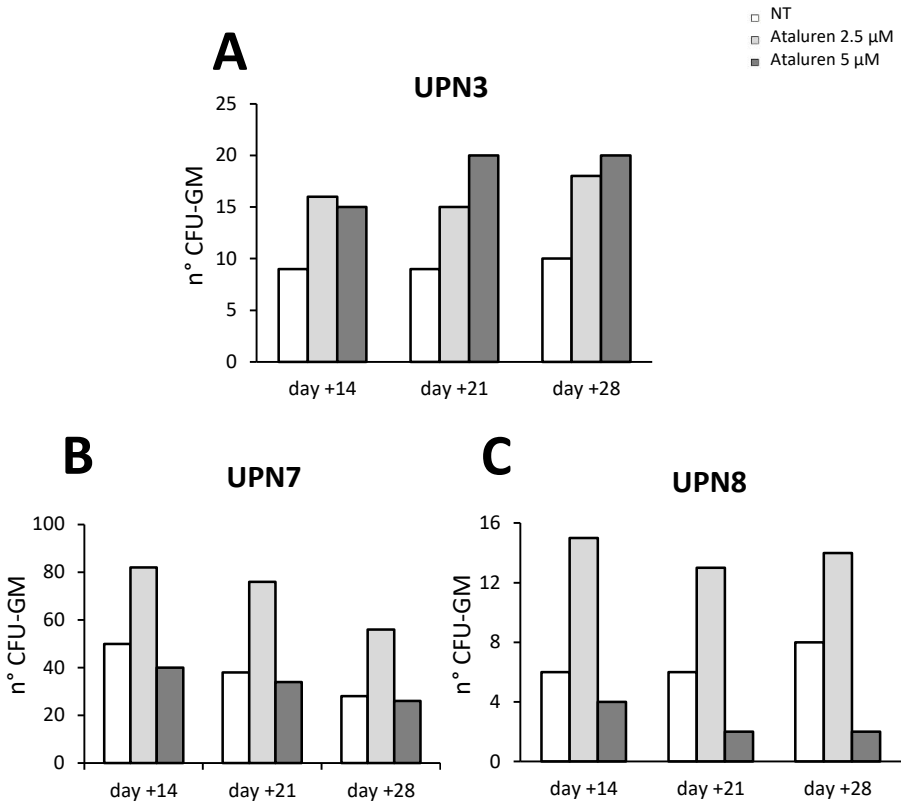


Figure 3.5: Ataluren can induce higher growth rate in CFU-GM colonies in some SDS patients. The seeding medium was supplemented at time zero with 2.5 and 5 µM of Ataluren or not (NT) and number of colonies was evaluated at different time points. Data, expressed as number of CFU-GM, indicate that Ataluren was able to duplicate number of colonies in three responding patients

Concerning the other five patients tested, we did not observed any significant effect of Ataluren on CFU-GM growth at any experimental condition or time point evaluated (Figure 3.6).

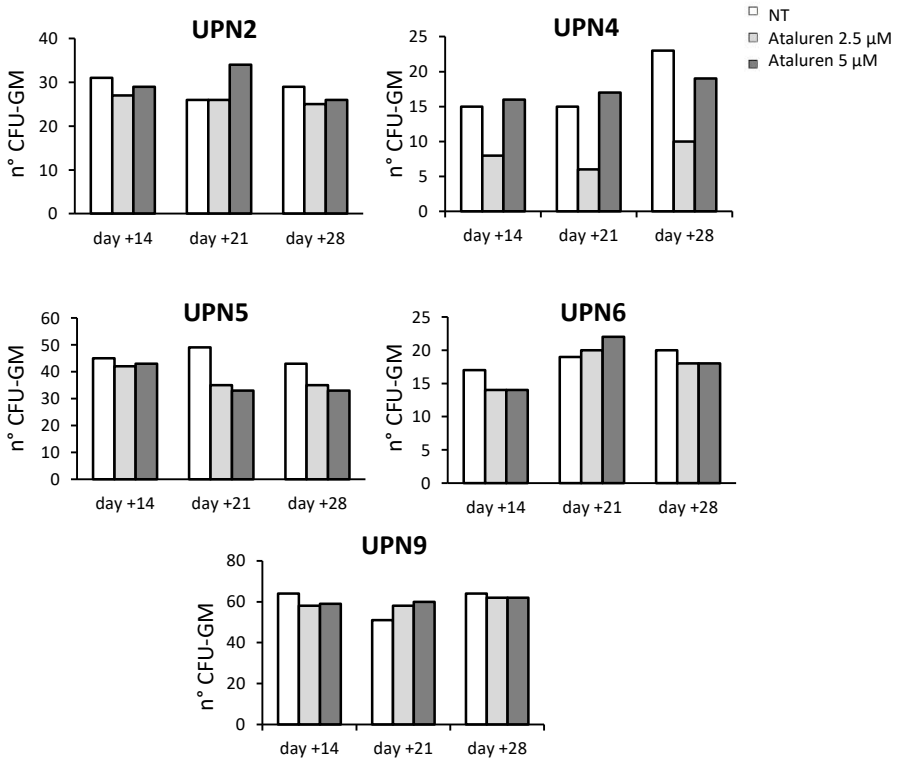
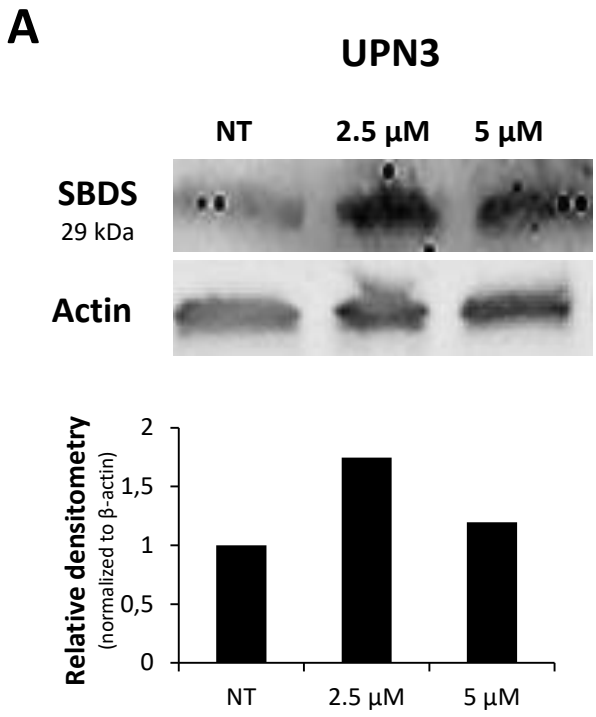


Figure 3.6: Controversial effect of Ataluren on CFU-GM growth. Data indicate that Ataluren in five patients did not improve colonies growth

Analyses of SBDS restoration after clonogenic assay

In addition, in order to investigate if the increase of colony number was effectively correlated with restoration in SBDS protein, at day 28 we analyzed protein lysates from colonies treated or not with the drug in five patients and then performed western blot analyses. We observed an upregulation of SBDS levels in colonies treated with Ataluren; in particular, UPN3 showed upregulated SBDS with Ataluren 2.5 μM, while levels of SBDS were comparable to control with Ataluren 5 μM (Figure 3.7, panel A). UPN7 was the patient who showed the strongest protein increase with both concentrations. Indeed, fold increase of the protein was greater than two either with Ataluren 2.5 μM and 5 μM compared to control (Figure 3.7, panel B). Similar to UPN3, UPN8 showed a slightly SBDS upregulation in presence of Ataluren 2.5 μM, whereas no augment was pointed out with the highest dose of drug (Figure 3.7, panel C).


A
UPN3
NT
2.5 µM
5 µM
SBDS
29 kDa
Actin
Relative densitometry
(normalized to β-actin)
2
1,5
1
0,5
0
NT
2.5 µM
5 µM
B
UPN7
NT
2.5 µM
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SBDS
29 kDa
Actin
Relative densitometry
(normalized to β-actin)
3
2
1
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NT
2.5 µM
5 µM

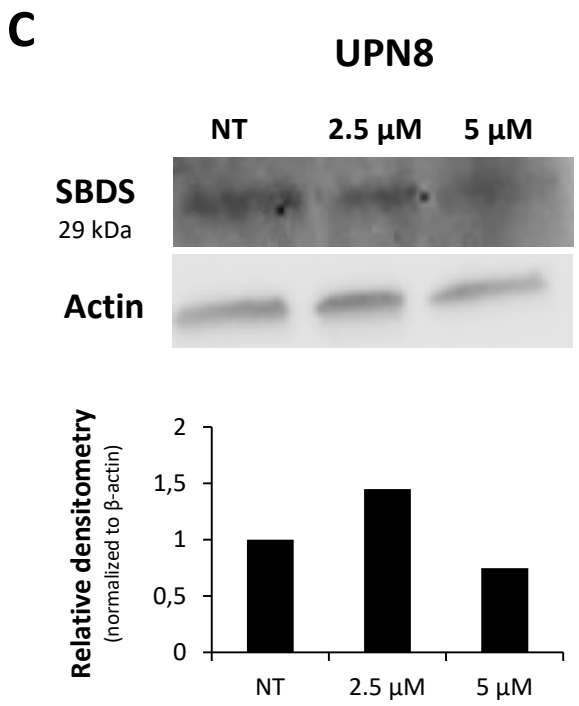

Figure 3.7: Western blot analyses on colonies lysates from three responding patients at day 28. Ataluren is able to restore in a variable manner the production of full-length protein. For each patient, densitometry analyses are shown in the lower graphs

Furthermore, we investigated protein restoration also in two other patients (UPN5 and UPN6) that did not show functional improvement in colony assay. Surprisingly, after Ataluren treatment, we found that also in these cases the drug was able to strongly restore SBDS production, even if this augment did not correlated with functional effect on colony number. In particular, both patients presented a restoration of protein production that was dose dependent, with a greater fold increase for Ataluren 5 µM (Figure 3.8).

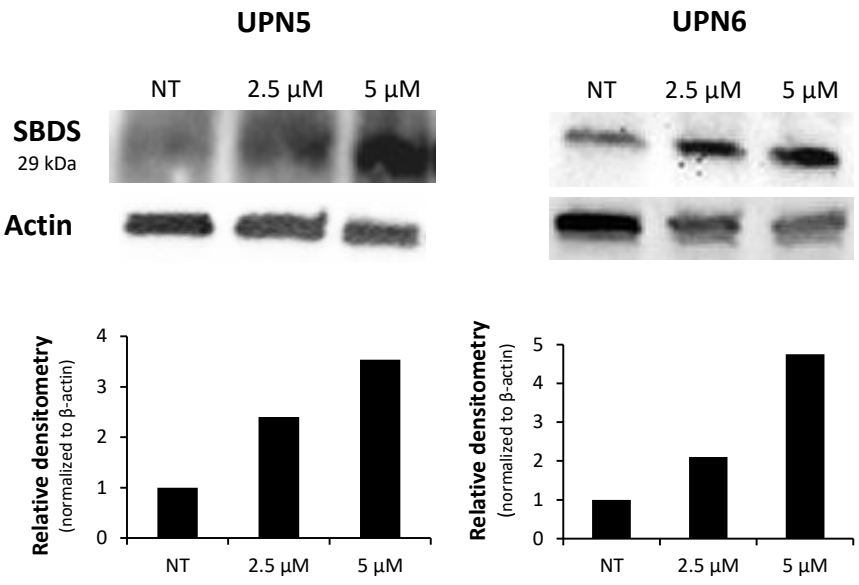

Figure 3.8: Western blot analyses on colonies lysates at day 28 on UPN5 and UPN6. Ataluren is able to restore in both cases the production of full-length protein, even if the functional effect (enhanced number of colonies) is lacking

3.4 Discussion

Shwachman-Diamond Syndrome is characterized by bone marrow failure, associated with bone marrow aplasia and severe neutropenia, also in young patients. Furthermore risk of developing leukemia or myelodysplastic syndrome is augmented in these patients, and in the Italian registry about 20% of SDS patients carrying 183-184TA>TC mutation on *Sbds* gene develops MDS or leukemia. The dinucleotide alteration 183-184TA>TC is a nonsense mutation which introduces a premature termination codon (PTC) resulting in the production of a truncated and no functional protein. Small molecules such as Ataluren are able to promote readthrough of premature codon stop and lead to translation of full length proteins and this drug is currently approved as therapy for codon stop diseases, such as Duchenne Muscular Dystrophy.

In this study we investigated the read-through capability of Ataluren in different cell types derived from SDS patients. Firstly, we found that Ataluren was able to rescue SBDS production *in vitro*, using LCLs derived from SDS patients carrying the nonsense mutation 183-184TA>TC. Cells treated for 24 hours with 5-10 μM Ataluren showed a restoration of full length SBDS production already at the lower dose of drug, as observed by western blot analyses.

Furthermore, we tested Ataluren on primary hematological cells deriving from patients, and in particular on mononuclear cells from bone marrow aspirates. MNCs derived from two SDS patients showed an increase in SBDS protein after 72 hours of treatment with Ataluren 5 μM. When in culture medium was added Phytohemagglutinin, the compound was *per se* able to augment SBDS production. PHA is a well-known mitogen activator and T-lymphocytes stimulator. Due to the fact that SBDS is involved in cell division, promoting mitotic spindle stability and chromosome segregation[13], it is feasible that stimulating cell proliferation could intrinsically represent the reason causing SBDS upregulation. Nevertheless, adding Ataluren 5 μM was a further catalyst to enhance SBDS full-length production and in both cases the ratios between the two conditions (PHA Alone or supplemented with Ataluren) remained significantly different in favor of the drug.

Since stromal population in bone marrow is a known regulator of hematopoiesis and can support or drive leukemic transformation[14], we analyzed the effect of Ataluren on Mesenchymal Stromal Cells derived from SDS patients. We treated MSCs deriving from three different patients with two drug concentrations and obtained cell lysates at different time points, to subsequently perform western blot analyses. We found that also in stromal compartment Ataluren is able to restore full-length protein production, at both concentration tested (2.5 and 5 μM). The upregulation seen in western blot is evident in all cases after 72 hours of treatment and not in the first

24-48 hours, probably due to a different metabolism and growth rate of MSCs compared to LCLs and MNCs. In fact, Ataluren acts during protein synthesis, a process that is enhanced during cell replication. Once obtained the increase of the protein observed at 72h, it was constantly maintained also in the 96h time point.

Colony assay is based on the capability of hematopoietic progenitors to proliferate and differentiate into GM-FU and BFU-E colonies in a semi-solid (methylcellulose) medium. Thanks to this assay, we can obtain information regarding the functional activation of stem cells, in particular following drug treatments.

Controversial results came from functional assay performed on MNCs from bone marrow aspirates. In fact, clonogenic assays performed on eight different SDS patients showed a different impact of Ataluren on these cells. First of all, we found in UPN1 that Ataluren was toxic at the 10 µM dose. This toxicity was confirmed also in other two patients (data not shown). Thus we lowered drug concentration and observed that Ataluren had different effects at the administered doses. In three patients (UPN3-UPN7-UPN8) 2.5 µM Ataluren had a strong effect on CFU-GM production, inducing an augment in colony formation, but two of them showed also a toxic effect at 5 µM dose. Interestingly, UPN3, who did not show any toxic effect at 5 µM, was the same patient able to strongly upregulate at the same concentration SBDS production in MNCs after 72 hours both in presence of PHA (increase of 25 times compared to control) or in absence (fold change of 4). In five other patients, Ataluren did not

show any effect, except for one patient (UPN4) in which had apparently a toxic effect at 2.5 µM. Given the fact that in this patient 5 µM concentration did not show toxic effect, this reduction in CFU-GM is probably due to a technical error, rather than a drug effect. Furthermore, in all tested patients no effects on BFU-E colony formation were observed. Sen *et al.* demonstrated that SBDS-deficient K562 cells retained the ability to enter into a differentiation program. In fact, although patients enrolled in their study showed reduced erythropoietic cells because of hypoplastic bone marrows, the percentage of erythroid marrow population was in the normal range[15]. It is likely that at this early differentiation stages SBDS lack do not affect the ability of precursor cells to form BFU-E colonies but defects may emerge in subsequent phases. Furthermore, Thada *et al.* reported variable efficiency of read-through therapy with Ataluren in different tissues, depending on tissue-specific variations in nonsense mutant transcript levels[16]. Moreover, also the different effects exerted by Ataluren in patients are congruent with data present in literature: phase II studies in Duchenne Muscular Dystrophy reported an increase in dystrophin expression in 61% of patients[17], while a varying response from 40 to 70% was reported in phase II prospective trial for cystic fibrosis[18]. Despite these contradictory functional results, we found in colonies lysates from responding or non-responding patients that in both cases, SBDS production was upregulated by Ataluren, indicating probably that the merely restoration of full-length protein production is not sufficient to exert also functional effects of colony formation.

Furthermore, the capability of Ataluren to read-through PTCs leads to insertion of a random amino acid to bypass the incorrect stop codon signal. Thus, the resulting full-length protein may be fully or partly functional, depending on the amino acid substitution for the stop codon. It is feasible that insertion of an amino acid with polarity different from the correct one (Lysine) could change the whole tertiary conformation of SBDS protein, causing a final lack of function due to incorrect protein folding. This single amino acid change cannot be revealed by western blot analyses, but on the contrary can lead to inefficacy and functional consequences shown in functional assays such as clonogenic one. Nonetheless, Thada and colleagues postulated that, at least in their mouse model, restore enzyme activity to only 4-5% of the normal levels can result in significant therapeutic benefits[16].

Since no specific therapies are currently available for SDS patients, the aim of our work was to test the efficacy of a new promising drug, Ataluren, in correcting protein expression and function in SDS patients carrying one of the most common mutation. In this study we tested for the first time, of our knowledge, the use of Ataluren in different cells types derived from Shwachman Diamond Syndrome patients carrying 183-184TA>TC mutation. We demonstrated the efficacy of this drug in restoring SBDS full-length production in all treated cells. Thanks to the fact that Ataluren has shown effects on different bone marrow cells, restoring SBDS production, and that had no toxic effect on these lineages, it could be considered feasible to

propone this drug for treatment of SDS patients. In fact, restoring SBDS production in bone marrow and stromal cells could ameliorate hematological phenotype and all linked effects (such as predisposition to infections) and even reduce risk of myelodysplastic transformation.

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CHAPTER FOUR

Summary, conclusions and future directions

Shwachman-Diamond Syndrome is an interesting human disease characterized by multiple manifestations and affecting different organs. Hematological defects, exocrine pancreatic insufficiency, bone marrow hypoplasia, skeletal abnormalities are all features that can characterize the phenotype of a SDS patient. Predisposition to develop leukemia and myelodysplastic syndrome in these patients seems to be one of the most serious complications of the pathology.

In the first part of our work we focused our attention on the role of SDS-MSCs in the bone marrow niche. It is now ascertained that interactions between MSCs and hematopoietic cells in marrow occur and that MSCs can favor cancer evolution and progression. In fact, in recent years, the paradigm that stromal components are just bystanders in the oncogenic process has changed. Microenvironment is thought to be a dynamic entity promoting cancer evolution, giving support and advantages to leukemic cells. SDS-MSCs turned out to be unable to recreate an appropriate *in vivo* niche, after transplantation of semi-cartilaginous pellets in a mouse model. Despite their *in vitro* resemblance to HD-MSCs, compared to healthy donors, SDS-MSCs displayed an impaired ability to form a complete ossicle that resembled the correct bone marrow architecture, defecting in osteocytes differentiation and vascularization. For the future we will better characterize by molecular and histological analyses which could be the critic step or molecules that can drive this *in vivo* failure. The complete elucidation of the all molecular characteristics and passages that undergo SDS-MSCs in this model may reveal crucial

defects in this lineage that an *in vitro* study cannot unravel. Discover new altered pathways in SDS-MSCs could help to better comprehend the biological bases of hematological defects in SDS patients and may also identify new therapeutic targets to prevent leukemic evolution.

The second part of our study was focused on testing a specific drug, Ataluren, able to act on nonsense stop codon mutation, one of the most diffuse alterations in SDS patients, linked to risk of developing myelodysplastic syndrome. Nowadays no therapy is available for SDS patients to cure their hematological defects, except bone marrow transplantation, or other symptoms. Partially restoring the production of SBDS protein could represent an intriguing strategy to treat SDS patients. In this work we successfully obtained restoration of SBDS protein in different cell lineages deriving from patients. Both hematological and non-hematological cells showed an increase in protein levels when treated with Ataluren at different concentrations. Protein restoration was also accompanied in some cases with an improvement of functionality. In particular, bone marrow mononuclear cells showed an augment in their ability to form colonies when cultured in a specific assay. This represents a powerful result, due to the potential clinical consequences related to possible therapeutic strategy. Indeed, given the fact that no toxic effects were observed and that Ataluren is currently under investigation in different clinical studies for other pathologies, SDS patients in future could take advantage of this drug to ameliorate their hematological defects and abolish other symptoms. For future

studies we intend to increase our patients cohort and better characterize Ataluren effects on different cells. Furthermore it will be interesting to investigate also in our *in vivo* model the effect of this drug. After treating with Ataluren SDS Mesenchymal Stromal Cells, we will generate implantable pellets and verify if, at the end of experimental time, SBDS restoration could have an effect on *in vivo* ossicles formation, giving advantages also to bone marrow niche and microenvironment. This could be a further step to strengthen the positive evidences regarding Ataluren effects on SDS patients and make this drug eligible for a therapy of this disease.

In this work we analyzed different key aspects of Shwachman-Diamond syndrome, by using different *in vitro* and *in vivo* approaches. Future studies are needed to further characterize our preliminary results and, hopefully, provide new insights into the pathogenesis and the treatment of this rare disease.

Other publications

The Chemerin/ChemR23 Axis Plays a Pivotal Role in the Pathogenesis of Intestinal Graft-versus-Host Disease

Paola Vinci[1], Donatella Bardelli[1], Erica Dander[1], Camilla Recordati[2], Valeria Fumagalli[1], Annalisa Del Prete[3], Claudia Cappuzzello[1], Andrea Biondi[1,4], Silvano Sozzani[3], Giovanna D'Amico[1]

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Manuscript in preparation

Autophagy and Glutamine Synthetase Induction Sustain ALL Blast Resistance to L-Asparaginase in Human Bone Marrow Mesenchymal Stromal Cells

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Manuscript in preparation