Mechanisms of Erythropoietic Failure in Shwachman Diamond Syndrome Caused by Loss of the Ribosome-Related Protein, SBDS

by

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Institute of Medical Sciences
University of Toronto

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Abstract

Anemia occurs in 60% of patients with Shwachman Diamond Syndrome (SDS). Although bi-allelic mutations in SBDS cause SDS, it is unclear whether SBDS is critical for erythropoiesis and what the pathogenesis of anemia is in SDS. I hypothesize that SBDS protects early erythroid progenitors from p53 family member mediated apoptosis by promoting ribosome biosynthesis and translation. SBDS deficiency by vector-based shRNA led to impaired cell expansion of differentiating K562 cells due to accelerated apoptosis and reduced proliferation. Furthermore, the cells showed general reduction of 40S, 60S, 80S ribosomal subunits, loss of polysomes and impaired global translation during differentiation. An upregulation of the pro-apoptotic p53 family member, TAp73, was found in resting SBDS deficient cells; however, not in differentiating cells. These results demonstrate SBDS plays a critical role in erythroid expansion by promoting survival of early erythroid progenitors and in maintaining ribosome biogenesis during erythroid maturation independently of p53 family members.

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List of Abbreviations

AML	Acute myelogenous leukemia
AA	Ascorbic Acid
BFU-E	Burst forming unit – erythroid
BM	Bone marrow
CB	Cord blood
CFU-E	Colony forming unit – erythroid
CHH	Cartilage hair hypoplasia
CIMFR	Canadian Inherited Marrow Failure Registry
CLP	Common lymphoid progenitor
CML	Chronic myelogenous leukemia
CMP	Common myeloid progenitor
c-PARP	Cleaved Poly-ADP Ribose Polymerase
DBA	Diamond Blackfan Anemia
DC	Dyskeratosis Congenita
dsRNA	Double stranded RNA
EPO	Erythropoietin
EPO-R	Erythropoietin receptor
FA	Fanconi Anemia
FBS	Fetal bovine serum
G-CSF	Granulocyte-colony stimulating factor
HSC	Hematopoietic stem cell
HSC/Ps	Hematopoietic stem cell and progenitors
HSCT	Hematopoietic stem cell transplantation
IL-3	Interleukin-3
IMDM	Iscoe's Modified Dulbecco's Medium
IMFS	Inherited marrow failure syndrome
i(7q)	Isochromosome 7
MCV	Mean corpuscular volume
MDS	Myelodysplastic syndrome
MPP	Multipotent progenitor

NAC	N-acetylcysteine
ORF	Open reading frame
RBC	Red blood cell
RNAi	RNA interference
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription and quantitative real time polymerase chain reaction
SBDS	Shwachman Bodian Diamond syndrome gene
SBDSP	Pseudogene of SBDS
SCF	Stem cell factor
SDS	Shwachman Diamond Syndrome
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
TPO	Thrombopoietin

CHAPTER I

GENERAL INTRODUCTION

1.1 Shwachman Diamond Syndrome

Shwachman Diamond Syndrome (SDS, OMIM 260400) is an inherited marrow failure syndrome (IMFS), first described by two reports in 1964.^{1,2} The autosomal recessive disorder is characterized most commonly by bone marrow dysfunction, exocrine pancreatic insufficiency and an increased risk of developing myelodysplasia, particularly acute myelogenous leukemia (AML).³⁻⁵ Patients show a broad range of additional clinical features including skeletal abnormalities, immune dysfunction, oral disease, renal complications, liver disease, insulin-dependant diabetes mellitus, and cognitive impairment.⁶⁻¹¹ The syndrome has an estimated incidence of 1 in 77, 000¹² and has no gender or ethnic predilection.^{1,8,13-15} Data from the Canadian Inherited Marrow Failure Registry (CIMFR) show that SDS is the third leading inherited marrow failure syndrome, next to Diamond-Blackfan Anemia, and Fanconi Anemia¹⁶ and typically presents either during infancy or early childhood.^{16,17} The estimated median survival of SDS patients is more than 35 years.¹⁸ Since its first description, no unifying pathogenic mechanism has been shown to be responsible for SDS. Significant advancements, however, have been made in the last several years regarding the genetic basis of this disorder. In 2003, a landmark study by Boocock et al. showed that approximately 90% of patients have hypomorphic mutations in the *Shwachman Bodian Diamond syndrome* gene, *SBDS*.¹⁴ Further exploration has revealed that SBDS may have diverse functions in ribosome biogenesis¹⁹⁻²² and other non-ribosomal processes.²³⁻²⁶ As such, SDS is increasingly characterized as a ribosome biogenesis disorder.²⁷ However, how loss of SBDS affects ribosomes and leads to the heterogenous clinical features of SDS is unknown.

1.1.1 Hematological abnormalities

Bone marrow failure in SDS affects one or more of the myeloid cell lineages leading to the suppressed production of erythrocytes, megakaryocytes and neutrophils. Anemia is a common cytopenia in SDS, as defined by a hemoglobin concentration of 2 standard deviations below the median for a healthy population of the same age and sex. A seminal study on the hematological component of SDS analyzing 21 cases, reported normochromic and normocytic anemia in a majority of patients (66%), elevated fetal hemoglobin and reduced reticulocyte counts in 75% of patients.²⁸ A recent analysis of 31 cases of SDS who were registered on the CIMFR as of September 2007 reported that 90% of the SDS patients have erythropoietic defects; reduced marrow erythroid precursors, elevated fetal hemoglobin and high red blood cell (RBC) mean corpuscular volume (MCV)¹⁶ resulting in anemia in more than 60% of the patients.¹⁶ The increased fetal hemoglobin may be an indication for ineffective erythropoiesis and is also associated with a high apoptosis rate of primitive erythroid progenitors in myelodysplastic syndrome (MDS).²⁹ To date, the role of SBDS in erythropoiesis and how it contributes to the underlying mechanism of anemia in SDS has never been investigated.

Neutrophils are another myeloid lineage known to be affected in SDS. Neutropenia, as defined by $<1.50 \times 10^9$ cells/L, is found to occur in 87-100% of patients.^{16,30} Intermittent neutropenia occurs more commonly than persistent neutropenia.^{30,31} As neutrophils play an important role in the host defense against pathogens, there is an increased risk of patients developing severe and recurrent infections. Mortality in these cases is a concern as some patients have died from pneumonia, septicemia and respiratory infection.^{28,32} Studies on SDS patient neutrophils show impaired mobility, migration and chemotaxis.³³ Previous studies have suggested the chemotactic defect to be associated with altered function of the cytoskeleton in SDS neutrophils.³⁴ Recent

investigation showed that SDS neutrophils have delayed F-actin polymerization upon chemoattractant induction and reported SBDS co-localizing with F-actin in human neutrophils.³⁵ A postulated role for SBDS in neutrophil chemotaxis has been suggested; however, there is little yet known about how SBDS contributes to normal neutrophil development. Knockdown of *SBDS* in the murine myeloid 32Dcl3 cell line showed normal neutrophil maturation, but reduced survival of granulocyte precursor cells,³⁶ suggesting a pro-survival function for SBDS in immature cells of the neutrophil lineage.

Although usually mild, thrombocytopenia, as defined by a platelet count $<150 \times 10^9/L$ has been reported in 30-70% of SDS patients and can lead to fatal bleeding.^{8,16,37,38} Pancytopenia has also been reported in approximately 10-65% of patients, with some developing severe aplastic anemia.³⁹⁻⁴¹ As such, an innate stem cell defect has been suggested, supported by the findings that SDS patients show reduced numbers of CD34+ hematopoietic stem cells, resulting in decreased production of various hematopoietic colonies *in vitro*.³⁷ Although the myeloid cell lineage abnormalities are more prominent, B cell, T cell and natural killer cells also show either reduced frequency or abnormal function.⁹ Variable bone marrow findings have also been reported including hypocellular, normocellular and hypercellular marrow which do not correlate with the severity of the cytopenias.⁴²

1.1.2 Non-hematological abnormalities

In addition to bone marrow dysfunction, SDS is also characterized by exocrine pancreatic insufficiency. The pathophysiology of the pancreatic defect in Shwachman syndrome is believed to be due to the replacement of pancreatic acinar cells with fatty tissue, as shown by imaging studies on the pancreas.⁴³ Patients exhibit impaired enzyme output with low serum trypsinogen

and low serum isoamylase levels.³⁸ The pancreatic deficiencies lead to malabsorption, steatorrhea, failure to thrive and low levels of fat-soluble vitamins A, D, E, and K. Exocrine pancreatic insufficiency is most significant between birth and two years of age, but spontaneously improves in about 50% of patients, yet it is unclear why this occurs.^{30,32}

Short stature and skeletal abnormalities are observed in SDS patients, but their severities vary with age. Burke et al. was the first to report the association of metaphyseal chondrodysplasia with SDS.⁶ Further investigation has reported abnormal development of SDS growth plates and metaphyses, rib cage and digit abnormalities, progressive spinal deformities and pathological fractures.^{44,45} SDS has also been associated with low turnover osteoporosis.⁴⁶ Less common clinical features in SDS patients include tooth enamel defects, renal tubular dysfunction, underlying liver abnormalities and developmental delay.^{8,31}

Multilineage cytopenias, hypoplasia of pancreatic acinar tissue, and short stature are the predominant clinical manifestations of SDS; however, how loss of SBDS contributes to this phenotype is unknown. The overall reduced growth and development phenotype may be explained by a defect in protein biosynthesis, similar to the dominant 'Minute' mutant phenotypes observed in *Drosophila melanogaster*. The *Minute* loci encode ribosomal proteins and also ribosomal components;⁴⁷ thus mutants result in prolonged development, low fertility and viability, reduced body size and abnormally short bristles.⁴⁸ As SBDS may encode a postulated ribosomal associated protein, it is plausible that loss of SBDS leads to translational insufficiency; the consequences of which are more prominent in tissues requiring a higher demand of protein synthesis. For example, highly proliferative erythroid progenitors may be particularly sensitive to the effects of diminished ribosome production as they require increased hemoglobin synthesis

needed for normal red blood cell function. Thus, specific cell types that are unable to upregulate ribosome biogenesis at critical stages of development may show particular sensitivity and lead to the clinical features observed in SDS.

The clinical heterogeneity of SDS is further compounded as SDS patients have an increased risk for myeloid transformation into MDS and acute myeloid leukaemia (AML), estimated as high as 36% by 30 years of age.⁴⁹ A previous literature search revealed among 54 SDS cases diagnosed with MDS/AML, 37 cases with clonal marrow cytogenetic abnormalities at a median age of 8 years (range, 2–42 years).⁵⁰ The most common of these in SDS particularly involve chromosome 7 (i(7q) and monosomy 7) and del(20)(q12). The clinical significance of many clonal abnormalities is unclear, because isolated i(7q) is not associated with a risk of progression and can sometimes regress spontaneously, whereas 42% of patients with additional or other abnormalities in chromosome 7 have progression to advanced MDS or AML.^{38,42,50}

Inherited marrow failure syndromes that are also thought to be ribosome disorders include SDS, Diamond Blackfan anemia, Dyskeratosis congenita and Cartilage-hair hypoplasia (See Table 1) are all characterized by cancer predisposition.²⁷ It remains unclear how the ribosomal defects directly contribute to increased cancer risk; however, there is an association between ribosome production and neoplastic transformation.⁵¹ Several tumor suppressors and proto-oncogenes directly regulate either ribosome production or the initiation of protein synthesis, including p53 and nucleophosmin which regulate RNA polymerase I and III activity and ribosome biogenesis respectively.⁵¹ Additionally, studies with zebrafish show that mutations in genes encoding ribosome proteins predispose the mutants to tumorigenesis.⁵² Moreover, deficiency of RPS14 was found in myelodysplastic patients with 5q- syndrome further connecting ribosomal

deficiency and cancer predisposition.⁵³ Taken together, mutations in SBDS likely predispose patients to cancer by altering ribosome biogenesis and/or translation.

Table 1: Genetic disorders linked to cancer predisposition and defects in ribosomal biogenesis

Disease	Gene Defect	Functional Role	Clinical Features
Shwachman Diamond syndrome	<i>SBDS</i>	Ribosome related protein with unknown molecular function	Bone marrow failure, pancreatic insufficiency, short stature, cancer predisposition
Dyskeratosis congenita	<i>DKC1, TERC, TERT, NOP10, NHP2, TINF2</i>	Dyskerin; A pseudouridyl synthase involved in rRNA modification; component of other ribonucleoprotein complexes including telomerase TERC, TERT, NOP10, NHP2; components of telomerase holoenzyme TINF2; component of telomerase shelterin complex	Abnormal skin pigmentation, nail dystrophy, bone marrow failure, cancer predisposition
Cartilage-hair hypoplasia	<i>RMRP</i>	Encodes the RNA component of ribonuclease mitochondrial RNA processing; cleaves rRNA precursors, processes RNA primers used in mitochondrial DNA replication, processes turnover of cell cycle related mRNAs	Skeletal defects and short stature, hypoplastic anemia, lymphoma
Diamond Blackfan anemia	<i>RPS17, RPS19, RPS24, RPL5, RPL11, RPL35a</i>	Structural ribosome proteins	Bone marrow failure, craniofacial abnormalities, cancer predisposition
5q- syndrome	<i>RPS14</i>	Structural ribosome protein	Severe macrocytic anemia, normal or elevated platelet counts, normal or reduced neutrophil counts, and erythroid hypoplasia in the bone marrow, cancer predisposition

1.1.3 Current treatment

The need for new treatment strategies for SDS patients is critical. Hematopoietic stem cell transplantation (HSCT) is the only curative therapy for SDS.^{42,54} However, for yet unknown reasons, the mortality and morbidity of SDS patients treated with HSCT are higher than patients with acquired aplastic anemia. Poor outcome with HSCT is related to a high rate of graft failure, neurological complications, pulmonary complications, excessive cardiac and other organ toxicity from the preparative therapy.^{40,49,55,56} Alternatives to HSCT include a chronic transfusion program and a cytokine stimulation program, however these are non-curative treatments. For example, granulocyte-colony stimulating factor (G-CSF) has been administered to induce a beneficial response to the severe neutropenia in SDS patients, by stimulating granulopoiesis and reducing infections. Acute adverse effects, however, have been reported with the therapy including headaches, musculoskeletal symptoms, bone pain and osteopenia.⁵⁷ Furthermore, an increased risk of myelodysplasia or acute leukemia has been reported with G-CSF treatment in SDS and other inherited marrow failure syndromes including severe congenital neutropenia and Kostmann's neutropenia.⁵⁸⁻⁶⁰ Additional therapies include the use of immunosuppressants such as corticosteroids to improve hematological abnormalities⁵ and oral pancreatic enzyme supplements for management of exocrine pancreatic insufficiency. Although several therapies have been administered to patients to improve various features of SDS, new approaches to disease management are needed. It is essential that the function of SBDS be elucidated in both hematological and non-hematological tissues. Beginning with studying the function of SBDS in a single cell lineage affected in SDS, such as the erythroid cell lineage, we aim to understand the defective cellular and molecular mechanisms involved in anemia. It is conceivable that SBDS functions in basic cellular processes in erythropoiesis that are common with granulopoiesis and megakaryopoiesis, such that we can identify improved treatment strategies targeting common

cytopenias in this disorder. This approach will not only lead to an improved understanding of the biology of this syndrome, but also provide further insight into new and efficacious therapeutic strategies for the management of SDS.

1.2 Erythropoiesis and erythroid differentiation models

1.2.1 Erythropoiesis

Erythropoiesis is a continuous, multi-step process beginning with the long-term hematopoietic stem cell (HSC) which undergoes a series of cellular divisions to terminally differentiate into the mature erythrocyte. According to a classical model of hematopoiesis,⁶¹ the long-term HSC gives rise to a short-term HSC and then to the multipotent progenitor (MPP). The latter gives rise to either the common myeloid progenitor (CMP) or the common lymphoid progenitor (CLP). Cells of the myeloid compartment are comprised of the erythroid, megakaryocytic, granulocytic and macrophage lineages whereas CLPs give rise to B and T lymphocytes.

A fraction of the CMPs develop into megakaryocyte/erythroid progenitors (MEPs) which, once committed to the erythroid lineage, mature into burst-forming unit erythroid progenitors (BFU-E) then into colony-forming unit erythroid progenitors (CFU-E), followed by progression to the proerythroblast, basophilic normoblast, polychromatophilic normoblast, orthochromatic normoblast, reticulocyte and finally mature erythrocytes (Fig. 1).

The steps involved in this complex differentiation process are numerous, but generally can be divided into two major stages of development comprising of separate compartments of erythroid precursors. Initially, the effective production of mature erythrocytes requires a rapid proliferative expansion of early precursors involving proerythroblasts to polychromatophilic erythroblasts.^{64,65} Following expansion, dynamic cellular changes occur including increased ribosome biogenesis needed for hemoglobin synthesis, loss of organelles and enucleation. This involves mainly the nonproliferative erythroid cell compartment consisting of polychromatophilic erythroblasts to mature red blood cells through the reticulocyte stage. Such transformations require the action of multiple cytokines, the two most important being the glycoproteins erythropoietin (EPO) and stem cell factor (SCF).⁶⁶ EPO stimulates hemoglobin synthesis, is essential for terminal differentiation and protects erythroid progenitor cells from apoptosis by activating anti-apoptotic proteins, including Bcl-X(L).^{67,68} Erythropoiesis is directly dependent on the interaction of EPO with its single transmembrane receptor (Epo-R) which is maximally expressed in late erythroid progenitors (CFU-E) and proerythroblasts (Fig. 1). SCF acts synergistically with this lineage-specific factor by promoting DNA synthesis and activating MAP kinase (ERK1/2), and hence, correlates with a proliferative effect on erythroid progenitor cells.^{66,69,70} The receptor for SCF (c-kit) is expressed in CD34+ hematopoietic progenitors and its expression is maintained at high levels during the stages of differentiation from BFU-E to CFU-E; c-kit expression progressively declines at later stages of differentiation during the maturation of CFU-E, and disappears in polychromatophilic and orthochromatic erythroblasts⁶³ (Fig. 1). As such, EPO and SCF are necessary to drive the proliferative stage of erythroid differentiation.

Erythropoietic differentiation is orchestrated at the molecular level by a complex network of transcription factors. Of critical importance in the erythroid lineage is the function of GATA-1,

GATA-2 and SCL/Tal-1. GATA-1 is a zinc-finger transcription factor necessary for erythropoiesis as GATA-1 null erythroid cells from chimeric mice fail to mature beyond the proerythroblast stage and undergo apoptosis.⁷¹ GATA-1 plays a direct role in erythroid cell survival by activating transcription of Epo-R,⁷² which leads to upregulation of Epo signaling, known to be important for the survival of erythroid progenitors.⁷³ The highest levels of GATA-1 are observed in CFU-Es and proerythroblasts⁷⁴ (Fig. 1). GATA-2 is another important regulator of hematopoiesis. In chicken erythroid progenitors, GATA-2 is implicated in promoting proliferation at the expense of differentiation.⁷⁵ GATA-2 is also suggested to play a role in regulating the self-renewal capacity of early erythroid progenitor cells.⁷⁵ SCL expression is essential for the early development of the primitive and definitive hematopoietic systems. SCL expression is strictly required for proper erythroid and megakaryocytic differentiation and is associated with proliferation of early erythroid cells.⁷⁶

Normal erythropoiesis is associated with the sequential expression of transmembrane proteins which are useful cell surface markers. The earliest identifiable HSC in humans express CD34⁷⁷ and CD133.⁷⁸ As the cells mature, the expression of these markers progressively decline (Fig. 1). The cell surface markers that reliably distinguish erythroid development include glycophorin A and the transferrin receptor, CD71. While CD71 is upregulated during mid stages prior to hemoglobin biosynthesis, glycophorin A is highly expressed in the later stages of erythropoiesis (Fig. 1).

Upon disruption of either the proliferation or maturation stages of erythroid development, anemia can ensue. Anemia is recognized as one of the world's largest public health problems, affecting over 1.2 billion people in both the developed and developing countries.⁷⁹ In addition to

nutritional causes of anemia, inherited disorders also cause anemia as observed in SDS, Diamond-Blackfan anemia and Fanconi anemia. Although advances in the normal erythroid maturation process have been made by the identification of growth factors, signaling pathways and transcription factors involved in different stages of erythroid development, an in-depth understanding of the underlying molecular mechanisms that regulate erythropoiesis and specifically erythroid cell expansion are not fully understood. Furthermore, the pathogenesis of anemia due to erythropoietic failure in both genetic and acquired disorders is largely unknown. There is a major need to better understand the anemia in inherited marrow failure syndromes as anemia or its only curative therapy, bone marrow transplantation, have substantial morbidity and mortality in these patients.

1.2.2 Erythroid differentiation model using erythroleukemic cell lines

K562 cells are the most common erythroleukemia cell line used to study determinants of erythropoiesis. The cells are derived from a patient with chronic myelogenous leukemia (CML).⁸⁰ Andersson et al. first noted the cells as multipotent hematopoietic progenitors, which exhibit properties in common with erythroid cells upon stimulation with various agents.⁸¹ K562 cells contain glycophorin and spectrin in the membrane and synthesize low amounts of hemoglobin.^{81,82} Rutherford et al. later reported a marked increase in hemoglobin synthesis in K562 cells by the action of hemin, which induces erythroid development to a normoblast stage over approximately 5 days.⁸³ As such, erythroid differentiation of K562 cells represents development from the megakaryocytic/erythroid progenitor (MEP) stage to normoblast stage (Fig. 1). Hemin, a ferric chloride salt of heme, acts to selectively accelerate embryonic and fetal globin gene synthesis and thus synchronizes the synthesis of hemin and globin in the maturing erythroid cell.⁸⁴ The precise mechanism by which hemin acts as an inducer is unclear; however, it has been suggested that hemin, a lipophilic compound, diffuses into K562 cells readily and binds to intracellular and nuclear proteins called hemin binding proteins.⁸⁵ Once in the nucleus, hemin modulates the interactions of known transcriptional factors like NF-E2, Oct-1, and GATA-1 by acting selectively on the γ -globin gene promoter.⁸⁶ Hemin-induced erythroid differentiation of K562 cells, therefore, represents an important *in vitro* model for basic studies of human erythropoiesis. In addition, K562 are an excellent model to study conditions with multilineage defects since they are bipotential in nature and can be stimulated to undergo both erythroid and megakaryocytic differentiation.^{87,88} K562 cells have also been used extensively in studies of inherited marrow syndromes⁸⁹ and cancer.⁹⁰

1.2.3 Erythroid differentiation model using CD34+ or CD133+ HSC/Ps

The most commonly used cell surface marker to isolate early hematopoietic stem and progenitor cells (HSC/Ps) is CD34/podocalyxin belonging to a single transmembrane sialomucin protein family.⁷⁷ It was first identified as a hematopoietic cell-surface antigen in KG-1a cells, an early human myeloblastic cell line,⁹¹ which highly expresses CD34 and displays a strong potential for myeloid colony-forming cells.⁹² CD34 comprises a subset of immature hematopoietic cells and is able to reconstitute all hematopoietic lineages.^{93,94} CD34 is expressed on 1–5% of the nucleated cells in normal human bone marrow (BM) aspirates.⁹⁵ A cell surface antigen initially identified as AC133,⁹⁶ and now referred to as CD133 or Prominin-1,⁹⁷ has been described as a marker of primitive HSC/Ps^{78,98} expressed in a subpopulation of CD34+ HSC/Ps. The CD133+ HSC/Ps, similar to CD34+ HSC/Ps, can reconstitute the entire hematopoietic system in lethally irradiated mice,⁹⁸ differentiate into all lineages *in vitro*^{78,98,99} and *in vivo*.^{100,101} Bone marrow and umbilical cord blood are common sources used for isolation of CD133+ and CD34+ HSC/Ps.

1.3 Characterization of *Shwachman Bodian Diamond syndrome* gene

In 2003, Boocock et al.¹⁴ reported 90% of patients with SDS had mutations in the *Shwachman-Bodian-Diamond Syndrome (SBDS)* gene, which was subsequently confirmed in other studies.¹⁰²⁻¹⁰⁴ These findings demonstrate that biallelic mutations in the *SBDS* gene are the primary cause of SDS. Approximately 75% of the mutations are a result of gene conversion with the adjacent pseudogene, *SBDSP*, which shares 97% homology to *SBDS* but contains deletions and nucleotide changes that prevent the generation of a functional protein.¹⁴ Numerous mutations in *SBDS* have been identified, yet a genotype-phenotype relationship in SDS has not been reported to exist.¹⁰⁵ The most common mutations occur in exon 2 (183-184TA > CT) and the splice site of intron 2 (258 + 2T > C) causing premature protein truncation (Fig. 2). Homozygous 183-184TA > CT

mutations have not been identified in patients suggesting such an occurrence leads to lethality. *SBDS* has been mapped to the q11 centromeric region of chromosome 7 and consists of 5 exons which encode a 250 amino acid protein, predicted to be 28.8 kiloDaltons (kD) (Fig. 2).¹⁴ The crystal structure of the archael *SBDS* ortholog *Archaeoglobus fulgidus*, revealed a three domain structure (Fig. 2) with the N-terminal FYSH domain consisting of a novel α - β fold, with unknown function. The middle domain shows a common winged-helix-turn-helix fold associated with DNA binding¹⁰⁶ while the C-terminal domain shows closest structural similarity to domain V of the yeast translation elongation factor 2.^{107,108} Additionally, by determining the X-ray structure of the *SBDS* ortholog from *Methanothermobacter thermautotrophicus* (mthSBDS) it was reported that the FYSH and C-terminal domains rotate with respect to the central domain suggesting *SBDS* proteins are highly flexible.¹⁰⁹ Furthermore, *SBDS* is expressed in a broad variety of tissues. Wide occurrence of *SBDS* in archaea, plants, yeast and vertebrates suggest a fundamental and evolutionarily conserved function,¹⁴ However the biochemical function of *SBDS* and its specific role in erythropoiesis are unknown.

1.4 Postulated Functions of SBDS

Several possible functions of SBDS have been hypothesized including a role in mitotic spindle stabilization and chemotaxis,^{25,26,33} however the two best supported functions in mammalian cells involve a role in cell survival^{23,24,110} and ribosomal biogenesis.^{19,21,22}

1.4.1 Cell survival

1.4.1.1 Role of SBDS in cell survival of human cells

Our lab has reported a reduced frequency of CD34+ HSC/Ps in the bone marrow of SDS patients and subsequent reduction of various hematopoietic colonies *in vitro*.³⁷ Our lab has also shown that hematopoietic progenitors from SDS patients undergo accelerated apoptosis through a Fas mediated pathway.¹¹¹ Loss of SBDS, using a HeLa cell model, directly led to a slower growth phenotype, hypersensitivity to Fas ligand and increased Fas localization to the plasma membrane.^{23,24} Further study showed the SBDS-deficient cells underwent increased apoptosis compared to controls.²³ Although increased Fas-mediated apoptosis plays a role in the growth of SBDS-deficient cells, it is unknown how SBDS deficiency affects erythroid development and whether loss of SBDS leads to a defect in the proliferative capacity of erythroid progenitors.

1.4.1.2 Cell Survival studies of SBDS in yeast and animal models

Knock-out of the yeast ortholog, *Sdo1p*, and double knockout of the murine ortholog, *SBDS*, reveal it is developmentally essential.¹¹² Although a viable SDS murine model has not yet been generated, a zebrafish model for SDS was explored. By micro-injection of morpholino modified antisense oligonucleotides against the *SBDS* gene into one-cell stage embryos, Venkatasubramani et al. showed a defect in the exocrine pancreas and granulocytes of developing zebrafish.¹¹³ In addition, Rawls et al. have established that *SBDS* is required for

normal hematopoiesis in mice as murine hematopoietic cells deficient in *SBDS* showed a trend toward reduced red blood cell counts, reduced myeloid progenitor production and defects in granulocytic differentiation.¹¹⁴ This landmark study is the first to demonstrate that loss of *SBDS* is sufficient to cause abnormal hematopoiesis. Further investigation into the role of *SBDS* during neutrophil differentiation show it is not necessary for the terminal maturation of neutrophils but plays a role in maintaining the viability of granulocytic precursors.³⁶ Although previous studies have addressed abnormalities in granulopoiesis in SDS, to date, there is little known about *SBDS* protein function during erythroid differentiation.

1.4.1.3 Role of p53 and its family members in mediating cell survival

Interestingly, SDS marrows show overexpression of p53.¹¹⁵ p53 is characterized as a sequence-specific nuclear transcription factor which induces cell cycle arrest and apoptosis following cellular stress and DNA damage.¹¹⁶ Its function is critically regulated by MDM2.¹¹⁷ Several p53 related proteins have been identified, including p73 and p63. Three major functional domains are shared by the p53 family members including: the NH₂-terminal transactivation domain; the central core sequence-specific DNA-binding domain; and the COOH-terminal oligomerization domain. Of these, the central DNA-binding domain is highly conserved across the family. There are several different isomers of p53 family members resulting from alternative promoter utilization and mRNA splicing. The isomers of p73 and p63 that contains an N-terminal transactivation (TA) domain are termed TAp73 and TAp63 respectively, while those lacking the TA domain are identified as Δ N isomers. In addition, the TAp73 gene expresses at least eight C-terminal isoforms (α , β , γ , δ , ϵ , ζ , η , and Φ)^{118,119} while the TAp63 gene expresses three C-terminal isoforms (α , β , γ) due to alternative splicing.^{119,120} TA isomers transactivate p53 target

genes and induce apoptosis, whereas the ΔN isomers are dominant inhibitors of the p53-responsive gene expression.^{120,121}

1.4.1.4 Activation of p53 and its family members in ribosome disorders

A number of ribosome biogenesis disorders have been linked to p53 activation. A study on Diamond Blackfan anemia, a syndrome characterized as a hypoplastic congenital anemia developing from mutations in structural ribosomal proteins (Table 1) including RPS19 showed activation of p53 family members lead to defective erythropoiesis in RPS19-deficient zebrafish.¹²² Using this model, it was suggested that $\Delta Np63$, a truncated isoform of p63 which opposes the pro-apoptotic functions of its family member p53, alleviates the anemia-related RPS19 protein-deficient zebrafish erythropoietic phenotype,¹²² and that an imbalance between p53/ $\Delta Np63$ may lead to a defective erythropoietic phenotype. Furthermore, a study on Treacher Collins syndrome, a congenital disorder of craniofacial development arising from mutations in *TCOF1*, which encodes the nucleolar phosphoprotein Treacle found to affect ribosomal RNA transcription, also showed increased p53 levels and activation of p53 target genes in murine *Tcof1*^{+/-} embryos compared to wild type controls.¹²³ Inhibition of p53 function using the chemical inhibitor of p53-dependent transcription and apoptosis, pifithrin- α ,¹²⁴ led to reduced apoptosis of the *Tcof1*^{+/-} embryos and rescue of cranioskeletal defects,¹²³ indicating p53 inhibition as a potential therapeutic treatment for Treacher Collins syndrome. The association between p53 family members and ribosome biogenesis disorders suggests a common potential mechanism involved in the pathogenesis of these diseases and warrants investigation in SDS.

The function of other p53 family members, such as p73, has recently been studied in erythropoiesis. In K562 cells, increased expression of TAp73 α , TAp73 β and $\Delta Np73$ was shown

during hemin treatment.¹²⁵ While stable expression of Δ Np73 in K562 cells showed a slower growth rate, knockdown of Δ Np73 in K562 cells led to reduced TAp73 expression and repression of erythroid differentiation genes, suggesting Δ Np73 expression works as a positive regulator of erythroid differentiation and that both isoforms are necessary to produce a stable erythroid phenotype in K562 cells.¹²⁵ Furthermore, *in vivo* studies of p73 knockout mice showed moderated anemia and reduced expression of GATA-1 and other erythroid related genes suggesting p73 has a physiological role in erythropoiesis.¹²⁵ Indeed, as p73 interacts with MDM2 and shares structural similarities with p53, it transactivates an overlapping set of p53-target genes implicated in apoptotic cell death.^{126,127} Further investigation into p53 and its family members in the setting of SBDS deficiency is needed to understand whether erythroid cell survival of SBDS-deficient cells is mediated through this pathway.

1.4.2 Ribosome biogenesis

Eukaryotic ribosome biogenesis is a complex process beginning in the nucleolus with the synthesis of the 28S, 18S, and 5.8S rRNAs by RNA polymerase I and 5S rRNA by RNA polymerase III (Fig. 3).^{128,129} Approximately 80 ribosomal proteins and 150 accessory proteins are involved in enabling rRNAs to form pre-ribosomal complexes.^{130,131} The complexes are then transported to the cytoplasm where final maturation events occur. Each mature 80S eukaryotic ribosome consists of a 60S (large) subunit and a 40S (small) subunit (Fig. 3). The protein synthesis mediated by ribosomes requires a coordinated effort of numerous proteins and can be divided into initiation, elongation and termination steps. The process of translation is crucial to cell expansion and adaptation to changing environments such as nutrient deprivation and stress.¹³²

1.4.2.1 Role of SBDS in ribosome biogenesis

Although the precise functions of SBDS are unknown, strong evidence supports a fundamental role of SBDS in ribosomal biogenesis. Firstly, SBDS concentrates in the nucleolus during G1 and G2 cell cycle phases.²² Moreover, the archaeal ortholog is located in an operon that encodes other RNA-processing enzymes.¹³³ Although the crystal structure of human SBDS has not been solved, the *Archaeoglobus fulgidus* ortholog is characterized with a C-terminal region showing structural homology with a predicted RNA binding motif.¹⁰⁷ Furthermore, an additional archaeal ortholog, mthSBDS, was recently reported to interact with ribosomal proteins RPL2, RPL1, RPL14.¹⁰⁹ The yeast ortholog, Sdo1p co-precipitates with pre-rRNAs (27S and 23S), mature rRNAs (5.8S, 25S and 18S), a nucleolar rRNA-processing factor (Nip7p) and a translation elongation factor (Eft2p), indicating that Sdo1p likely binds to pre-ribosomal subunits in the nucleus and remains attached during ribosomal maturation in the cytoplasm.¹³⁴ A recent study has reported that human SBDS interacts with several ribosomal proteins of both the large and small ribosomal subunits including RPL3, RPL4, RPL5, RPL6, RPL7a, RPL7, RPL8, RPL12, RPL13, RPL14, RPL18, RPS3¹³⁵ and nucleophosmin, a multifunctional protein implicated in ribosome biogenesis, leukemogenesis, and centrosomal amplification.²¹ Interestingly, microarray expression analysis of SBDS-deficient marrow mononuclear cells from our lab revealed downregulation of several ribosomal protein genes with which SBDS interacts including RPL4, RPL5, RPL6, RPL12, RPL13, RPL14.¹⁹ Notably, RPS27L, which mediates p53-dependent induction of apoptosis, was the only ribosomal protein gene upregulated in SBDS-deficient patient marrow cells.¹⁹ Furthermore, functional studies in yeast show loss of Sdo1p led to inhibition of Tif6p release, a nucleolar shuttling factor, from nascent 60S ribosomal subunits preventing association with the 40S subunit (Fig. 4).²⁰ Furthermore, Sdo1p deficiency led to reduced 60S subunit levels in ribosome profiles, indicating a role of Sdo1p in 60S subunit

maturation.²⁰ This is consistent with a study that demonstrated human SBDS migrates with the 60S large subunit and co-precipitates with the 28S ribosomal RNA.²¹ However, SBDS-deficient fibroblasts did not show a corresponding reduction of 60S subunit in ribosomal profiling experiments suggesting ribosome subunit processing in human cells is still unclear.²¹

Ribosome biogenesis is the most energy-consuming process in growing cells and needs the coordinated efforts of over 150 non-ribosomal factors and 100 small non-coding RNAs for normal function.¹³⁶ In addition to critical roles in ribosome assembly and function, several ribosome-related factors perform important extra-ribosomal functions, including roles in cellular stress response, DNA repair, transcriptional regulation, proliferation, and apoptosis.¹³⁶⁻¹⁴⁰ Functional loss of many ribosome-related factors in yeasts and *Drosophila* lead to severe growth defects while the de-regulation of various ribosome-related factors is associated with several human diseases, including the inherited marrow failure syndromes, SDS, Diamond Blackfan anemia, Dyskeratosis congenita, and Cartilage-hair hypoplasia. Therefore, although there is strong evidence supporting a central role for SBDS in ribosome biogenesis, SBDS may well have additional cellular roles that are currently uncharacterized but which contribute to the clinical heterogeneity of SDS.

Fig. 3: Ribosomal RNA processing and assembly

The mammalian ribosome is a complex structure composed of four ribosomal RNAs (rRNAs) and requires over 80 ribosomal proteins (RPs) and about 150 accessory proteins for normal assembly. Most steps in ribosome biogenesis are temporally and spatially organized within the nucleolus, where the 45S rRNA precursor is transcribed and processed alongside 5S rRNA. Immature 40S and 60S complexes form with the aid of the ribosomal and accessory proteins. After further modification, the pre-ribosomal complexes translocate to the cytoplasm and additional maturative events occur to generate the mature 80S ribosome as described in ^{130,141}.

Fig. 4: Sdo1 is necessary for Tif6 release

Binding of Sdo1 to pre-60S subunits allows for release of the nucleolar shuttling factor, Tif6, thus allowing for subsequent association of the 60S subunit with the 40S subunit for 80S ribosome formation. In the absence of Sdo1, Tif6 blocks the association of the large and small subunits preventing 80S ribosome formation as described in ²⁰.

1.5 shRNA-mediated approach to study SBDS function

A widely accepted approach to study gene function is through RNA interference (RNAi). This methodology first generated interest when it was discovered that the introduction of dsRNA¹⁴² into *Caenorhabditis elegans* lead to gene-specific inhibition much more efficiently than either sense or antisense RNA.^{143,144} The general principle behind RNA interference involves introduction of a 19–21 nt double stranded RNA fragment or a shRNA designed specifically against a targeted gene. The dsRNA or intracellularly transcribed hairpin RNA is processed by Dicer, a dsRNA-specific endonuclease, and the generated functional siRNAs are incorporated into RNA-induced silencing complex (RISC). The multi-enzyme complex then unwinds the siRNA and continues to degrade the messenger RNA whose sequence is complementary to that of the siRNA guide strand.¹⁴⁵⁻¹⁴⁷ In 2001, siRNAs were first reported to mediate sequence-specific gene silencing in mammalian cells.¹⁴⁸ Since then, the use of RNAi to study the molecular basis of human disease has flourished.¹⁴⁹⁻¹⁵¹ Indeed, the use of RNAi to target disease-specific genes such as SBDS in murine cells to study SDS¹¹⁴ and RPS19 in TF-1 cells¹⁵² to study DBA has proven to be widely successful.

There are several advantages to transfecting mammalian cells with vector based shRNAs over transient transfection by siRNA. First, the RNAi effect can sustain knockdown longer and is more pronounced. Plasmid delivery into a cell can produce large numbers of shRNAs and subsequent siRNA molecules, while synthetically synthesized siRNAs are not amplified or inherited in mammalian cells.¹⁵³ Furthermore, viral vector based shRNAs are the most efficient method for gene delivery. Lentiviral vectors compared to retrovirus vectors have the advantage of transducing dividing and non-dividing cells and are more efficient at shRNA delivery to cells that are difficult to genetically modify such as hematopoietic stem cells. In this study, both

vector based shRNA and lentiviral-mediated shRNA were used to silence *SBDS* to elucidate its role during erythropoiesis.

1.6 Objectives of study

1.6.1 Rationale

Bone marrow failure in Shwachman-Diamond Syndrome (SDS) is characterized by multilineage cytopenias affecting erythrocytes, neutrophils and/or megakaryocytes. Although hematological abnormalities are the leading cause for mortality and morbidity in these patients, the molecular and cellular mechanisms leading to failed hematopoiesis have not been well characterized. Indeed, bi-allelic mutations in *SBDS* cause SDS; however, how loss of *SBDS* contributes to hematopoietic failure is unknown. Lentiviral-mediated RNAi of *SBDS* in mice revealed a trend towards a reduction in the red blood cell count, defective granulocytic differentiation *in vitro* and reduced production of myeloid progenitors *in vivo*.¹¹⁴ Furthermore, knockdown of *SBDS* in the murine myeloid 32Dcl3 cell line showed normal neutrophil maturation but reduced viability of granulocyte precursor cells.³⁶ Together, these studies provide evidence that loss of *SBDS* is sufficient to cause abnormal hematopoiesis. While preliminary investigation into *SBDS* function in granulopoiesis has been studied in mice, the erythroid and megakaryocytic lineages in SDS remain largely uncharacterized. To enable an in depth analysis of the chain of events leading to cytopenia in SDS, I studied one cell lineage as a model. I chose the erythroid cell line since erythropoietic defects such as reduced marrow erythroid progenitors, large RBCs and high fetal hemoglobin occur in up to 90% of the SDS patients and anemia in over 60%.^{8,16,42} As there is little known about *SBDS* protein function during erythroid differentiation, I used an erythroid differentiating *SBDS*-knockdown model to identify defective pathways leading to erythropoietic failure and then studied the molecular link between these processes. As anemia or the

underproduction of RBCs can be due to either slow cell expansion or a block in differentiation I initially determined whether SBDS deficiency led to defects in either of these processes.

Although the function of SBDS has been under intense investigation over several years with postulated effects on cell survival, ribosome biogenesis, and other cellular processes, how defects in these processes lead to erythropoietic failure are unknown. Although a potential role of SBDS in cell survival has been suggested through studies in SDS marrows showing reduced colony formation³⁷ and SBDS-deficient HeLa cells reporting slower growth and increased Fas-mediated apoptosis,¹¹¹ these studies have only been performed using non-differentiating cells and may not accurately represent what occurs during erythropoietic development. Similarly, the plausible role of SBDS in promoting ribosome biogenesis has not been previously characterized in erythroid differentiating cells. A previous study showed that SBDS is localized predominantly in the nucleolus during G1 and G2 phases in non-differentiating HeLa cells,²² and interacts with various ribosomal-associated factors and rRNAs^{20,135} in a non-differentiated state. However, how SBDS functions in resting cells compared to maturing cells is highly likely to represent two dynamic scenarios. Furthermore, studies in both yeast and humans have explored ribosome biogenesis, with conflicting results between models on 60S subunit processing, but these studies were done with resting cells, which may not be relevant in erythropoiesis. Cellular processes of non-differentiating cells likely are regulated by a different mechanism compared to maturing cells. As such, SBDS deficiency in erythroid differentiating cells may lead to defects in pathways specifically involved in erythropoietic maturation. One such process that may particularly show sensitivity during erythroid differentiation includes ribosome biogenesis, as erythroid cells are highly proliferative and require increased ribosome synthesis for the high demand of hemoglobin synthesis. To better understand the potential roles of SBDS in promoting

erythroid cell survival, ribosome biogenesis and translation during erythropoiesis, I used K562 cells, an erythroleukemic cell line that is induced to undergo erythroid development with hemin and erythroid differentiating CD133+ HSC/Ps. These cells are an excellent model to study whether basic cellular processes caused by SBDS deficiency, such as ribosome biogenesis and translation, are exacerbated during erythroid cell expansion. Furthermore, I studied whether the reduced erythroid cell expansion phenotype are specifically due to abnormalities in ribosome biogenesis and translation.

Having identified defective pathways leading to erythropoietic failure, I then assessed a potential mechanistic link between the processes. A possible link between abnormalities in ribosome biogenesis and erythroid cell expansion through p53 induction has been described.^{122,154} For instance, activation of p53 occurs as a result of cellular stress where several ribosomal proteins including RPL5 and RPL11, inhibit MDM2-mediated ubiquitination of p53.¹⁵⁵ Furthermore, an imbalance between p53 and $\Delta Np63$ may affect the ability of blood cell progenitors to proliferate and differentiate into erythrocytes.¹²² Indeed, suppression of p53 and $\Delta Np63$ in RPS19-deficient cells alleviates the defective erythropoietic phenotype.¹²² As p53 protein is reported to be overexpressed in SDS bone marrow cells¹¹⁵ and since the p53 family members also play a role in the upregulation of Fas, they are excellent candidates which can be activated in SDS and lead to slow erythroid cell expansion. In addition, although the role of p73, a member of the p53 family, in bone marrow failure disorders has not been studied, the pro-apoptotic isoforms of p73 play a role in erythropoiesis by promoting differentiation;¹²⁵ and therefore warrants further investigation in SDS.

1.6.2 Hypothesis

Defects in ribosome biogenesis and cell survival are central to the pathogenesis of SDS, but what remains to be elucidated is just how defects in these processes lead to erythropoietic failure. A hypothetical model of erythropoietic failure in SDS, due to loss of SBDS, is shown in Fig. 5. I hypothesized that SBDS protects early erythroid progenitors from apoptosis and promotes their proliferative capacity at a critical stage of erythroid maturation, in which loss of *SBDS* function results in reduced ribosome biosynthesis and leads to translational insufficiency. During erythropoiesis the p53 family members might be over-activated in SBDS-deficient cells and inhibit normal cell expansion.

1.6.3 Specific aims

1. To characterize the defects in erythroid cell expansion due to SBDS-deficiency.
2. To determine whether reduced cell expansion in SBDS-deficient cells is due to abnormalities in ribosomal biogenesis and translation efficiency during erythroid differentiation.

CHAPTER II

MATERIALS AND METHODS

2.1 Cell culture

2.1.1 Cell lines and induction of erythroid differentiation

K562 human erythroleukemia cells were purchased from ATCC (Manassas, VA). The K562 cells were maintained in Iscove's Modified Dulbecco medium (IMDM), (Invitrogen, Carlsbad, CA) supplemented with 10 % fetal bovine serum (FBS) and geneticin (Invitrogen, Carlsbad, CA) at 37°C in a humidified 5 % CO₂ atmosphere. In the erythroid differentiation experiments, the cells were seeded at a density of 2×10^5 cells/ml and cultured for 5 days in the presence or absence of 25 µM hemin solution (Sigma, St. Louis, MO) prepared as previously described.¹⁵⁶

Cord blood (CB) samples were collected into single blood-pack units, containing anticoagulant citrate phosphate dextrose solution. Mononuclear cells were separated using Ficoll-paque, as previously described,³⁷ and then used fresh. Cells were incubated with the antibody CD133/2 (293C3)-PE (Miltenyi Biotec) according to the manufacturer's instructions and separated using EasySep PE selection Cocktail (Stem Cell Technologies, Vancouver, BC). The studies were approved by the institutional Research Ethics Board, and informed written consent was obtained from controls prior to sample collection. CB CD133+ cells were maintained in serum-free StemSpan H3000 medium (Stem Cell Technologies, Vancouver, BC) for 10 days and then transferred to Iscove's modified Dulbecco medium supplemented with 20 % fetal bovine serum (FBS) for the remainder of culture. Cells were given 50 ng/ml stem cell factor, 50 ng/ml FLT-3, 50 ng/ml thrombopoietin (TPO), 20 µU/ml insulin, and 2 ng/ml interleukin-3 (IL-3) immediately after isolation and on day 2 of culture. Cells were then induced to undergo erythroid

differentiation on day 4 with the addition of 2 U/ml EPO, 50 ng/ml stem cell factor and 20 μ U/ml insulin, followed by addition of 2 U/ml EPO every two days.

2.1.2 Short hairpin RNA expression cassettes and generation of *SBDS*-knockdown cells

Two *SBDS* (Genbank sequence #AY169963) shRNAs consisting of nucleotides 585-603 of the *SBDS* open reading frame (ORF) (S-1) and nucleotides 137-156 after the open reading frame (S-3) were cloned into a pSEC/Neo plasmid (Ambion, Austin, TX), as previously described.²³ A scrambled control sequence was also synthesized and cloned into a pSEC/Neo plasmid (Ambion, Austin, TX).²³ These sequences were as follows:

S-1: GGTCATAGAAAGTGAAGAT

S-3: GGCTTTCCTACATAAGTAT

SCR: CTACCGTTGTTATAGGTGT

The plasmids were transfected into K562 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and 24 hours post-transfection, cells were plated in ClonaCell™-Transfection Cell Selection medium (Stem Cell Technologies, Vancouver, BC) containing geneticin (Invitrogen, Carlsbad, CA). The cells were incubated at 37°C in 5 % CO₂ and after 14 days, isolated colonies were picked. Positive cultures were selected by geneticin (Invitrogen, Carlsbad, CA) and maintained in IMDM (Invitrogen, Carlsbad, CA) supplemented with 10 % FBS. According to the shRNA expression construct, we termed the lines K562/sh*SBDS*-1, K562/sh*SBDS*-3 and K562/SCR. *SBDS*-knockdown was confirmed by immunoblotting using a chicken anti-*SBDS* antibody as described.²³

2.1.3 Morphological analysis by benzidine and Wright-Giemsa staining

Samples of control CD133+ or CD34+, K562 and shRNAi infected cells were prepared on glass slides using the cytopspin centrifuge for 3 minutes at 500 x g. Morphologic features were evaluated with use of May-Grunwald and Wright-Giemsa staining. Benzidine staining¹⁵⁶ was used to detect the pseudoperoxidase activity of hemoglobin in K562 and bone marrow (BM)/CB cells. K562 cells were treated with or without 25 μ m hemin for 5 days, while CD133+ or CD34+ cells were stimulated to undergo erythroid differentiation using the cocktail mentioned above. Samples were taken each day for staining for K562 cells and periodically for CD133+ or CD34+ cells. Cells were cytopspun at 500 x g for 3 minutes and incubated in the benzidine solution containing 0.66% (w/v) 3,3'-dimethoxybenzidine (Sigma, St. Louis, MO) and 0.0024 % (v/v) hydrogen peroxide prepared at room temperature. Benzidine-positive cells were enumerated by light microscopy.

2.1.4 Cell expansion assays

Cell expansion was monitored using two complementary methods. Hemin-induced SBDS-deficient K562 and control cells were seeded at 2.0×10^5 cells/ml and viable cell counts were determined using trypan blue dye exclusion for a period of 5 days. Cell expansion was also determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (ATCC, Manassas, VA), according to the manufacturer's instructions, at different time points after plating depending on the specific experiment.²⁴ In these experiments, SBDS-knockdown and control cells were plated into 96-well plates in triplicate wells. Absorbance was measured at 570 nm in a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). The cells were assayed daily to evaluate growth following stimulation with 600 μ g/ml L-leucine (Sigma, St.

Louis, MO). The cells were seeded at 2.0×10^4 cells/well, treated with or without 600 $\mu\text{g/ml}$ L-leucine and assayed every 24 hours for 4-5 days.

2.2 Lentiviral production and transduction

2.2.1 Cloning of shRNA-SBDS in pFCYsi-YFP vector

Two pairs of antisense oligonucleotides used to generate shRNA complementary to the *Homo sapiens* SBDS and a non-specific scrambled control sequence were generated similar to the shRNA constructs used in K562 cells. The oligonucleotides contained an additional AgeI sequence at the 5' end and a BamHI sequence at the 3' end to allow for directional cloning into the pFCYsi vector (a generous gift from Dr. Dan Link, Washington University School of Medicine). The pFCYsi vectors contained a cytomegalovirus (CMV) promoter driving expression of a yellow fluorescent protein cassette and a U6 promoter used to drive shRNA expression. Accuracy of cloning was confirmed by sequencing. Additional plasmids for lentiviral production were obtained (a generous gift from Dr. Eyal Grunebaum, Hospital for Sick Children). Plasmids were purified by using the Maxi Prep kit (Qiagen).

2.2.2 Lentiviral production in 293FT cells and transduction of cord blood CD133+ and CD34+ cells

293FT cells (Invitrogen, Calsbad, CA) were seeded at 5×10^6 cells overnight and co-transfected with the respective pFCYsi plasmids and *Env*, *Gag/pol*, *Rev* and *Tat* expression constructs. Human CB CD133+ or CD34+ HSC/Ps, pre-activated for 24 h in the already described liquid culture conditions, were transduced by three cycles of overnight infection with viral supernatant in the presence of polybrene (8 $\mu\text{g/ml}$). Transduced YFP+CD133+ cells were sorted flow cytometry.

2.3 RNA isolation and real time PCR

The expression of SBDS in cells undergoing erythroid differentiation was measured by quantitative real-time PCR (RT-qPCR) using SYBR green technology as previously described.¹⁵⁷ Total RNA was isolated by using the RNeasy minikit (Qiagen). cDNA was synthesized with Omniscript reverse transcriptase (Qiagen) by using oligo(dT) primer. The primer pairs used were as follows: SBDS gene forward primer: ATC GCC TGC TAC AAA AAC AAG, reverse primer: TTG GCA ACC TGA CCT TTA GAA. β -globin gene Forward primer: GGC ACC CAG CAC AAT GAA GAT C, Reverse primer: AAG TCA TAG TCC GCC TAG AAG CAT.

2.4 Western blot analysis

2.4.1 Antibodies

Custom chicken anti-human SBDS antibody was generated by immunization of animals with the C-terminal of SBDS and purified by Invitrogen (Carlsbad, CA)²³ and used in a dilution of 1:100. A rabbit anti-human cleaved Poly ADP-ribose polymerase (c-PARP) (Asp214) antibody (Cell Signaling Technology Inc, Danvers, MA) was used as a marker for apoptosis and used at a dilution of 1:1000. A mouse anti-human β -actin antibody (Sigma, St. Louis, MO) was used as an internal control for protein loading and was purchased from Sigma (St. Louis, MO) and used at a dilution of 1:10,000. Antibodies against TAp73 (GC-15, Pharmingen, San Jose, CA) and Δ Np73 (IMG-313, Imgenex, San Diego, CA) were generous gifts from Dr. Meredith Irwin, Hospital for Sick Children.

2.4.2 Western blotting analysis

Hemin induced and uninduced cells were lysed in CytobusterTM (Novagen, San Diego, CA) solution containing protease inhibitor cocktail (Sigma, St. Louis, MO). Lysates were centrifuged

at 15,000 x g for 5 minutes at 4°C and the protein concentration was measured using BCA protein assay solution. Lysate proteins (25 µg) were separated by 12 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The blots were blocked for 1 hour with 5 % (w/v) non fat dry milk in TBS-T followed by incubation with primary antibodies. The blots were then incubated with the following secondary antibodies for 1 hour at room temperature: rabbit anti-chicken IgY- horseradish peroxidase conjugate (1:3000), donkey anti-rabbit IgG-horseradish peroxidase conjugate (1:5000) and goat anti-mouse IgG-horseradish peroxidase conjugate (1:10000). Antibody-reactive proteins were detected using enhanced chemiluminescence reagents (Amersham Biosciences, UK). Quantification of protein signals was performed using computer-assisted densitometry.

2.5 Flow cytometry

2.5.1 Apoptosis, cell proliferation and cell cycle assay

Double staining for Ki-67 and determination of apoptotic events by DNA content analysis was performed as previously described²³ with minor modifications. In brief, 5×10^5 cells were washed with phosphate-buffered saline (PBS), fixed with 70 % ethanol, and stored at -20 °C until further analysis. Cells were re-washed and incubated with mouse anti-Ki-67 (Dako Canada Inc., Mississauga, ON) at 4°C for 30 minutes in the dark. After washing with PBS, the cells were incubated with FITC-conjugated IgM (H+L) antibody for 30 minutes. Cells were washed again and incubated an additional 1 hr with propidium iodide, RNaseA and Triton X-100. DNA content was then analyzed by flow cytometry and the sub-G1 cell population was quantified, which indicates apoptotic cells, while Ki-67-FITC expression was analyzed in viable cells.

2.6 Sucrose gradient density ultracentrifugation

Extracts of hemin induced and uninduced SBDS-deficient K562 and control cells for ribosome profile analysis were adapted from^{158,159}. Briefly, cells were incubated at 37°C for 10 minutes after treating with 100 µg/ml cycloheximide. Cells were pelleted and washed twice in ice cold PBS containing 100 µg/ml cycloheximide and then resuspended in ice-cold TMK100 lysis buffer (1 M Tris, 100 mM KCL, 5 mM MgCl₂, 10 mM HEPES, pH 7.4, 100 µg/ml cycloheximide, 0.5% (v/v) Triton X-100, and 250 U/ml RNaseOUT), followed by passage through a 27.5-gauge needle three times. Nuclei were removed by centrifuging at 12,000 rpm for 5 mins. Total RNA was measured at A260 nm. Lysates were layered on a 5% to 45% (w/v) sucrose gradient in ribosome profile sucrose gradient buffer (100 mM KCl, 5 mM MgCl₂, and 10 mM HEPES, pH 7.4), and centrifuged in a SW41 rotor (Beckman Coulter, Fullerton, CA) for 2.5 hours at 40,000rpm. For dissociated conditions, cells were lysed in ice-cold TEK100 lysis buffer (1 M Tris, 100 mM KCL, 10 mM EDTA, 10 mM HEPES, pH 7.4, 100 µg/ml cycloheximide, 0.5% (v/v) Triton X-100, and 250 U/ml RNaseOUT (Invitrogen, Carlsbad, CA) and centrifuged for 3 hours at 40,000 rpm. All sucrose gradients were fractionated using the Brandel gradient fractionator system (Brandel, Gaithersburg, MD). Absorbance was monitored at 254 nm with an ISCO UA-6 flow cell (Teledyne ISCO, Lincoln, NE). Fractions were snap-frozen and stored at –80°C. Chart records were digitized using Adobe Photoshop (Adobe Systems, San Jose, CA).

2.7 Evaluation of global translation

2.7.1 Incorporation of ³⁵S methionine/cysteine

Metabolic labeling of K562 knockdown and control cells with [³⁵S]-methionine was adapted from procedures published in.¹⁵⁹ Cells (treated with and without 25 µM hemin or 600 µg/ml leucine) were rinsed and incubated for 15 minutes in methionine-free medium supplemented with

10% dialyzed FBS (Wisent). Cells were then given 1000 μ l of methionine-free medium containing 150 μ Ci/ml [35 S]methionine and placed for 30 min at 37°C. The labeling medium was removed, and cells were rinsed twice with ice-cold phosphate-buffered saline, followed by a 5 minute incubation at 25°C in CytobusterTM reagent. Protein concentration was determined using the BCA assay (Novagen, San Diego, CA). [35 S]methionine incorporation was determined by trichloroacetic acid (TCA) precipitation of 25 μ g aliquots of the supernatants. Counts per minute (cpm) were obtained using a scintillation counter (Beckman Coulter LS 6500).

2.8 Re-introduction of *SBDS* into *SBDS*-deficient K562 using retrovirus

FLYRD18 cells¹⁶⁰ stably transfected with pLNCX retroviral vectors expressing *SBDS*-YPET and control YPET, previously generated in our laboratory, were used to transduce K562/sh*SBDS*-3 cells. K562/sh*SBDS*-3 cells were infected for 6 hours each day for 3 days with viral supernatant and polybrene (8 μ g/ml). Expression of YPET was noted by confocal microscopy. Cell expansion and translation of cells transduced with the retrovirus was measured, as described above.

2.9 Statistics

Means and standard errors were used to describe the results concerning cell growth, apoptosis, proliferation, cycling, and global translation output. Student's t-test was used to determine the statistical significance of differences between two means. A one-way analysis of variance (ANOVA) followed by Dunnett's test or Tukey's test of multiple means was performed to determine statistical significance of differences between multiple means.

CHAPTER III RESULTS

3.1 *SBDS* is expressed early during erythroid differentiation

Various erythroid associated genes are expressed at different stages of erythroid development correlating with their critical function. For example, Epo-R shows maximal expression during the CFU-E stage, leading to the proliferation and differentiation of erythroid progenitors¹⁶¹ by upregulating GATA-1, an erythroid-specific transcription factor. To understand whether *SBDS* behaves similarly during erythroid development in that its expression correlates with a stage in which it has a prominent role in erythropoiesis, *SBDS* expression was studied by reverse transcription and quantitative real-time PCR (RT-qPCR). Early CD133+/HSC/Ps isolated from umbilical cord blood showed high expression of *SBDS*, followed by a decline in expression as erythroid differentiation progressed (Fig. 6a). Evaluation of *SBDS* expression in K562 cells, which were induced to undergo erythroid differentiation by treatment with hemin was also performed. The cells showed a significant upregulation of *SBDS* 24 hours after induction with 25µm hemin, with expression returning to basal levels for the following 4 days (Fig. 6b). Erythroid differentiation of K562 cells comprises the development from the megakaryocyte/erythroid precursor to the normoblast stage. Since the upregulation of *SBDS* appears to be short-lived, the initial increase in *SBDS* expression at day 1 in K562 cells (Fig. 6a) may correspond with an upregulation of *SBDS* in a stage of erythroid culture of CD133+ HSC/Ps that was not tested, such as day 1 after stimulation with cytokines necessary for erythroid development. Alternatively, the increased expression of *SBDS* seen on day 0 (Fig.6b) of CD133+/HSC/Ps culture may have occurred since these cells have already been exposed to growth factors and other differentiating signals *in vivo* unlike in K562 cells. Protein expression of *SBDS* in differentiating K562 cells was assessed by western blotting and constant expression

was detected during erythroid differentiation (Fig. 6c). There was a mild trend of an increase in the protein level on day 1 (Fig. 6c), but the difference was not statistically significant. It is possible that the semi-quantitative nature of immunoblotting limits its ability to detect small biological differences. In addition, a change in mRNA levels may not necessarily correlate with subsequent changes in protein levels, as the latter also depends on rate of translation and decay. The half-life of SBDS protein is greater than 25 hours,¹⁶² as such, transient changes at the mRNA level may not be easily detectable at the protein level. These are the first studies to assess SBDS expression during erythroid development and suggest *SBDS* mRNA levels are increased early during erythroid commitment and present at reduced levels during differentiation.

Fig. 6: Analysis of SBDS expression during erythroid differentiation.

A) Real-time qPCR was used to analyze *SBDS* mRNA expression in erythroid-committed CD133+/HSC/Ps, where early upregulation of *SBDS* is observed by one experiment performed in triplicate and B) *SBDS* mRNA expression in hemin-induced K562 cells by RT-qPCR is shown. A significant increase in SBDS is detected 24 hours after hemin induction in control K562 cells, followed by a return to baseline levels of expression. Three separate experiments were conducted in triplicate. Statistically significant values are marked with an asterisk, ($p < 0.05$) determined by t-test. C) SBDS protein expression in hemin-induced K562 cells by western blotting showing constant expression. Three independent experiments were performed.

3.2 Establishment of SBDS-deficient cell models using shRNA in hematopoietic stem cells and progenitors and K562 myeloid cells

To further characterize the cellular role of SBDS and, in particular, to ascertain whether it has any functional significance during erythroid differentiation, our lab has established a stable line of K562 cells with *SBDS* gene knockdown using a shRNA expressing system. As our lab had previously generated two successful *SBDS*-knockdown HeLa cell lines,^{23,24} the same two shRNA sequences targeting *SBDS* were used to knockdown *SBDS* in K562 cells (See Methods Section 2.1.2). A non-specific shRNA sequence also previously generated was used as the scrambled (SCR) control cell line (Fig. 7a). The shRNA plasmids that showed successful knockdown of human SBDS expression were termed K562/shSBDS-1 (S-1) and K562/shSBDS-3 (S-3). Western blotting was used to confirm knockdown and revealed S-3 cells showed approximately 90% reduction of protein levels, while the S-1 cell line retained 10-15% of SBDS protein expression in K562 cells in comparison to controls (Fig. 7b). As such, the S-3 cell line consistently showed a stronger phenotype indicating dose-dependant knockdown model similar to what was shown previously in SBDS-deficient HeLa cells.^{23, 24}

A similar approach was then used to knockdown *SBDS* in CD133+ and CD34+ HSC/Ps through a shRNA lentiviral cloning and expression system (Fig. 8). After lentiviral transduction of CD133+ HSC/Ps, cells were sorted for YFP and purity after sorting was confirmed over 90% for both control and *SBDS*-knockdown cells using flow cytometry (Fig. 9a) and confocal microscopy (Fig. 9b). To confirm that YFP+ HSC/Ps are depleted of *SBDS*, western blot analysis was performed (Fig. 9c). Limited number of HSC/Ps and only 10ug of protein were obtained; however, no SBDS band was detected for the HSC/Ps transduced with pFCYsi-SBDS. These results show successful generation of an *SBDS*-knockdown model in human HSC/P cells isolated from CB.

3.2.1 Re-introduction of *SBDS* into stable *SBDS*-knockdown K562/sh*SBDS*-3 cells

Although the use of two effective shRNA sequences targeting *SBDS* allows for proper validation against non-specific and off-target effects of the shRNAs, I wanted to confirm the various phenotypes observed upon silencing were specifically due to *SBDS* deficiency. As such, constructs of *SBDS* fused to YPET (pLNCX/*SBDS*-YPET) and of YPET alone (pLNCX/YPET) were generated and cloned into a retroviral pLNCX vector. Viral supernatant from FLYRD18 cells expressing the constructs were harvested, concentrated and used to transduce K562/sh*SBDS*-3 cells. This knockdown cell line expresses a shRNA sequence targeting nucleotides 137-156 after the ORF, therefore re-introduction of *SBDS* was possible as pLNCX/*SBDS*-YPET constructs only contained the *SBDS* ORF sequence. Transduction efficiency was above 80% as assessed by analysis of *SBDS*-YPET and control YPET expressing cells using confocal microscopy (Fig. 10a). Western blotting analysis also showed the presence of a band at 56kDa, the approximate size of the *SBDS*-YPET fusion protein, in S-3 cells transduced with *SBDS*-YPET (Fig. 10b), with no such band present in YPET transduced cells, indicating successful re-introduction of *SBDS* into K562/sh*SBDS*-3 cells.

A.

S-1: GGTCATAGAAAGTGAAGAT

S-3: GGCTTTCCTACATAAGTAT

SCR: CTACCGTTGTTATAGGTGT

B.

Fig. 7: pSEC/Neo plasmids used for *SBDS*-knockdown in K562 cells

A) Stable shRNA-mediated knockdown of *SBDS* was achieved by transfecting K562 cells with the respective pSEC/Neo plasmids (Ambion, Austin, TX) and selecting positive colonies grown in ClonaCell-TCS medium with geneticin. The pSEC/Neo plasmid, modified from¹⁶³ and shRNA sequences targeting *SBDS* and a non-specific control sequence are shown. **B)** Western blot confirmed knockdown of *SBDS*, performed using 30μg of protein from K562 cells. S-1 and S-3 show *SBDS* depletion compared to controls.

Fig. 8: pFCYsi lentiviral plasmids

Lentiviral-mediated knockdown of *SBDS* in CD133+ HSC/Ps was achieved through transduction of viral particles produced through co-transfection of respective pFCYsi plasmids (a generous gift from Dr. Dan Link, Washington University School of Medicine), and pRev, pGag/pol, pTAT, and pVSV-g (a generous gift from Dr. E. Grunebaum, Hospital for Sick Children) from 293FT cells.

A. **B.** **C.**

Fig. 9: Purity of YFP sorted HSC/Ps and knockdown of *SBDS* in transduced cells

A) CD133+ HSC/Ps isolated from CB were transduced with lentivirus expressing control or shRNA targeting *SBDS* and sorted by YFP. Post sorted results showed greater than 95% purity in both control and knockdown cells **B)** Confocal microscopy showing YFP sorted cells **C)** Lentiviral-mediated knockdown of *SBDS* was confirmed by western blotting in HSC/Ps from CB, using 10 μ g of protein. In this case, positively selected CD34 cells were used, instead of CD133+ cells, to obtain adequate protein amounts required for western blotting. The blot shows efficient inhibition of *SBDS*.

3.3 SBDS-deficiency impedes cell expansion during erythroid maturation, but does not interfere with normal erythroid differentiation

3.3.1 Impaired expansion in hemin induced SBDS-deficient K562 cells

During erythropoiesis, progenitors undergo prominent cell expansion; however, SDS bone marrows are frequently hypocellular with an overall reduction in all myeloid cell lineages including erythrocytes.^{8,16,37} Therefore, I hypothesized that during differentiation the SBDS-deficient cells would expand slower than normal cells. To study the effect of SBDS-deficiency on mammalian erythroid-committed cells, the cell expansion of human K562/shSBDS-1 and K562/shSBDS-3 cell lines were compared to control and K562/SCR cell lines. Cells were induced to undergo erythroid differentiation for a period of five days once stimulated with 25µM hemin and cell counts were assessed daily by trypan blue exclusion. The expansion of the *SBDS*-knockdown K562 cells was markedly reduced compared to cells from both the normal and SCR cell lines (Fig. 11). Both S-1 and S-3 cells showed significant impairment of cell expansion after 4 days of hemin-induction. Preliminary studies on lentiviral-mediated knockdown of *SBDS* in YFP sorted CD133+ HSP/Cs also show impaired cell expansion over 17 days of erythroid culture and reduced viability compared to controls (Fig. 12). To decipher whether the reduced cell expansion phenotype was a direct consequence of SBDS-deficiency, *SBDS* was reintroduced into K562/shSBDS-S3 cells by the pLNCX/*SBDS*-YPET retroviral vector and cell expansion was assessed by trypan blue exclusion during erythroid differentiation. Cell expansion improved to levels better than controls (Fig. 13), confirming the growth impairment was specifically due to SBDS deficiency. As such, these results show that SBDS-deficiency in human K562 cells leads to impaired cell expansion and represents a successful model for studying erythropoietic failure in SDS.

3.3.2 SBDS-deficient K562 cells retain an ability to undergo hemoglobinization during erythroid development

Although bone marrow samples from SDS patients are deficient in erythroid cells, all stages of erythroid maturation are seen and demonstrate normal differentiation.^{16,157} This suggests that erythroid progenitors are able to undergo differentiation to completion. Hence, I hypothesized that SBDS-deficient cells would show a normal ability to undergo morphological differentiation and hemoglobinization. During erythroid commitment, K562 cells demonstrate increased expression of hemoglobin and a reduction in cell size,¹⁶⁴ as shown in (Fig. 14). To understand whether erythroid differentiation potential is intact in SBDS-deficient cells, hemoglobinization and morphology of hemin-induced K562 cells was characterized. Control and *SBDS*-knockdown cells were treated with 25 μ m for five days and daily aliquots were obtained to detect hemoglobinization using benzidine staining. As assessed by the counting of percent-positive benzidine cells in each cell line, SBDS-deficient cells showed normal hemoglobinization with S-3 cells showing generally higher levels of benzidine positive cells during erythroid differentiation (Fig. 15), though not significant. As SBDS-deficient cells did not expand at the same rate as control cells and dead undifferentiated cells do not stain for benzidine, the increased hemoglobinization in knockdown cells likely represents cells primarily driven to undergo differentiation while control cells represent both proliferating, undifferentiated cells and differentiating cell populations. These novel data suggest that at least the major erythroid differentiation property, hemoglobinization, is intact in SDS, a finding that is further supported by the presence of normal erythrocytes in SDS bone marrows.

Fig. 11: Impaired cell expansion of SBDS-deficient K562 cells induced with hemin.

K562 cells were plated at 2×10^5 cells/ml and treated with $25 \mu\text{M}$ hemin for five days. Cell expansion was assessed by trypan blue exclusion. The data represents the mean $\pm$ SE of 5 independent experiments. Statistically significant results using one-way ANOVA and Tukey's test for multiple means are marked with an asterisk ($p < 0.05$) against both wild type and SCR controls. (WT, K562/WT; SCR, K562/SCR; S-1, K562/shSBDS-1; S-3, K562/shSBDS-3)

Fig. 12: Lentiviral-mediated knockdown of SBDS in CD133+HSC/Ps impairs erythroid cell expansion

A) YFP-sorted CD133+HSC/Ps transduced with lentivirus expressing control or shRNA targeting *SBDS* were counted by trypan blue exclusion during erythroid culture. Reduced cell expansion of *SBDS*-knockdown cells compared to the control are observed. Results of a preliminary experiment are shown. **B)** Viability of the control and knockdown cells were assessed by trypan blue exclusion during erythroid culture in a preliminary study.

Fig. 13: Reduced cell expansion in stable *SBDS*-knockdown cells is specifically due to deficiency of *SBDS*.

K562 cells were plated at 2×10^5 cells/ml and treated with 25 μ m hemin for five days. Cell expansion was assessed by trypan blue exclusion. The data represents the mean $\pm$ SE of 3 independent experiments. Statistically significant results using one-way ANOVA and Tukey's test for multiple means are marked with an asterisk ($p < 0.05$) against both S-3 + YPET and S-3 cells. (SCR, K562/SCR; S-3, K562/shSBDS-3; S-3 + YPET, K562/shSBDS-3 + pLNCX/YPET; S-3 + SBDS/YPET, K562/shSBDS-3 + pLNCX/SBDS-YPET)

Multipotent/undifferentiated → Intermediate progenitors → Normoblast

Fig. 14: Erythroid commitment of K562 cells stimulated with 25µm hemin.

Normal K562 cells in different stages of erythroid development marked by an increased presence of hemoglobin as detected by benzidine staining and reduction in cell size.

Fig. 15: Erythroid differentiation potential is maintained in SBDS-deficient cells.

Benzidine stain was used to assess hemoglobinization of hemin-induced K562 cells. At least 200 cells were counted in triplicate for each data point under light microscopy showing evidence of intact erythroid differentiation potential. Normal levels of hemoglobinization were observed in *SBDS*-knockdown cells as assessed by one-way ANOVA and Tukey's test for multiple means ($p < 0.05$) against both wild type and SCR controls. The data represents the mean $\pm$ SE of 3 independent experiments.

3.4 SBDS-deficiency results in marked increase in apoptosis and a mild reduction in proliferation during erythroid differentiation

My results show that SBDS-deficiency leads to impaired erythroid cell expansion. The underlying mechanisms responsible for reduced cell expansion can be either accelerated apoptosis or reduced proliferation or a combination of both. Previous studies from our laboratory showed that SBDS-deficient HeLa cells undergo spontaneous Fas-mediated apoptosis.^{23,24} Also, clonogenic assays of CD34+ HSC/Ps from SDS marrows show a reduction in BFU-E production compared to controls.³⁷ Therefore, I hypothesized that during erythroid differentiation, SBDS-deficiency leads to both increased apoptosis and defects in proliferation.

To understand whether apoptosis is a factor leading to the reduced cell expansion phenotype noted in Figure 11, protein was harvested from K562 control and SBDS-deficient cell lines for each of the five days of hemin-induced erythroid development and analyzed for caspase-3 activation by poly (ADP-ribose) polymerase (PARP) cleavage. PARP is involved in the process of DNA repair in response to environmental stress and is a cleavage target for the effector caspase-3 during the execution of apoptosis.¹⁶⁵ Using an antibody recognizing only the carboxy-terminal catalytic domain of PARP (cleaved PARP at 89 kDa) and not full length PARP (116 kDa) or other PARP isoforms, through western blot analysis I found a more prominent 89 kDa immunoreactive band corresponding to the cleaved PARP product, in both K562/shSBDS-1 and K562/shSBDS-3 compared to control K562 cells during both non-differentiating (Day 0 in Fig. 16) and differentiating conditions (Days 1-5 in Fig. 16). These results show SBDS-deficient cells undergo increased apoptosis compared to control cells during erythroid differentiation, suggesting SBDS has a pro-survival function in erythroid differentiating cells.

To further study apoptosis and to determine whether proliferation was also affected by SBDS-deficiency, knockdown and control K562 cells were analyzed by flow cytometry after dual staining with propidium iodide and Ki-67. Propidium iodide has been used widely to analyze the DNA content for identification of pre G0/G1 (apoptotic cells), G0/G1, S phase and G2/M cells (Fig. 17a),^{23,166} while Ki-67 is a protein expressed only in cycling cells in G1, S and G2/M phase.¹⁶⁷ As shown in Figure 17b, as highlighted by the blue boxes, the apoptotic cell populations of the control and knockdown cell lines for five days of erythroid differentiation were analyzed and quantified. Non-differentiating *SBDS*-knockdown cells were slightly apoptotic than controls; however, they became markedly apoptotic after induction of differentiation (Fig. 18a). Both knockdown cell lines underwent significantly accelerated apoptosis during day 4 and 5 of differentiation (Fig. 18a).

Although impaired cell expansion can be related to delayed progression through the cell cycle or cell cycle arrest, I also analyzed the percentages of cells in each of the cell cycle phases in *SBDS*-knockdown and controls, but a significant accumulation of cells in G1/G0 of the cycle was not found. To assess the effect of *SBDS*-deficiency on proliferation, viable cells as highlighted by green boxes in Figure 17b, (which represent all cells excluding the pre G0/G1 cell population) were gated on and of the viable cells, the cell population that were expressing Ki-67 were quantified. The proliferation rate among the live S-3 cells were significantly reduced compared to controls by day 2 of differentiation (Fig. 18b) but S-1 cells showed normal proliferation, suggesting the defect in proliferation capacity is not as prominent as the increase in apoptosis which affected both knockdown cell lines. As such, to further compare the two processes, the fold increase of apoptotic cells were compared to the fold decrease in proliferating cells of the control and knockdown cell lines during differentiation (Fig. 18c). The increase in

apoptosis was 3.6 fold compared to only a 0.71 fold decrease in proliferation by day 5 of differentiation. This novel and critical result suggests that apoptosis, as opposed to a defect in proliferation, plays a more important role in mediating the slow cell expansion in SBDS-deficient K562 cells.

Fig. 16: Increased c-PARP expression in *SBDS*-knockdown cells.

SBDS-knockdown and control cells were induced to undergo erythroid differentiation with hemin. Replicate cultures were harvested and analyzed for PARP cleavage using an antibody that recognizes the large fragment (89 kDa) of human PARP produced by caspase cleavage. The figure shows more dense bands in the knockdown S-1 and S-3 cells compared to the wild type and scrambled controls. The values underneath the blots represent c-PARP/β-actin ratios. A representative diagram of three independent experiments is shown.

Fig. 18: Apoptosis is the prominent mechanism of reduced cellularity in *SBDS*-knockdown cells

A) Enumeration of the apoptotic cells (which are to the left of the peak of G0/G1). Apoptosis in the *SBDS*-knockdown cell lines dramatically increases after induction of differentiation (day 1-5). **B)** Quantification of viable cells expressing the Ki-67 proliferation marker as assessed by flow cytometry. There is significant reduction in proliferation of K562/sh*SBDS*-3 cells. **C)** Quantification of cells in G1/G0. No significant accumulation of cells in G1/G0 is observed for the *SBDS*-deficient cells during differentiation. The data from A, B, and C represent the mean $\pm$ SE of 4 independent experiments. Statistically significant results using one-way ANOVA and Tukey's test for multiple means are marked with an asterisk ($p < 0.05$) against both wild type and SCR controls. **D)** Comparison of the fold apoptosis versus fold proliferation, showing apoptosis to be the main mechanism of reduced cellularity in *SBDS*-knockdown cells. The values for apoptosis and proliferation were normalized to the value obtained for each day in control K562 cells, and assigned a value of 1.0 for proliferating cells and apoptotic cells.

3.5 SBDS-deficient K562 cells manifest abnormal ribosomal profile during erythroid differentiation but not in an undifferentiated state

3.5.1 Ribosomal profiles of SBDS-deficient K562 cells show loss of polysomes and reduced 80S subunits during erythroid differentiation

In the above described experiments, I showed that loss of SBDS during erythroid differentiation results in reduced erythroid cell expansion due to an increase in apoptosis and, to a lesser degree, due to a reduction in proliferation. It is unknown, however, what events trigger apoptosis or inhibit proliferation during differentiation. As SBDS has recently been proposed to play a role in ribosome biogenesis^{20,107,135} and since expanding cells devote most of their energy to this process, I hypothesized that defects in basic cellular processes caused by SBDS deficiency, such as ribosome biogenesis, would be exacerbated during erythroid cell expansion.

To determine whether SBDS deficiency impaired ribosome biogenesis during differentiation, I investigated sucrose gradient polysome profiles of normal and differentiating *SBDS*-knockdown and control cells. In this method 40S, 60S, and 80S ribosome subunits and polysomes were separated based on their density by ultracentrifugation (Fig. 19a). Non-differentiating *SBDS*-knockdown cells showed slightly reduced amounts of 80S subunits compared to controls, but no further differences were observed in the relative amounts of the 40S, 60S, and polysomes (Fig. 19b). By contrast, during erythroid differentiation *SBDS*-knockdown cells showed markedly impaired ribosome profiles. After 72 hours, both knockdown cell models manifested markedly reduced polysomes (Fig. 19c). K562/shSBDS-3 cells, which express lower levels of SBDS, also showed global reduction in all ribosome subunits including 80S, 40S and 60S subunits. These results indicate that SBDS has a more prominent role in promoting ribosome biogenesis during erythroid differentiation than in non-differentiated conditions.

3.5.2 Dissociation of ribosomal subunits show SBDS deficiency results in general reduction of 40S and 60S subunits during hemin-induced erythroid differentiation

The main abnormality seen in the ribosome profile studies of SBDS-deficient cells during differentiation was marked reduction in levels of the polysomes. The limitation of this method is the difficulty in accurately evaluating the amounts of and the ratio between 40S and 60S subunits.¹⁶⁸ This information is valuable since SBDS was implicated in the biogenesis of the 60S subunit in yeast²⁰ and disruption in either 60S or 40S subunits can lead to relative reduction in the level of 60S subunit.¹⁶⁹ As such, ribosome profiles were prepared in the presence of EDTA to allow for dissociation of subunits. This approach showed that non-differentiating *SBDS*-knockdown cells had normal levels of 40S and 60S subunits (Fig. 20a) and upon differentiation, there was also no significant reduction in the 60S/40S ratio in knockdown cells. Instead, a general reduction of 40S and 60S subunits was observed, particularly in K562/shSBDS-3 (Fig. 20b). These results are consistent with human SBDS-deficient fibroblasts also showing no reduction in the relative ratio between the 60S and 40S subunits,²¹ suggesting loss of SBDS in human cells contributes to a global defect in ribosome biogenesis as opposed to impaired biogenesis of a specific subunit.

To confirm that the abnormalities in the ribosome profiles of SBDS-deficient cells were an effect of differentiation, and not due to an apoptotic cell phenotype, dead cells were removed from the cell medium by the Dead Cell Removal Kit (Miltenyi Biotec, Auburn, CA). Profiles were then prepared from SCR control and K562/shSBDS-3 knockdown cells, which showed 97% viability by trypan blue exclusion (Fig. 21). General reduction of 80S subunits and loss of polysomes were observed in the *SBDS*-knockdown cells compared to the control, indicating that the ribosome defects observed were due to the effect of SBDS-deficiency during stimulation of erythroid differentiation and not simply a reflection of apoptotic cells.

Fig. 20: Ribosome profiles of SBDS-deficient and control K562 cells under EDTA dissociating conditions.

A) The ribosomal profiles of undifferentiated control and SBDS-deficient K562 cells were analyzed by sucrose gradient density centrifugation under conditions that allowed dissociation of the 40S and 60S subunits. A representative diagram of two independent experiments is shown. **B)** Ribosome profiles after 48 hours of hemin-induction under dissociating conditions. A representative diagram of two independent experiments is shown. On day 0 (no differentiation) and day 2, cells were harvested using TMK100 lysis buffer with cycloheximide and EDTA. Equal amounts of RNA were layered on 5-35% sucrose gradients, as measured by A260nm. Absorbance of each fraction at A254 was done by the Brandel Density Gradient Fractionator. During differentiation, both knockdown cell lines showed general reduction in 40S and 60S subunits, but no significant reduction in the ratio between 60S and 40S subunits, as calculated by measuring area under the curve, using Adobe Illustrator.

Fig. 21: Ribosome profiles of differentiating K562 cells after removal of dead cells.

To study whether the abnormalities in the differentiating SBDS-deficient K562 cells were an effect of an apoptotic cell phenotype, dead cells were removed using the Dead Cell Removal Kit (Miltenyi, Auburn, CA) and ribosome profiles of K562/SCR and K562/shSBDS-3 were obtained. Reduced 80S subunits and loss of polysomes (as highlighted in red) were observed in viable cells of the knockdown S-3 cell line, compared to SCR control cells.

3.6 SBDS-deficient K562 cells exhibit reduced global translation, which is heightened during erythroid stimulation

3.6.1 Global translation in hemin-induced and uninduced SBDS-deficient K562 cells is reduced compared to controls

As a generalized reduction in the ribosome profiles during differentiation of *SBDS*-knockdown cells was identified, I next investigated whether these defects led to ribosome dysfunction by assessing global translation. Incorporation of ^{35}S -methionine/cysteine into equal amounts of newly synthesized trichloroacetic acid–precipitable peptides was measured as precipitable counts per minute (cpm) in non-differentiating and differentiating K562/SCR and K562/shSBDS-3 cells. Global protein synthesis of non-differentiating K562/shSBDS-3 cells was significantly reduced to 72.3% of K562/SCR control cells (Fig. 22 – Day 0). After 48 hours of hemin-induced erythroid differentiation, the reduction of protein synthesis became more prominent as translation in K562/shSBDS-3 cells was reduced to 42.5 % of K562/SCR control cells (Fig. 22 – Day2). To further assess the specific role of SBDS-deficiency in translation, *SBDS* was re-introduced into K562/shSBDS-3 cells by the pLNCX/SBDS-YPET retroviral vector. Re-introduction of SBDS led to restoration of global translation during hemin-induced erythroid differentiation (Fig. 23). These results show SBDS is necessary for normal protein translation, particularly during erythroid differentiation.

3.6.2 Leucine improves translation of SBDS depleted K562 cells and shows significantly increased cell expansion.

To determine whether differentiating SBDS-deficient K562 cells expand slowly because of translation insufficiency, I tested the ability of a protein synthesis stimulator to rescue the slow cell expansion phenotype. Protein synthesis can be stimulated by agents such as leucine that activate the mTOR pathway.¹⁷⁰⁻¹⁷⁵ Indeed, a previous study tested the efficacy of nutritional supplementation with the amino acid leucine as a potential therapy for Diamond Blackfan

anemia and showed improved erythropoiesis in one patient.¹⁷⁶ To determine an optimal leucine concentration, I incubated K562/shSBDS-3 cells with escalating doses of leucine (0-1 mg/ml) in both differentiating and non-differentiating conditions. The optimal leucine concentration identified for further studies was determined to be 600 μ g/ml (Fig. 24a). Leucine treatment significantly improved global translation efficiency in both non-differentiating K562/SCR and K562/shSBDS-3 cells, although not completely to normal levels (Fig. 25). Subsequent studies on the effect of leucine on cell expansion of non-differentiating K562/shSBDS-3 cells showed significant improvement of cell expansion to near normal levels when treated with 600 μ g/ml of leucine (Fig. 26). However, leucine alone did not have the same positive effect on differentiating *SBDS*-knockdown K562 cells (Fig. 27). It is possible that during differentiation, more prominent improvement in translation is necessary by additional agents such as insulin or insulin growth factor, which stimulate protein synthesis in part by activation of the PI3K/mTOR pathways¹⁷⁷ and support proliferation and terminal differentiation of erythrocytes.¹⁷⁸ These studies show translational insufficiency in non-differentiating *SBDS*-deficient cells cause reduced cell expansion, however the defects in the expansion of maturing *SBDS*-deficient cells is more complex as leucine was not sufficient to rescue the impaired growth phenotype.

Fig. 22: SBDS deficiency leads to insufficient translation which becomes more prominent during erythroid differentiation.

Translation in S-3 is reduced to 72.3% of the control in non-differentiating K562 cells, while after 48 hours of hemin-induced erythroid differentiation, translation in S-3 cells is reduced to 42.5% of controls. Protein synthesis was studied by measuring incorporation of ³⁵S-methionine/cysteine into equal amounts of newly synthesized TCA-precipitable peptides. The data represents the mean $\pm$ SE of 3 experiments. Statistically significant results using t-test are marked with an asterisk, ($p < 0.05$).

Fig. 23: Reduced global translation in stable *SBDS*-knockdown cells is specifically due to deficiency of *SBDS*.

Translation in S-3 cells is improved after re-introduction of *SBDS*. Protein synthesis was measured by detecting incorporation of ³⁵S-methionine/cysteine into equal amounts of TCA-precipitated peptides. The data represents the mean $\pm$ SE of 3 experiments. Statistically significant results using one-way ANOVA and Tukey's test for multiple means are marked with an asterisk ($p < 0.05$) against both S-3 + YPET and S-3 cells.

Fig. 25: Leucine improves translation of non-differentiating K562 cells.

Translation in S-3 cells and SCR cells is improved with the addition of 600 $\mu\text{g/ml}$ leucine. Protein synthesis was studied by measuring incorporation of ^{35}S -methionine/cysteine into equal amounts of newly synthesized trichloroacetic acid-precipitable peptides. The data represents the mean $\pm$ SE of 3 experiments. Statistically significant results using t-test are marked with an asterisk, ($p < 0.05$).

Fig. 26: Cell expansion improves in SBDS-deficient cells treated with leucine.

MTT assay was used to measure cell expansion of non-differentiating K562/SCR and K562/shSBDS-3 cells treated with 600 $\mu\text{g/ml}$ of leucine. Cells were plated at 2×10^4 cells/well in triplicate and daily assessment for cell growth was measured for four days. The data represents the mean $\pm$ SE of 3 separate experiments. Statistically significant results using t-test are marked with an asterisk, (day 2 $p < 0.005$, day 3 $p < 0.01$, day 4 $p < 0.01$).

Fig. 27: Leucine is not sufficient to improve cell expansion defects in differentiating *SBDS*-knockdown cells

MTT assay was used to measure cell expansion of differentiating K562/SCR and K562/shSBDS-3 cells treated with 600 µg/ml of leucine. Cells were plated at 2×10^4 cells/well in triplicate and daily assessment for cell growth was measured for five days. The data represents the mean $\pm$ SE of triplicate wells.

3.7 SBDS-deficient K562 cells express higher levels of TAp73 than control cells only in the non-differentiated state

To study pathways that can mechanistically link ribosome and translation defects, accelerated apoptosis and failed erythropoiesis, I investigated the expression of p53 and related family members. K562 cells are deficient in p53 as the gene is inactivated by bi-allelic nonsense mutations.¹⁷⁹ Furthermore, p63 isoforms were not expressed in K562 cells by western blot analysis. However, several isoforms of p73 were detected. Interestingly, the level of the pro-apoptotic isoform, TAp73, was higher in non-differentiating S-3 cells compared to the K562/SCR control cells (Fig. 28a). There was also an accumulation of Δ Np73 in the S-3 cell line likely needed to stabilize the high expression of TAp73.¹²⁵ During differentiation, however, TAp73 expression in the knockdown cell line was dramatically reduced compared to the K562/SCR control cells (Fig. 28b). These data suggest that since the pro-apoptotic TAp73 isoform of p73 was not activated during hemin-induced erythroid differentiation of SBDS-deficient K562 cells the increased apoptosis of these cells is mediated independent of p73 isoforms.

CHAPTER IV DISCUSSION

The present study was aimed at characterizing the mechanism of anemia in SBDS-deficient cells using human erythroleukemic K562 cells and CD133+ HSC/Ps isolated from CB. Our lab successfully generated cellular models of anemia in SDS through shRNA-mediated suppression of the SBDS gene in K562 cells undergoing erythroid differentiation. Using these models, I showed that SBDS-deficiency leads to impaired erythroid cell expansion, a significant increase in apoptosis, and a moderate decrease in proliferation. Furthermore, I showed that during erythroid differentiation, the characteristic abnormalities in the ribosomes and global translation of resting SBDS-deficient K562 cells became more prominent. Importantly, the addition of the translation stimulator, leucine, to non-differentiating SBDS-deficient erythroid cells restored normal cell growth, indicating that the impaired cell growth of SBDS-deficient cells is caused by translation insufficiency. However upon differentiation, the reduced cell expansion phenotype was not rescued with leucine treatment. As p53 and p63 have been reported to mediate apoptosis upon ribosomal stress, I asked whether p53 family members mediated apoptosis in the SBDS-deficient cells. K562 cells do not express p53¹⁷⁹ and were found to express p63 either. In addition, pro-apoptotic TAp73 was not upregulated in differentiated SBDS-deficient K562 erythroid cells. Taken together, these studies indicate the role of SBDS changes during erythroid maturation and that SBDS plays a critical role in erythroid development as a promoter of survival of early erythroid progenitors and in maintaining ribosome assembly and function during erythroid maturation through a p53/p63/p73-independent pathway. A model summarizing these findings is shown in Fig. 29.

Fig. 29: Model for the mechanisms of erythropoietic failure caused by loss of SBDS

The role of SBDS changes during the transition from a non-differentiated to a differentiated cell and thus represents two distinct scenarios. In the non-differentiated cell, SBDS deficiency leads to reduced 80S subunits, and a reduction in global translation, which may be sufficient to activate p53 family members, subsequently leading to slower cell expansion. Upon differentiation, SBDS deficiency leads to an increase in apoptosis, a reduction in proliferation and a more prominent defect in ribosome biogenesis and translation, all contributing to the reduced erythroid cell expansion defect, through a pathway independent of p53 family members.

4.1.1 *SBDS* expression in erythroid differentiating cells

Expression analysis can provide important insight into the stages in development in which gene function is critical. As such, *SBDS* expression was analyzed in differentiating erythroid cells derived from CB CD133+/HSC/P cells and K562 cells. Up-regulation of *SBDS* was detected in freshly isolated CD133+/HSC/P cells followed by a gradual decrease during erythroid commitment, with a moderate increase in expression on day 7 of differentiation. An up-regulation of *SBDS* expression was also observed after 24 hours of hemin treatment in K562 cells followed by a subsequent return to baseline levels for the next 4 days. The initial surge in *SBDS* expression in K562 cells is probably triggered by the hemin-induced differentiation signal, while the cultured primary HSC/P cells are already exposed to differentiating cytokines *in vivo* and continue the differentiation process *in vitro* and hence already show increased expression. Alternatively, up-regulation of *SBDS* shortly after hemin treatment in K562 cells may correspond with the upregulation at a time point not collected, such as day 1 of differentiation of CD133+/HSC/P cells. Although comparison of erythroid differentiation of cultured HSC/P cells and K562 has limitations because the cells represent different stages of erythroid development, these results do suggest that *SBDS* is required for early erythroid differentiation and is present in lower levels throughout differentiation. Indeed, protein expression of SBDS during differentiation of K562 cells showed consistent level of expression throughout differentiation with no significant changes in expression, which may in part be a reflection of the long half-life of SBDS,¹⁶² such that transient changes at the mRNA level were not detected at the protein level.

This study is the first to describe *SBDS* expression in erythroid differentiating cells. Interestingly, the results are consistent with the expression pattern of SBDS seen in murine myeloid 32Dcl3 cells undergoing neutrophil differentiation. SBDS showed a similar early upregulation followed

by a decline in expression during neutrophil commitment.³⁶ In this study, I did not focus on differentiation into granulocytic and megakaryocytic lineages, which are also affected in SDS. However, the early high expression of *SBDS* in the stage of CD133+ cells, which comprise long-term HSC/Ps, short-term HSC/Ps and common multipotent progenitor cells, may explain why deficiency leads to defects in all lineages.

Comparing the expression pattern of *SBDS* during erythropoiesis to other inherited marrow failure genes may be useful, particularly if they share similar expression patterns, since they may share similarities in the pathogenesis of anemia. Indeed, the murine model of Fanconi anemia showed the fanconi anemia C (*FANC*) gene is expressed in a broad variety of tissues¹⁸⁰ and its expression is high in actively dividing progenitor cells but is down-regulated in differentiated cells.¹⁸¹ In addition, the expression analysis of *RPS19*, a gene found mutated in 25% of Diamond Blackfan anemia patients,^{182,183} showed a gradual reduction in mRNA and protein levels during terminal erythroid differentiation of FAV cells.¹⁸⁴ A consistent trend in all three inherited marrow failure genes is downregulation during late stages of differentiation, suggesting early defects prior to terminal differentiation contribute to the respective disease states.

4.1.2 *SBDS*-deficiency results in reduced erythroid cell expansion but normal erythroid maturation.

After identifying that *SBDS* is expressed throughout erythroid development with an upregulation during the early maturation stage, our lab generated two successful K562 *SBDS*-knockdown cell lines to study the *SBDS* function during differentiation. Both K562 *SBDS*-knockdown cell lines showed similar phenotypes. However, K562/sh*SBDS*-3 cells, whose *SBDS* expression was lower than K562/sh*SBDS*-1, manifest a more severe phenotype. *SBDS* knockdown in both the primary HSC/P cells and K562 cells showed impaired cell expansion. Defects in cell expansion

mimic the anemia seen in 60-80% of SDS patients;^{16,37} validating our *SBDS*-knockdown model for studying erythropoietic failure in SDS. Furthermore, re-introduction of *SBDS* into K562/sh*SBDS*-3 cells fully rescued the reduced cell expansion phenotype, indicating that *SBDS*-deficiency is the cause of the observed impaired cell expansion phenotype, and that overexpression of *SBDS* can improve cell expansion. This is the first study to prove that *SBDS* has a direct role in erythroid cell expansion and further corroborates studies in *SBDS*-deficient HeLa cells showing slow growth.^{23,110}

Importantly, cell expansion was significantly impaired upon *SBDS*-deficiency in K562 cells, yet differentiation potential was maintained. Although further studies on differentiating *SBDS*-deficient CD133+ HSC/Ps are required to confirm this result, it does suggest that *SBDS* has a primary role in promoting cell expansion and does not affect differentiation capacity. This finding is consistent with studies in *SBDS*-deficient 32Dcl3 cells undergoing neutrophil differentiation, which also show normal terminal maturation of neutrophils.³⁶ Furthermore, it has been reported that CD34+ HSC/P cells from patients lacking *SBDS* could differentiate into functional neutrophils.^{22,103} In contrast, although also affected with reduced erythroid cell proliferation, both lentiviral-mediated and zebrafish models for RPS19-deficient Diamond Blackfan anemia show reduced hemoglobinization and decreased erythroid-committed progenitors and mature erythroid cells.^{152,185,186} In addition the RPS14-deficient model of the 5q⁻ syndrome, which is characterized by severe macrocytic anemia, normal or elevated platelet counts, normal or reduced neutrophil counts, and erythroid hypoplasia in the bone marrow, also shows blocked production of terminally differentiated erythroid cells and an increase in the ratio of immature-to-mature erythroid cells⁵³ suggesting alternative hematopoietic and molecular mechanisms are responsible for the failed erythropoiesis in Diamond Blackfan anemia and 5q-

syndrome in comparison to SDS. Since SBDS-deficient K562 cells showed normal morphological differentiation and hemoglobinization, I focused my investigation on the mechanism of the slow cell expansion SDS disease phenotype.

4.1.3 Accelerated apoptosis and reduced proliferation limits the cell expansion capacity of SBDS-deficient erythroid cells

Several underlying reasons may be the cause of impaired cell expansion observed in erythroid differentiating SBDS-deficient cells. As previous studies have reported SDS marrows and SBDS-deficient cells show accelerated Fas-mediated apoptosis,^{23,24,111} and reduced clonogenic potential of CD34+ cells from SDS marrows, I hypothesized increased apoptosis, reduced proliferation or a combination of both are responsible for the cell growth defects in differentiating SBDS-deficient cells. Some features marking activation of the apoptotic pathway include caspase-3 activation, blebbing of the plasma membrane, DNA fragmentation, and exposure of phosphatidyl serine to the plasma membrane. As such, apoptosis was analyzed by detecting caspase-3 activation by PARP cleavage and DNA fragmentation was assessed by DNA content analysis to study apoptotic cells in the pre-G1/G0 phase. Furthermore, proliferation was assessed by analysis of cycling cells expressing Ki-67 during G1, S, G2, and M phase. I showed that during erythropoiesis, *SBDS* knockdown cells undergo accelerated apoptosis but also manifest a mild reduction in proliferation. This approach suggests that the decreased cell growth observed in erythroid differentiating *SBDS* knockdown cells is predominantly caused by apoptosis and, to a lesser extent, decreased proliferation. However, further examination of the apoptotic pathway is necessary to prove the knockdown cells do show increased apoptosis by studying caspase-3 cleavage, as well as Annexin V/PI staining, to study exposure of phosphatidyl serine to the plasma membrane, and thereby determining the relative contribution of necrosis and apoptosis in cell death during erythroid differentiation.

As SBDS has a postulated role in ribosome biogenesis and in promoting global translation, this would suggest loss of SBDS results in major defects in both apoptosis and proliferation. However, in depth studies on the effects of inhibiting ribosome biogenesis or translation on apoptosis or proliferation in SBDS-deficient cells have yet to be investigated and hence whether reduced proliferative capacity is a major or minor defect is still up for debate. It is not surprising that our results support apoptosis to be central in the pathogenesis of erythropoietic failure in SDS, as previous reports from our lab on SDS marrows and SBDS-deficient HeLa cells demonstrate accelerated Fas-mediated apoptosis.^{23,24,111} Indeed, induction of apoptosis plays a more prominent role in the pathogenesis of other ribosome biogenesis disorders compared to a defect in proliferation. For example, lymphocytes from Cartilage-hair hypoplasia patients show increases in apoptosis, mediated by the Fas/FasL signaling pathway.¹⁸⁷ Furthermore, studies on two lymphoblastoid cell lines from Dyskeratosis congenita patients showed increased apoptosis, but normal rRNA transcription and proliferative capability, indicating that the reduced growth rate of the Dyskeratosis congenita cell lines was not due to an impaired proliferative capacity or abnormal ribosomal biogenesis but to a progressive increase in programmed cell death.¹⁸⁸ Further investigation into the effect of general caspase inhibitors on apoptosis during induction of erythroid development in SBDS-deficient cells will provide additional insight into the relative contribution of apoptosis to the reduced erythroid cell expansion.

4.1.4 Abnormalities in ribosome biogenesis and function likely trigger downstream events leading to reduced erythroid cell expansion

As SBDS is postulated to function in ribosome biogenesis^{20,107,135} I next wanted to understand whether SBDS deficiency led to impaired erythroid cell expansion due to abnormalities in ribosome biogenesis and translational insufficiency. SBDS-deficiency led to marked loss of polysomes, and a general reduction of 40S, 60S and 80S subunits resulting in subsequent

translational defects during erythropoiesis. These results implicate SBDS in the maintenance of normal ribosome assembly and function particularly during erythroid development. As studies on yeast²⁰ and human cells²¹ show different levels of 60S subunit production, I investigated dissociated ribosome profiles in *SBDS*-knockdown cell lines and showed no consistent decrease in 60S/40S subunit ratios, consistent with what was reported in *SBDS*-deficient human fibroblasts.²¹ Although much of our current understanding of ribosome biogenesis comes from studies in yeast, pre-rRNA processing and assembly pathways between the two species are not identical and could well explain the observed differences.¹³⁰ The finding that during erythroid differentiation *SBDS* deficiency led to a general reduction in all ribosome subunits and polysomes has several important implications. With the genetic defect unknown in approximately 10% to 17% of SDS cases (unpublished data from CIMFR), this finding suggests that dysregulation or mutations of other ribosome related proteins affecting either the large or small subunit may also lead to SDS. This possibility is supported by a previous study in our lab showing *SBDS*-deficient marrows show dysregulation of ribosome proteins of both the large and small ribosome subunit.¹⁹ Alternatively, it is also plausible the general reduction in all ribosome subunits in differentiating *SBDS*-deficient cells begins at the level of RNA polymerase I resulting in reduced rRNA transcription. Supporting this possibility, Ganapathi et al. showed general reduction in rRNA synthesis in *SBDS*-deficient human skin fibroblasts.²¹ In terms of understanding the molecular pathogenesis of erythropoietic failure in SDS, the finding of globally reduced ribosome biogenesis by loss of *SBDS* adds significant support to the hypothesis that *SBDS* has a critical role in ribosome synthesis during erythroid development.

An important question that remained was whether abnormalities in ribosome biogenesis directly caused cellular growth deficiencies by triggering apoptotic pathways or whether a loss of *SBDS*

directly initiated apoptosis and/or inhibited proliferation which then led to secondary effects on ribosomal and other cellular activities. To answer this question, I used the protein synthesis stimulator, leucine, to determine whether impaired cell growth was directly due to abnormalities in ribosome biogenesis. Leucine improved not only translation but also cell growth of non-differentiating K562/shSBDS-3 cells and, hence, provides some support for the former hypothesis. Under differentiating conditions, however, leucine alone did not have a similar response in K562 cells. It is possible that during differentiation of this cell model, additional factors or a combination of factors including insulin or insulin growth factor are required for leucine to rescue the cells. Alternatively, translational insufficiency may not be the sole cause for reduced erythroid cell expansion defects. Further study into the effect of leucine and additional translation stimulating agents on erythroid-committed SBDS-deficient HSC/P cells and SDS patient cells will provide additional insight into the involvement of translational insufficiency in impaired erythroid cell expansion.

4.1.5 Differentiating SBDS-deficient erythroid cells undergo accelerated apoptosis independent of the p53 family members

As both defects in ribosome production and accelerated apoptosis of SBDS-deficient K562 cells were demonstrated, investigation into p53 and its family members were approached as possible candidates linking the abnormalities. In K562 cells, the p53 gene is inactivated by bi-allelic nonsense mutations,¹⁷⁹ and hence its expression was not detected. Expression of p53 and any of the p63 isoforms were not detected in the wild type and in the *SBDS*-knockdown K562 cells; thereby excluding their role in activating apoptosis in this model. Interestingly, expression of TAp73, which transactivates p53 target genes and induces apoptosis,¹⁸⁹ was elevated in non-differentiating K562/shSBDS-3 cells. Although TAp73 and its direct link in ribosomal disorders have yet to be studied, the p73 isoform has been shown to interact with MDM2, a negative

regulator of p53 and transactivates an overlapping set of p53 target genes.^{126,127} As such, the results are consistent with studies that showed altered pre-ribosomal assembly triggers activation of the tumor suppressor p53 *in vitro*¹⁹⁰⁻¹⁹² and *in vivo*.^{190,193,194} Furthermore, SDS marrows overexpress p53¹¹⁵ while SBDS-deficient SDS marrows show specific upregulation of a p53 target gene, RPS27L, which is a ribosomal protein that contributes to p53-induced apoptosis.¹⁹⁵ Taken together, in non-differentiating conditions, the p73 member of the p53 family might be activated as a result of *SBDS* deficiency, and promote apoptosis through effects at the transcriptional level.

Interestingly, the expression of TAp73 was lost in differentiating *SBDS*-knockdown K562 cells, suggesting that apoptosis in differentiating cells is regulated, at least in part, in a TAp73-independent manner. It is possible that in addition to the loss of TAp73, a decrease in Δ Np73 increased the sensitivity of the knockdown cells to alternative apoptotic pathways. This is consistent with a previous study on differentiating K562 cells showing knockdown of Δ Np73 reduced TAp73 levels and showed slower cell growth.¹²⁵ It is possible that re-introduction of Δ Np73 would rescue accelerated apoptosis seen in erythroid differentiating *SBDS*-deficient cells. In addition, as K562 cells are an erythroleukemic cell line, various signaling pathways may already be dysregulated. Therefore, further studies in lentiviral-mediated knockdown of *SBDS* in CD133+/HSC/Ps and SDS patient cells will provide a better understanding of the role of p53 and its family members in relation to *SBDS* deficiency.

4.1.6 Extra-ribosomal functions and alternative mechanisms of erythropoietic failure in SDS

A central, unresolved question is how mutations in *SBDS* or other inherited marrow failure syndrome genes cause more severe hematological phenotypes than non-hematological

phenotypes despite ubiquitous expression and involvement in fundamental processes such as ribosome biogenesis. Although differential requirement for translational activity is one plausible explanation for tissue specificity, the loss of unknown extra-ribosomal functions of SBDS is another possibility. Multiple functions have been identified for a number of inherited marrow failure genes, including *RPS19*, *RNase MRP* and *DKC1*.^{152,196-198} For example, *RPS19* interacts with the growth factor FGF-2,¹⁹⁹ the protein kinase PIM-1,²⁰⁰ and a protein of unknown function named S19-BP.²⁰¹ It is certainly not beyond the scope of SBDS to have several cellular functions as SBDS has already been shown to interact with nucleophosphomin, a multifunctional protein implicated in ribosome biogenesis, leukemogenesis, and centrosomal amplification²¹ and several proteins involved in DNA metabolism including DNA-dependent protein kinase (DNA-PK) and Replication protein A 70 kDa (RPA-70).¹³⁵ As the interaction of SBDS with these proteins are further characterized, more clues for the tissue-specific cellular functions for SBDS will be elucidated. Interestingly, expression analysis by microarray of SBDS-deficient SDS marrow cells showed dysregulation of several ribosomal proteins including down-regulation of RPS9, RPS20, RPL5, RPL6, RPL15, RPL22, RPL23 and RPL29 and up-regulation of RPS27L.¹⁹ Indeed, ribosomal proteins are reported to have extra-ribosomal functions.¹³⁸ Disruption of the extra-ribosomal functions of the dysregulated RPs in SBDS-deficient SDS bone marrow cells may well play a mechanistic role in the tissue specific phenotype of SDS.

The finding of reduced levels of pro-apoptotic p73 isoforms and lack of presence of other p53 family members in differentiating SBDS-deficient K562 cells, suggests alternative molecular mechanisms could be involved in the pathogenesis of anemia in SDS. Various studies have shown that reactive oxygen species (ROS) are important signaling molecules that at low levels can promote cell survival and growth, but at higher levels can be detrimental leading to induction

of apoptosis.²⁰⁶ As such ROS has been shown to play an important role in the regulation of internal and receptor-mediated apoptosis including the Fas pathway.²⁰²⁻²⁰⁵ As previous studies report that loss of SBDS results in increased Fas-mediated apoptosis and slower cellular growth, it is possible that the aforementioned abnormalities are mediated by defects in ROS regulation. Interestingly, elevated ROS levels and increased spontaneous apoptosis are observed in SBDS-deficient HeLa cells (unpublished data from our lab). Upon the addition of antioxidants ascorbic acid (AA) and N-acetylcysteine (NAC), ROS levels decrease, apoptosis is reduced and cell growth enhanced (unpublished data from our lab), indicating that cell growth defects due to loss of SBDS may be an affect of oxidative stress.

As red blood cells are under constant exposure to ROS and oxidative stress due to their role as O₂ and CO₂ transporters, it is plausible that excessive oxidative stress due to SBDS deficiency results in reduced survival of SDS erythrocytes. As the present study has shown SBDS deficiency leads to abnormal ribosome biogenesis during erythroid development, and since SBDS-deficiency results in the dysregulation of RPs,¹⁹ it is possible that SBDS has a role in protecting ribosome assembly and ribosomal proteins against oxidative damage, which becomes more prominent during erythropoiesis.

Another clinical feature of SDS is exocrine pancreatic insufficiency. As such, low levels of pancreatic enzymes, such as trypsinogen, are reported in SDS patients.³⁰ As a result, it is plausible that insufficient amino acids are circulating in the body and cells may be constantly subjected to nutrient starvation. Interestingly, it has been reported that nutrient starvation stimulates ROS production²⁰⁶ and induces autophagy.²⁰⁷ Indeed, a recent study showed elevated ROS levels are essential for the induction of autophagy as treatment with antioxidants showed

impaired autophagosome formation.²⁰⁶ As reduced polysomes were a defining feature of differentiating SBDS-deficient K562 cells, aberrant autophagy and more specifically, altered ribophagy, a selective form of autophagy targeting ribosomes,²⁰⁸ may be a pathway warranting further investigation into the underlying cellular pathogenesis of SDS. Ribophagy may be involved as a compensatory mechanism that allows survival of cells during prolonged periods of starvation, as degradation of excessive ribosomes serves to shut down protein translation rapidly and provide an important source of new building blocks to maintain cellular homeostasis.²⁰⁸ Further investigation on ROS levels and ribophagy in differentiating SBDS-deficient K562 cells, and the effect of NAC or AA on ribosome biogenesis will elucidate whether SBDS is a protector against oxidative stress during erythropoiesis. As well, studies on ROS levels in other cell types affected in SDS patients including neutrophils, pancreas and skeletal tissues are necessary to further examine the pathologic process of SDS.

4.2 Potential Limitations of this study

A number of limitations may have influenced this study. Ideally, the use of SBDS-deficient SDS patient CD133+/HSC/P cells cultured in erythroid differentiating conditions would provide a wealth of information about erythropoiesis in SDS. However, due to the problem of limited availability of cells from SDS patients, K562 cells were used for this study. Although K562 cells are of human origin and can be used to study a process of differentiation and cell division characteristic of normal human erythropoiesis, they are a neoplastic cell line and, hence, simply represent a model for better understanding the underlying molecular mechanisms involved in the pathogenesis of anemia in SDS. Further characterization shows that K562 cells are aneuploidic and are Philadelphia chromosome positive (t9:22), hence showing dysregulated constitutive expression of *BCR-ABL*.²⁰⁹ As such, dysregulation of various pathways involved in

differentiation, proliferation and apoptosis are likely already occurring in such transformed cells and, hence, do not replicate exactly the underlying signaling pathways of normal hematopoietic cells or diseased SDS cells. Furthermore, the observation of early stages in erythropoiesis from the long-term HSC to the BFU-E stage is not possible with K562 cells; nor is terminal differentiation into mature erythrocytes achieved with this cell line. As such, the use of K562 cells in this study represents a specific period of erythroid development from the megakaryocyte-erythroid progenitor (MEP) to late normoblast. Furthermore, parallel studies using an additional cell line such as TF-1 cells, which can also be induced to express erythroid properties,²¹⁰ may further validate the results shown with K562 cells.

This study used the powerful tool of vector-based RNA interference to study SBDS function. Although this approach has been used extensively for functional genomics, it has its limitations. For instance, RNAi not only degrades mRNA transcripts and induces translational inhibition, but is also capable of influencing mRNA transcription by induction of DNA methylation²¹¹ and heterochromatin formation.²¹² By designing shRNA sequences to target *SBDS* and generating a non-specific shRNA to use as a negative control, I ensured the results of the shRNA were specific to *SBDS* and did not lead to off-target effects. Nevertheless, additional methods of inhibiting SBDS function would be beneficial to validate the results of this study using vector-based RNA interference. For example, using a specific pharmacological agent to inhibit SBDS would be extremely beneficial. However, as of yet, no such agent has been identified.

4.3 Future directions

To further study whether the abnormalities in apoptosis, proliferation and ribosomes of differentiating SBDS-deficient K562 cells is specifically due to SBDS deficiency, re-

introduction of SBDS into K562/shSBDS-3 cells will be done. Apoptosis and proliferation of these and YPET only control cells will be assessed by Ki-67/PI staining by flow cytometry. By re-introducing SBDS, it is expected that there will be a reduced proportion of apoptotic cells compared to the YPET control cells. Further analysis of the ribosome profiles of the knockdown cells in which SBDS has been re-introduced in comparison to YPET only controls by sucrose density gradient centrifugation will also be performed. It is expected that there will be normal levels of ribosomal subunits and polysomes in the SBDS expressing cells, showing that indeed the abnormalities observed in the SBDS-deficient cells were caused by loss of SBDS.

Studies with the erythroid differentiating SBDS-deficient K562 cells provided a strong basis for understanding general cellular defects in apoptosis, proliferation and translation. However, further studies in SBDS depleted CD133+/HSC/Ps would provide a better model for understanding both the cellular and molecular abnormalities leading to anemia in SDS. Currently, we are in the process of generating a lentiviral vector-based RNA interference approach to knockdown *SBDS* in CD133+/HSC/P cells. Parallel to our studies in K562 cells, lentiviral *SBDS*-knockdown primary HSC/P cells will be used to primarily assess erythroid cell expansion, apoptosis, proliferation and translational defects. More specifically, as leucine did not rescue the reduced erythroid cell expansion phenotype in K562 cells, a study assessing the effect of leucine and additional protein synthesis stimulators such as insulin and insulin growth factor on translation and cell expansion of differentiating SBDS-deficient K562 and CD133+/HSC/P cells will be examined. It is hypothesized the combination of leucine and insulin will show improved translation and cell expansion. To further identify if translational defects are the main cause for reduced cell expansion, clonogenic assays on SDS patient HSC/P cells treated with leucine will be performed.

An important question that remains to be answered in this study is the chain of events leading to the reduced cell expansion of erythroid cells in SDS. Do translational defects lead to induction of apoptosis due to loss of SBDS or does SBDS play a direct role in cell survival or proliferation, which then affects its functions in ribosome assembly and translation? To answer this question, we will non-selectively inhibit apoptosis with the general caspase inhibitor ZVAD while inducing erythroid differentiation of *SBDS*-knockdown and control K562 and CD133+ cells and study improvement over time in cell expansion, ribosome profiles and translation. Additionally, we will use a protein synthesis inhibitor such as cycloheximide or actinomycin D and measure whether it increases sensitivity to apoptosis or reduces proliferation to understand the relative contribution of translation insufficiency on erythroid cell expansion of *SBDS*-knockdown and control K562 and HSC/P cells.

The CD133+/HSC/P cells will also be used to obtain a closer examination of the role of p53 and its family members in erythropoietic failure upon SBDS depletion. Furthermore, if p53 and/or its family members are overexpressed in these cells during differentiation, p53 will be silenced by shRNA and cell expansion, apoptosis and proliferation will be evaluated. If $\Delta Np63$ is downregulated, we will overexpress it in *SBDS*-knockdown CD133+ cells before induction of differentiation and evaluate cell expansion, apoptosis and proliferation of differentiating cells.

Alternatively, if it is found that the differentiating SBDS-deficient CD133+ cells are independent of p53 mediated apoptosis, we will further investigate the role of ROS regulation and ribophagy in cell expansion of differentiating SBDS-deficient K562 and CD133+ cells. ROS levels can be measured by using 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) as previously reported.²¹³ The conversion of DCFH-DA to its fluorescent derivative in the presence of ROS

can be measured by flow cytometry. Furthermore, we will test whether cell expansion of differentiating SBDS-deficient cells can be rescued by the addition of antioxidants NAC and AA. As ROS production is reported to be essential for autophagy, and as erythroid differentiating SBDS-deficient cells show reduced polysomes, it is of interest to examine whether SBDS deficiency induces polysome loss through ribophagy. Ubiquitin specific protease 10 (USP10) and G3BP, a Ras-GAP SH3 binding protein and modulator of USP10, have been identified to be involved in the ribophagy pathway.²⁰⁸ As such, initial expression of USP10 and G3BP will be analyzed by Western blotting in differentiating SBDS-deficient cells. If elevated levels of USP10 are detected, we will test the efficacy of known autophagy inhibitors such as wortmannin and 3-methyladenine,²¹⁴ on correcting cell expansion defects seen in the differentiating SBDS-deficient cells.

4.4 Significance

These studies strengthen the current understanding of processes regulating erythroid proliferation, apoptosis and translation due to SBDS deficiency. Furthermore, this study deepens our grasp of the underlying mechanism of erythropoietic failure in SDS and will have implications towards common hematological cytopenias such as neutropenia and thrombocytopenia also observed in SDS. By furthering our knowledge about the specific role of SBDS on cell survival and ribosome biogenesis in erythropoiesis we can uncover the specific biochemical pathway that SBDS functions, which may lead to the identification of new genes, implicated in bone marrow failure. Furthermore, understanding the cause of the defects in cell expansion could suggest new potential therapeutic targets, such as enhancing protein synthesis by using agents such as leucine. Lastly, the relationship between ribosome dysfunction and accelerated apoptosis in hematopoietic progenitors likely will provide broader relevance to

several human bone marrow failure and cancer predisposition syndromes well beyond the unique model of SDS.

CHAPTER V CONCLUSION

The present study was aimed at characterizing the mechanism of erythropoietic failure in SBDS-deficient cells using human erythroleukemic K562 cells and CD133+ HSC/Ps isolated from CB. With the successful generation of cellular models of anemia in SDS through shRNA-mediated suppression of the SBDS gene in K562 cells, this study suggests that SBDS plays a critical role in erythroid cell expansion through promoting survival of early erythroid progenitors by mainly preventing apoptosis and to a lesser extent promoting proliferation in these cells. The underlying defects leading to reduced erythroid cell expansion were hypothesized to be distinct in differentiating cells compared to non-differentiating cells, and as such this study showed during erythroid maturation SBDS plays a more prominent role in maintaining ribosome assembly and translation than in non-differentiated cells. Importantly, translational insufficiency was shown to cause reduced cell expansion in non-differentiating SBDS-deficient cells but not in erythroid-committed cells as leucine was not sufficient to correct the cell expansion defects. Furthermore, during erythropoiesis, SBDS-deficiency in K562 cells did not lead to increased activation of p53 family members unlike in non-differentiated conditions. This suggests SBDS function in maturing cells is more complex than in resting cells and that future studies on maturing cells are required to further delineate additional pathways involved in the pathogenic process of anemia in SDS caused by loss of SBDS. The results deepen our grasp of the underlying mechanism of erythropoietic failure in SDS, and may have broader relevance to other common causes of anemia due to bone marrow failure.

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