Monosomy 7 or deletion of 7q (del(7q)) are common clonal cytogenetic abnormalities associated with high grade myelodysplastic syndrome (MDS) arising in inherited and acquired bone marrow failure. Current non-transplant approaches to treat marrow failure may be complicated by stimulation of clonal outgrowth. To study the biological consequences of del(7q) within the context of a failing marrow, we generated induced pluripotent stem cells (iPSCs) derived from patients with Shwachman Diamond Syndrome (SDS), a bone marrow failure disorder with MDS predisposition, and genomically engineered a 7q deletion. The TGFβ pathway was the top differentially regulated pathway in transcriptomic analysis of SDS versus SDSdel(7q) iPSCs. SMAD2 phosphorylation was increased in SDS relative to wild type cells consistent with hyperactivation of the TGFbeta pathway in SDS. Phospho-SMAD2 levels were reduced following 7q deletion in SDS cells and increased upon restoration of 7q diploidy. Inhibition of the TGFbeta pathway rescued hematopoiesis in SDS-iPSCs and in bone marrow hematopoietic cells from SDS patients while it had no impact on the SDSdel(7q) cells. These results identified a potential targetable vulnerability to improve hematopoiesis in an MDS-predisposition syndrome, and highlight the importance of the germline context of somatic alterations to inform precision medicine approaches to therapy.
Therapeutic discovery for marrow failure with MDS predisposition using pluripotent stem cells

Melisa Ruiz-Gutierrez1,2, Özge Vargel Bölükbaşı1, Gabriela Alexe1,3,4, Adriana G. Kotin5,6,7, Kaitlyn Ballotti1, Cailin E. Joyce8, David W. Russell9, Kimberly Stegmaier1,2,3, Kasiani Myers10, Carl D. Novina3,8, Eirini P. Papapetrou5,6,7# and Akiko Shimamura1,2*#
Abstract

Monosomy 7 or deletion of 7q (del(7q)) are common clonal cytogenetic abnormalities associated with high grade myelodysplastic syndrome (MDS) arising in inherited and acquired bone marrow failure. Current non-transplant approaches to treat marrow failure may be complicated by stimulation of clonal outgrowth. To study the biological consequences of del(7q) within the context of a failing marrow, we generated induced pluripotent stem cells (iPSCs) derived from patients with Shwachman Diamond Syndrome (SDS), a bone marrow failure disorder with MDS predisposition, and genomically engineered a 7q deletion. The TGFβ pathway was the top differentially regulated pathway in transcriptomic analysis of SDS versus SDSdel(7q) iPSCs. SMAD2 phosphorylation was increased in SDS relative to wild type cells consistent with hyperactivation of the TGFβ pathway in SDS. Phospho-SMAD2 levels were reduced following 7q deletion in SDS cells and increased upon restoration of 7q diploidy. Inhibition of the TGFβ pathway rescued hematopoiesis in SDS-iPSCs and in bone marrow hematopoietic cells from SDS patients while it had no impact on the SDSdel(7q) cells. These results identified a potential targetable vulnerability to improve hematopoiesis in an MDS-predisposition syndrome, and highlight the importance of the germline context of somatic alterations to inform precision medicine approaches to therapy.
Introduction

Monosomy 7 or del(7q) frequently arise in the context of inherited and acquired bone marrow failure (1, 2). The appearance of this cytogenetic abnormality is associated with high grade MDS and leukemic transformation with poor prognosis. The current treatment of choice for bone marrow failure is hematopoietic stem cell transplantation, but outcomes are limited by regimen-related toxicities and donor availability. The development of non-transplant approaches to treat bone marrow failure without promoting outgrowth of malignant clones is limited by the paucity of disease models. Modeling in mice is challenging because the syntenic regions of human chromosome 7q map to several different murine chromosomes. It is currently unknown whether the surrounding failing marrow provides a contextual relative fitness advantage for the monosomy 7/del(7q) clone or whether the propensity to develop this cytogenetic abnormality in BMF results from a cell-intrinsic process.

Here we developed a human iPSC model of del(7q) in the context of bone marrow failure. We derived iPSCs from patients with Shwachman Diamond syndrome (SDS), a bone marrow failure syndrome characterized by pancreatic dysfunction, skeletal abnormalities and a propensity to developing MDS and Acute Myeloid Leukemia (AML) (3, 4). A recent genomic analysis of somatic mutations in MDS revealed that a significant subset (4%) of young MDS patients had SDS, suggesting that SDS is likely more prevalent than currently recognized (5). Biallelic mutations in the Shwachman-Bodian-Diamond Syndrome (SBDS) gene are the most common genetic cause of SDS (6). Monosomy 7 or del(7q) frequently arise in SDS (4). SDS-derived iPSC have been previously shown to phenocopy bone marrow failure (7). Therefore, we engineered a deletion of 7q in SDS iPSC and studied the molecular and biological consequences.
of del(7q) with the goal of identifying a potential therapeutic strategy to improve bone marrow failure in an MDS-predisposition syndrome.

Results

Generation of SDS iPSC and SDSdel(7q) iPSCs.

We generated iPSCs from bone marrow mononuclear cells of two patients with SDS (SDS1 and SDS2). Both patients carried homozygous c.258+2T>C SBDS mutations, the most common SBDS mutation found on at least one SBDS allele as noted in the North American SDS Registry (8) (Figure 1A, Supplemental Figure 1). To model progression of SDS to MDS, we engineered del(7q). The long arm of chromosome 7 was deleted by targeted insertion of two inverted loxP sites into the long arm of chromosome 7 followed by transient expression of Cre-recombinase as previously published (9) (Supplemental Figure 2A-C). Multiple clones were screened for the deletion of 7q by qPCR and FISH (Supplemental Figure 2D, E). The deletion of 7q was verified by karyotype analysis and mapped by aCGH to span region 7q11.23-7q36.3, which encompasses the MDS-associated common deleted region (CDR) (10), (11), (12) (Figure 1B and C). All SDS iPSC lines were verified to retain the endogenous SBDS mutations (Supplemental Figure 1B and 2F), and express scant levels of SBDS protein similar to the reduced levels found in patients (Figure 1D). All iPSC lines were confirmed to be pluripotent as determined by expression of markers of pluripotency (SSEA3, SSEA4, Tra-1-60, Tra-1-81) and by formation of teratomas in mice containing all three embryonic germ layers (Figure 1E and F).

Hematopoiesis from SDS and SDSdel(7q) iPSCs.

We investigated the hematopoietic differentiation potential of the SDS and SDSdel(7q) iPSCs.
All clones tested from SDS1 (SDS1.2, SDS1.3 and SDS1.5) and SDS2 (SDS2.2 and SDS2.5) iPSCs demonstrated impaired hematopoiesis with decreased generation of CD34+ cells (Figure 2A, B top panel) and reduced differentiation to CD45+ cells (Figure 2A, B bottom panel) compared to normal iPSCs. The SDS iPSCs also demonstrated impaired differentiation to the CD33+ myeloid population compared to normal iPSCs (Figure 2). Deletion of 7q further reduced the production of CD34+ cells. The CD34+ cells with del(7q) showed markedly impaired differentiation to CD45+ cells and myeloid CD33+ cells (Figure 2 A-D). The cell growth and cell cycle profiles were not significantly different between the SDS and SDSdel(7q) cells for all clones tested (Supplemental Figure 3).

As previously reported in del(7q) iPSCs in a normal (non-SDS) background (9), we observed spontaneous acquisition of an extra chromosome 7 in SDSdel(7q) iPSCs (Figure 2F and Supplemental Table 1). Upon spontaneous duplication of chromosome 7, hematopoietic differentiation and myeloid differentiation improved in three independent lines tested (SDS1.5Cre4.9+7#1, SDS1.5Cre4.9+7#3, SDS1.5Cre4.9+7#4) (Figure 2A-D), when compared to the parental SDSdel(7q) cells. Specifically, a 2-fold increase in CD34+ cells (8% +/- 2% for SDSdel(7q) vs. 17% +/- 4% for SDSdel(7q)+7), a 2.2 fold increase in CD45+ cells (32% +/- 11% for SDSdel(7q) vs. 70% +/- 5% for SDSdel(7q)+7) and a 1.2 fold increase in CD33+ cells (20% +/- 4% for SDSdel(7q) vs. 30% +/- 9% for SDSdel(7q)+7) was observed upon spontaneous duplication of chromosome 7. The SDSdel(7q)+7 iPSCs expressed low SBDS protein levels similar to those in SDS and SDSdel(7q) cells (Figure 2G). Thus 7q haploinsufficiency severely impaired hematopoiesis in the context of a failing marrow.
Differential activation of the TGFβ pathway in SDS versus SDSdel(7q)

To explore the biological consequences of del(7q) in SDS, we conducted RNAseq analysis of the SDS1 (SDS1.5Cre6), SDSdel(7q) (SDS1.5D5Cre4.9) and SDSdel(7q)+7 (SDS1.5D5Cre4.9+7#1) iPSCs (Supplemental Table 2 and Supplemental Figure 4). 726 genes were downregulated and 634 genes upregulated following deletion of 7q in SDS iPSCs (Figure 3A). Gene set enrichment analysis (GSEA) identified differential expression of inflammatory pathways, including decreased expression of the TGFβ pathway in SDSdel(7q) relative to SDS (Figure 3B and C, Table 1). Six of the top 16 upstream regulators identified by Ingenuity Pathway Analysis (IPA) to be downregulated in SDSdel(7q) relative to SDS iPSCs belonged to the TGFβ family (Figure 3D and Supplemental Table 3). As predicted from pathway analysis, downstream TGFβ targets quantified by qRT-PCR analysis demonstrated decreased expression in SDSdel(7q) iPSCs relative to the SDS iPSCs (Supplemental Figure 4C). These findings were consistent for TGFβ targets located on the long arm of chromosome 7 as well as those located elsewhere in the genome (Supplemental Table 3). Expression of TGFβ targets was increased in the SDS iPSCs relative to that in normal iPSCs (Supplemental Figure 4C), demonstrating hyperactivation of the TGFβ pathway in SDS. Restoration of 7q diploidy in the SDSdel(7q) cells reactivated the TGFβ pathway (Fig 3E and Supplemental Figure 4C). In contrast, analysis of non-SDS del(7q) iPSC transcriptomes previously published (9), showed downregulation of DNA repair and splicing pathways and increase in the TGFβ pathway compared to normal donor iPSCs (Supplemental Figure 4D). Thus, the del(7q)-associated relative modulation of the TGFβ pathway was dependent on the germ-line genetic context of the deletion.

The TGFβ pathway is an important regulator of hematopoiesis. Either hyperactivation or
inhibition of the TGFβ pathway can impair hematopoiesis (13) (14) (15). Upregulation of the TGFβ pathway inhibits hematopoiesis in Fanconi Anemia (16). We therefore hypothesized that hyperactivation of the TGFβ pathway in SDS cells may contribute to bone marrow failure and that inhibition of the TGFβ pathway might present a targetable vulnerability to exploit for the treatment of BMF in MDS-predisposition syndromes.

**TGFβ pathway inhibition rescues hematopoiesis in SDS but not SDSdel(7q).**

To query the activation status of the canonical TGFβ pathway, we measured phosphorylated nuclear SMAD2 by immunofluorescence in the normal, SDS1, SDSdel(7q) and SDSdel(7q)+7 iPSCs. A significant increase in nuclear phospho-SMAD2 was observed in the SDS1 iPSCs compared to normal iPSCs (Figure 4A, B). Deletion of 7q reduced phospho-SMAD2 levels, and restoration of 7q diploidy upregulated phosphorylation of SMAD2 (Figure 4A, B). The increased phospho-SMAD2 signaling together with the transcriptome data were consistent with signaling through the canonical SMAD2/3 TGFβ pathway. To test whether the canonical TGFβ pathway plays a role in the hematopoietic impairment of SDS, we investigated the effect of knocking down *SMAD3* expression. Two shRNA constructs targeting different regions of SMAD3 were verified to reduce SMAD3 mRNA and protein levels (Supplemental Figure 5) (16). The number and size of hematopoietic colonies from primary bone marrow mononuclear cells from three different SDS patients were improved following knockdown of *SMAD3* expression (Figure 4C and D). No consistent effect of SMAD3 knockdown was observed in healthy donor control bone marrow cells (Figure 4C and D).

We next used the small molecule inhibitor SD208, an ATP-mimetic which blocks the kinase
activity of TGFβ receptor 1 and thereby inhibits downstream signaling (Supplemental Figure 6A) (17). Hematopoietic colony formation was decreased in SDS iPSC compared to normal iPSCs, consistent with the bone marrow failure phenotype of SDS (Figure 4E). Both erythroid and myeloid colony number were improved in the SDS iPSCs with SD208 treatment. In contrast, no significant improvement in hematopoietic colony number was observed following TGFβ inhibition of normal or SDSdel(7q) cells (Figure 4E). Increased colony size of SDS iPSC-derived erythroid and myeloid colonies was also readily visible following TGFβ inhibition (Supplemental Figure S6B, C). Addition of SD208 to the iPSC-derived CD34+ cells rescued myeloid differentiation of the SDS iPSCs without improving myelopoiesis of the normal or SDSdel(7q) cells (Figure 4F). Improved hematopoiesis upon treatment with either SD208 or AVID200, a ligand trap specific to TGFβ1 and TGFβ3 was also evident in primary marrow mononuclear cells from SDS patients (Shimamura Lab, unpublished observations). Thus, inhibition of the TGFβ pathway improves hematopoiesis selectively in the SDS cells wherein the TGFβ pathway is hyperactivated, but not in the SDSdel(7q) or normal non-SDS cells where the TGFβ pathway activity is relatively reduced (Figure 5).

**Discussion**

The molecular mechanisms leading to bone marrow failure in SDS remain poorly understood. SBDS-deficient cells exhibit impaired ribosome biogenesis (18) (19) (20) (21) (22), abnormal mitotic spindle dynamics (23), and increased responses to ER stress and DNA damage (24). Impaired hematopoiesis may result from either excessive activation or inhibition of the TGFβ pathway. Although hyperactivation of the TGFβ pathway inhibits hematopoiesis and affects stem cell quiescence,(13) (14) (25) the TGFβ pathway plays an important role in hematopoiesis
and knockout of the TGFβ pathway is also deleterious (14). Suppression of hematopoiesis by
hyperactivation of the TGFβ pathway has also been reported in Fanconi anemia, a bone marrow
failure syndrome with impaired DNA repair (16), and iPSC models of Diamond Blackfan
anemia, which is caused by mutations affecting ribosome homeostasis and protein translation
(26). Taken together, these data suggest that TGFβ likely exerts general effects on hematopoiesis
in addition to affecting possible disease-specific pathways such as homologous recombination
versus non-homologous end-joining in Fanconi anemia (16).

The selective pressures resulting in the frequent acquisition of monosomy 7 and del(7q) clones in
bone marrow failure disorders remain unclear. Previous studies demonstrated that deletion of 7q
failed to confer a proliferative advantage in iPSC MDS models (9). Our study extended these
findings to show that deletion of 7q failed to confer a relative growth advantage even in the
context of a failing marrow in this iPSC model. Indeed, other MDS-associated driver mutations
have also failed to confer a proliferative advantage in murine models (27, 28). Monosomy 7 can
be transient (28-31), suggesting that additional events may be required in addition to
chromosome 7 deletion for MDS development.

Here we demonstrate a hematopoietic cell-intrinsic inhibitory effect of TGFβ on blood cell
production; however, TGFβ effects are also context-dependent, and the additional contribution
of potential interactions between the bone marrow niche and hematopoietic cells remain to be
explored. TGFβ regulation by the bone marrow niche affects hematopoiesis (32). Deletion of
SBDS in the bone marrow niche promotes genotoxic stress in hematopoietic cells through
activation of inflammatory signaling in SDS mouse models (33). Increased levels of
inflammatory cytokines activating the TLR and TGFβ pathways have been implicated in MDS pathogenesis (34) (35) (36).

Taken together, our studies with iPSC models identified a differentially regulated pathway that could be therapeutically targeted in a bone marrow failure and MDS predisposition disorder with the goal of improving hematopoiesis. In this proposed model (Figure 5), TGFβ inhibition improves hematopoiesis of the SDS marrow, wherein the TGFβ pathway is hyperactivated, but further reduction of the already low TGFβ signaling in the del(7q) cells did not improve hematopoiesis of the del(7q) cells. Further studies are needed to understand the potential effects of TGFβ inhibition on the del(7q) clone. Activin receptor ligand traps to inhibit downstream TGFβ pathway signaling have shown promise in MDS models and in clinical trials of MDS (37). Additional studies to elucidate the mechanism(s) whereby the TGFβ pathway is activated in SDS and how this pathway impairs hematopoiesis will further inform treatment strategies. The distinct effects of del(7q) in SDS versus non-SDS stem cells highlights the importance of the germline context of somatic alterations to inform precision medicine approaches to therapy.

**Methods**

**iPSC generation.** Cryopreserved bone marrow mononuclear cells were cultured in StemPro-34 SFM media (Gibco) with 1% nonessential amino acids (NEAA, Gibco), 1 mM L-glutamine (Gibco) and 0.1mM β-mercaptoethanol (Gibco) and supplemented with 100 ng/mL stemcell factor (SCF), 100ng/mL Flt3 ligand (Flt3L), 100 ng/mL thrombopoietin (TPO) and 20 ng/mL IL-3 for 24 - 48hrs. Viral transduction with the excisable lentiviral vector CMV-fSV2A expressing OCT4, KLF4, c-MYC and SOX2, reprogramming, selection of colonies with hPSC morphology
and Cre-mediated vector excision were done as previously described (38). Characterization of pluripotency by flow cytometry and teratoma formation assay was conducted as previously described (9). Patients with SDS harbored biallelic SBDS mutations.

**Cell Culture.** Culture of human iPSCs on MEFs (MMC-treated, Applied Stem Cell, Inc) or in feeder-free conditions using matrigel (Gibco) was done as previously described (9). Normal human iPSC cell line niPS (female, gift from I. Bernstein) and 1157 (male, gift of G. Daley). HEK293T cells (gift of D.A. Williams) were cultured in Dulbecco’s Modified Eagle’s Medium (Gibco) supplemented with 10% fetal bovine serum (FBS). A549 cells (ATCC) are grown in F-12K (ATCC) media with 10% FBS (Sigma), 1% Pen/Strep (Gibco). Cells were treated with 1µM SD208 (Sigma) or 0.6µg/mL AVID200 (Formation Biologics) for 2 – 4 hrs at 37˚C/5%CO2 in the tissue culture incubator prior to harvest.

**AAV-mediated gene targeting and selection of clones with 7q deletions.** The AAV targeting vector was previously described (9). Puromycin-resistant colonies were selected and southern blot verification was conducted as previously described (9). Transduction with a Cre-expressing integrase-deficient lentiviral vector was done as previously described (9). Ganciclovir (Sigma) selection was performed at concentration of 150µM for 14 days.

**Karyotyping.** iPSCs were plated on matrigel as single cells at density of 400,000 cells in a T-25 flask. After 24 - 48hrs, cells were karyotyped by Cell Line Genetics (Madison, WI, USA).
Array CGH was performed on SurePrint G3 Human Genome CGH+SNP Microarray kit, high resolution array with SNPs included with average coverage every 25 KB with increased coverage in ISCA regions (5 KB) by Cell Line Technologies (Madison, WI, USA).

**Western blot analysis.** hiPSC cells were harvested as single cells with Accutase (Stem Cell Technologies) and lysed in RIPA buffer (Sigma) supplemented with protease inhibitors (Inhibitab, Roche) and phosphatase inhibitor cocktail 2 and 3 (Sigma). Protein concentrations were determined by colorimetric assay (BCA Protein, ThermoFisher) and 40µg of protein was loaded on 12% SDS-PAGE gels and blotted on PVDF membrane (Millipore). The membranes were blocked with 5% nonfat dry milk (VWR) diluted in Tris-buffered saline (Sigma) with 1% Tween-20 (VWR). Primary antibodies SBDS (23), GAPDH (Cell Signaling, clone 14C10), total Smad2 (ab40855, Abcam), Phospho-Smad2 (ab3849-I, EMD Millipore) were incubated overnight at 4C. After washing, membranes were incubated with HRP-conjugated secondary antibodies (GE Healthcare) and developed using SuperSignal West Pico Chemiluminescent substrate (ThermoFisher). Detection of bands was conducted in the Amersham Imager 600 (GE Healthcare).

**Flow cytometry.** For flow cytometry, the following antibodies were used: Alexa-Fluor-647 SSEA-3 (clone MC-631, Biolegend). Alexa-Fluor-647 SSEA-4 (clone MC-813-70, Biolegend). Alexa-Fluor-647 Tra-1-60 (clone Tra-1-80-R, Biolegend). Alexa-Fluor-647 Tra-1-81 (clone Tra-1-81, Biolegend), PECy7-CD34 (clone 8G12, BD Pharmigen), APC-CD45 (clone 2D1, BD Pharmingen), PE-CD33 (clone WM33, BD Pharmingen), PECy7-CD11b (clone ICRF44, BD Pharmingen), DAPI (Sigma), Propidium Iodide (BD Pharmingen). Flow cytometry conducted
**Hematopoietic differentiation of iPSCs.** hPSC colonies were collected and grown in ultra-low attachment dishes (Corning) and subjected to cytokine media changes for 18 days as previously described (9). At the end of embryoid body (EB) differentiation culture (Day 10, 14, 18) cells were dissociated with Accutase (Stem Cell Technologies) into single cells and analyzed by flow cytometry. For methylcellulose colony formation assays, the cells were dissociated at Day 12 and 1.5x10^4 cells were resuspended in StemPro-34 SFM, added to 3 mL of methylcellulose (H4434, Stem Cell Technologies) and 1 mL was plated in triplicate wells of 6 well Smartdish (Stem Cell Technologies). After 14 days of growth at 37°C/5% CO\(_2\), colonies were imaged and scored using STEMVision (Stem Cell Technologies). The score was averaged for triplicates wells. For myeloid differentiation, the EBs were dissociated at Day 18 and grown in media containing 100 ng/mL SCF (R&D Biosystems), 50 ng/mL granulocyte colony stimulating factor (GCSF) (R&D Biosystems), 50 µg/mL ascorbic acid (Sigma), 40ng/mL FLT3L (R&D Biosystems), 40 ng/mL IL-3, 20 ng/mL TPO (R&D Biosystems), and 20ng/mL IL-6 (R&D Biosystems), with media changes every 3-4 days. 50,000 cells were harvested by cytospin at 300rpm for 4 min (Shandon Cytospin 3) onto glass microscope slides (Fisher Scientific). Cells were allowed to dry prior to staining per manufacturer’s (NovaUltra Hema-Diff Stain kit, IHC World).

**Gene expression analysis by qRT-PCR.** RNA was isolated following manufacturer’s instructions for RNeasy Plus Mini Kit (Qiagen), RNA was eluted in 30 µL of water. 200ng-5µg
of RNA was used for reverse transcription with Superscript III First Strand Synthesis using oligo-dT primer (Invitrogen). qPCR was performed with iTaq Universal SYBR Green Supermix (Bio-Rad) using GAPDH as internal control with primers shown in Supplemental Table 4. Reactions were carried out in triplicate in a 7500 Fast Real-Time PCR System (Applied Biosystems) and analyzed using the ΔΔCT method.

**RNA-seq.** RNA quality was verified on an Agilent 22-TapeStation for a RIN >8. Library prep, quality control and sequencing with Illumina HiSeq 2500 platform performed at the Fred Hutchinson Cancer Research Center Genomic core. Reads were aligned using TopHat v2.13 against the hg19 assembly of the human genome. Counts for each gene were generated using htseq-count v0.6.1pl, genes with less than 1 count/million in at least 3 samples were removed. Gene expression was quantified as log2(1+FPKM) (Fragments Per Kilobase of transcript per Million mapped reads). Data were restricted to the genes with log2(1+ FPKM) expression >1 in at least one sample: 12,993 genes for SDS del(7q) and 11,576 genes for SDS del(7q)+7. EdgeR v3.12.1 (39) and DEseq2 v1.20.0 (40) were used to normalize data, conduct significance testing and pair samples from the same patient, with significance cut-offs: absolute fold change for log2(FPKM) expression ≥ 1.5, P ≤ 0.05, Benjamini-Hochberg false discovery rate ≤ 0.05. Genome-wide Gene Set Enrichment Analysis (GSEA v3.0 (41) (42)) of the different samples were compared to gene sets included in the Molecular Signature Database (MSigDB v6.2, (41) (43)), while disregarding the chr7 genes. Ingenuity Pathway Analysis v01-07 (Qiagen) was used for Upstream Regulators analysis. The RNA-sequencing data for this study have been deposited to NCBI’s Gene Expression Omnibus repository (44) and are accessible through the GEO series accession number GSE118378.
**Immunofluorescence.** Cells were grown on matrigel-covered coverslips at a density of $5 \times 10^5$ cells/coverslip for 24hrs. Cells were washed with PBS (Gibco), fixed with 4% (w/v) paraformaldehyde (Sigma) for 10 min at room temperature, washed three times with PBS, permeabilized with 0.3% Triton X-100 (VWR) for 10 minutes at room temperature, washed three times with PBS, blocked for 1 hour at room temperature in solution with 1% fetal bovine serum (Sigma) prior to overnight incubation at 4˚C with primary antibody (Phospho-SMAD2, 44-244G Thermo Fisher Scientific). The coverslips were washed three times with PBS prior to incubation with secondary antibody (AlexaFluor 488 goat anti-rabbit IgG, Life Technologies). Cells mounted with DAPI (Vector Laboratories) for nuclear counterstaining and were imaged with 63X oil immersion objective of confocal Leica SP5 microscope (DFCI core facility). Image analysis and quantification done by Fiji (ImageJ, [www.Fiji.sc](http://www.Fiji.sc)).

**Fluorescence in-situ Hybridization.** Cells were incubated 2X in fixative (3:1 methanol:acetic acid) for 15 min each. Cells were attached to glass coverslip (Fisher Scientific) by cytopsin at 2000rpm for 2 min (Shandon Cytospin 3). Cells were rehydrated in 2X SSC (Sigma) at 37˚C for 2 min followed by dehydration in a series of ethanol (70%, 80% and 95%) for 2 minutes each. Fluorescent probes (Cytocell) were added to coverslip, incubated at 75˚C for 2min and then overnight at 37˚C. Cells were washed for 2 min at 72˚C in 0.4X SSC followed by 2X SSC/0.5% Tween20 (Sigma) at room temperature for 30 seconds. Cells were mounted and imaged as described above. Fluorescent probes: chromosome 7 centromeric (Cytocell, D7Z1, 7p11.1-7q11.1, Aqua) and chromosome 7 subtelomeric (Cytocell, Texas-Red, LPT07QR/G-A).
Lentivirus Production and Transduction. Lentivirus was produced in HEK293T cells seeded at ~50% confluence 24 hours prior to transfection. Transfection was performed with PEI (Life Technologies). Virus was harvested 24 hours post transfection, filtered through 0.45µm membrane (Millipore) and concentrated by ultracentrifugation at 24,000rpm for 2 hours at 4°C. An MOI of 20 was used. Primary bone marrow-derived mononuclear cells were grown for 24h in StemSpan SFEM II (Stem Cell Technologies) supplemented with 100 ng/mL of SCF, TPO, Flt3L and 20 ng/mL of IL-3 (PeproTech, Rocky Hill, NJ). Cells were resuspended at 1x10^6 cells/mL with 8µg/mL polybrene (Sigma) and 200µL of cell suspension were used per reaction. After addition of virus, cells were centrifuged at 2,3000 rpm for 30min at room temperature. Puromycin (1µg/mL, Mirus) selection was conducted for 72 hrs before cells were harvested for methylcellulose assay as described above. Scrambled shRNA (CAACAAGATGAAGAGCACCAA) (45), SMAD3-shRNA1 (CTGTGTGAGTTCGCCTTCAAT) (16), and SMAD3 shRNA2 (CCCAGGCATAATAACTTGG) (16) were synthesized by Addgene (Watertown, MA).

Statistics. Statistical analyses were conducted with Prism 7 (Graphpad Software) using 2-tailed, unpaired Student’s T test.

Study Approval. Patient samples were obtained with informed consent under protocols approved by the Institutional Review Boards at Fred Hutchinson Cancer Research Center (Seattle, WA), Seattle Children’s Hospital (Seattle, WA), and Boston Children’s Hospital (Boston, MA).
Author Contributions:
M.R-G, O.V.B, E.P.P, K.S. and A.S designed experiments; D.W.R. and A.G.K. designed and produced the AAV targeting vectors, K.M. and A.S. collected clinically phenotyped patient samples; M.R-G, O.V.B and K.B. performed experiments; M.R-G, G.A and C.J. performed computational analyses; M.R-G, O.V.B, and A.S. analyzed data; M.R-G and A.S. wrote the manuscript; all authors provided critical reviews of the manuscript.

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References:


Figure 1. Generation of SDS iPSC and SDSdel(7q) iPSCs. (A, B) Representative iPSC colony morphology and karyotype for SDS patient-derived iPSC (SDS1.5) before (A) and after (B) deletion of the long arm of chromosome 7 (box) (SDS1.5D5Cre4). Karyotype analysis was performed for all iPSC lines. (C) Array CGH analysis showing deletion of the chromosome 7 region between bands q11.23 and q36.3 in one allele. (D) Western blot analysis of SBDS protein expression in SDS1 (SDS1.5) iPSC and SDSdel(7q) (SDS1.5D5Cre4.9#9) iPSC compared to normal (niPS) iPSC. Actin is shown as a loading control. Numbers below the bands indicate average densitometry quantitation of the SBDS band normalized to normal control sample value. (E) Flow cytometry of pluripotency surface markers SSEA3, SSEA4, Tra-1-60 and Tra-1-81 in SDS1 iPSCs (blue, SDS1.2), SDSdel(7q) iPSCs (green, SDS1.5D5Cre4.9#2) and non-pluripotent cell line (293T) (red). (F) The indicated iPSCs were injected into immunodeficient mice. Histology of representative teratomas derived from SDS1 (SDS1.5) iPSCs and SDSdel(7q) (SDS1.5D5Cre4.9) iPSCs show differentiation into all 3 embryonic germ layers (endoderm (left panels), mesoderm (middle panels), and ectoderm (right panels)). Scale bar 100µm.
Figure 2. Effect of del(7q) on hematopoiesis of SDS iPSCs. (A) iPSC-derived CD34+ and CD45+ cells at days 10, 14 and 18 of hematopoietic differentiation of normal, SDS1, SDSdel(7q) and SDSdel(7q)+7 iPSCs. (B) Graph summary of CD34 expression at day 10 of hematopoietic differentiation (top panel) and CD45 expression at day 18 of hematopoietic differentiation (bottom panel). Normal (N2.12 D1-1, 1157, n=5), SDS (SDS1.5, SDS2.5, n=4), SDSdel(7q) (SDS1.5D5Cre4.9#4, SDS1.5D5Cre4.9#9, n=4) and SDSdel(7q)+7 (SDS1.5D5Cre4.9+7#1, SDS1.5D5Cre4.9+7#3, SDS1.5D5Cre4.9+7#4, n=4). (C) iPSC-derived CD45+ and CD33+ cells at day 14 of myeloid differentiation for normal, SDS1, SDSdel(7q), and SDS del(7q)+7 iPSCs. (D) Comparative graph of percent CD33+ cells at day 14 of myeloid differentiation. Normal (N2.12 D1-1, 1157, n=4), SDS (SDS1.5, SDS2.5, n=3), SDSdel(7q) (SDS1.5D5Cre4.9#4, SDS1.5D5Cre4.9#9, n=3) and SDSdel(7q)+7 (SDS1.5D5Cre4.9+7#3, SDS1.5D5Cre4.9+7#4, n=3). For B and D all results are represented as means +/- s.d. (E) Representative Wright Giemsa stain of myeloid cells on day 7 of differentiation from normal (left panel, N2.12 D1-1) and SDS iPSCs (right panel, SDS1.5). Scale bar 20µm. (F) Representative karyotype of SDSdel(7q) iPSC after spontaneous acquisition of additional chromosome 7 (box). (H) Western blot analysis of SBDS protein in SDS1 (SDS1.5), SDSdel(7q) (SDS1.5D5Cre4.9#2) and SDSdel(7q)+7 iPSCs (SDS1.5D5Cre4.9+7#4). GAPDH is shown as a loading control. Numbers below the bands indicate average densitometry quantitation normalized to normal control sample values. Significance determined using unpaired two-tailed student’s t-test with p value <0.05 (*).
Figure 3. Differential activation of TGFβ pathway in SDS iPSCs vs SDSdel(7q) iPSCs. (A) Heatmap of RNA-seq transcriptomic analysis of 12,993 genes with log2FPKM expression >1 from SDS (SDS1.5) versus SDSdel(7q) (SDS1.5SDCre4.9) iPSCs. Genes ranked based on log2 fold change FPKM ≥ 1.5 between SDSdel(7q) versus SDS iPSC with P-value ≤ 0.05 in EdgeR. 634 genes were noted to have increased expression and 726 genes had decreased expression in SDSdel(7q) iPSCs relative to SDS iPSCs. (B) Subset of the genome-wide GSEA canonical pathways and experimental gene sets with decreased expression in SDSdel(7q) iPSCs compared to SDS iPSCs. (C) Volcano plot of GSEA canonical pathways and experimental gene sets. Pathways associated with TGFβ are noted with red dots. (D) Top sixteen upstream regulators identified by Ingenuity Pathway Analysis to be inhibited in SDSdel(7q) compared to SDS iPSCs with activation score ≤ -3.0. Upstream regulators of TGFβ pathways are highlighted (*). (E) Heatmap of TGFβ upstream regulators in SDSdel(7q) and SDSdel(7q)+7 iPSCs relative to SDS iPSCs for the canonical (left panel) and non-canonical (right panel) pathways.
Figure 4. The TGFβ pathway is upregulated in SDS iPSCs and modulated following 7q deletion. (A) Representative images of phospho-SMAD2 immunofluorescence (green) in normal, SDS1, SDSdel(7q), and SDSdel(7q)+7 iPSCs. Nuclei are stained with DAPI (blue). Scale bar 10 µm. (B) Mean signal intensity for nuclear phospho-SMAD2 signal was quantified in individual cells (n = 100 cells/line/experiment) of normal (1157), SDS1 (SDS1.5), SDSdel(7q) (SDS1.5D5Cre4.9#9) and SDSdel(7q)+7 (SDS1.5D5Cre4.9+7#2) iPSCs. (C) Relative methylcellulose hematopoietic colony formation per 10,000 cells plated for normal or SDS primary marrow mononuclear cells following lentiviral transduction of the indicated shRNA (scrambled (c), SMAD3 shRNA 1 (sh1), SMAD3 shRNA2 (sh2)). Cells were plated in triplicate for each of 3 independent experiments (n=3). (D) Representative images of erythroid (top panels) and myeloid (bottom panels) colonies from normal or SDS primary marrow with scrambled (c) versus SMAD3 shRNA (sh1). Scale bar 1mm. (E) Hematopoietic colony formation per 15,000 iPSC-derived cells plated in methylcellulose for normal (blue, N2.12 D1-1, n=3), SDS (orange, SDS1.5, n=3) and SDSdel(7q) (grey, SDS1.5D5Cre4.9#9, n=3) iPSCs in the presence or absence of 1 µM SD208. Mean +/- s.d. are shown for myeloid (top panel) and erythroid (bottom panel) colonies. (F) CD33 expression at day 7 of myeloid differentiation showing means +/- s.d. for normal (blue, N2.12 D1-1, n=4) and SDS (orange, SDS1.5, n=4). For (B,C,E and F) Significance determined using unpaired two-tailed student’s t-test with p value <0.05 (*).
Figure 5. Proposed model of TGFβ pathway activity in bone marrow failure and MDS.

Either excessive or insufficient TGFβ signaling is deleterious. In SDS, the TGFβ pathway is hyperactivated which inhibits hematopoiesis. TGFβ inhibitors relieve the hyperactivated state of TGFβ signaling in SDS resulting in improved hematopoiesis. Since the TGFβ pathway activity is relatively lower in SDSdel(7q), reduction of TGFβ signaling results in insufficient TGFβ pathway activity and inhibits outgrowth of these cells.
Table 1. TGFβ gene sets from the Molecular Signature Database (MSigDB) ordered by normalized enrichment score (NES). The number of genes included in each gene set (size) is noted.

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