Deficiency of Sbds in the Mouse Pancreas Leads to Features of Shwachman–Diamond Syndrome, With Loss of Zymogen Granules

MARINA E. TOURLAKIS,[*,‡] JIAN ZHONG,[‡] RIKESH GANDHI,[‡] SIYI ZHANG,[*,‡] LINGLING CHEN,[*,‡] PETER R. DURIE,[§] and JOHANNA M. ROMMENS[*,‡]

[*]Department of Molecular Genetics, University of Toronto; [‡]Program in Genetics & Genome Biology, Research Institute, The Hospital for Sick Children; [§]Program in Physiology & Experimental Medicine, Research Institute, Division of Gastroenterology and Nutrition, The Hospital for Sick Children, Department of Paediatrics, University of Toronto, Toronto, Ontario, Canada

See Covering the Cover synopsis on page 276.

BACKGROUND & AIMS: Shwachman–Diamond syndrome (SDS) is the second leading cause of hereditary exocrine pancreatic dysfunction. More than 90% of patients with SDS have biallelic loss-of-function mutations in the *Shwachman–Bodian Diamond syndrome* (*SBDS*) gene, which encodes a factor involved in ribosome function. We investigated whether mutations in *Sbds* lead to similar pancreatic defects in mice. **METHODS:** Pancreas-specific knock-out mice were generated using a floxed *Sbds* allele and bred with mice carrying a null or disease-associated missense *Sbds* allele. Cre recombinase, regulated by the *pancreatic transcription factor 1a* promoter, was used to disrupt *Sbds* specifically in the pancreas. Models were assessed for pancreatic dysfunction and growth impairment. **RESULTS:** Disruption of *Sbds* in the mouse pancreas was sufficient to recapitulate SDS phenotypes. Pancreata of mice with *Sbds* mutations had decreased mass, fat infiltration, but general preservation of ductal and endocrine compartments. Pancreatic extracts from mutant mice had defects in formation of the 80S ribosomal complex. The exocrine compartment of mutant mice was hypoplastic and individual acini produced few zymogen granules. The null *Sbds* allele resulted in an earlier onset of phenotypes as well as endocrine impairment. Mutant mice had reduced serum levels of digestive enzymes and overall growth impairment. **CONCLUSIONS: We developed a mouse model of SDS with pancreatic phenotypes similar to those of the human disease. This model could be used to investigate organ-specific consequences of *Sbds*-associated ribosomopathy. *Sbds* genotypes correlated with phenotypes. Defects developed specifically in the pancreata of mice, reducing growth of mice and production of digestive enzymes. SBDS therefore appears to be required for normal pancreatic development and function.**

Keywords: Acinar Cells; Recessive Inheritance; Genotype-Phenotype Correlation; Genetic Model.

Shwachman–Diamond syndrome (SDS; Online Mendelian Inheritance in Man entry 260400) is an autosomal-recessive disorder that presents in infancy with bone marrow failure and exocrine pancreatic dysfunction.[1–3] The hematologic complications can include depression of myeloid lineages, most often intermittent or persistent neutropenia,[4] as well as an increased risk of leukemia (15%–25% or higher).[5,6] The exocrine dysfunction, if untreated, results in failure to thrive with malabsorption and malnutrition. Patients can display steatorrhea, low levels of serum digestive enzymes, as well as a deficiency in pancreatic enzyme secretion.[1,7,8] More variable clinical features of SDS include skeletal problems[9] with short stature and other abnormalities.[1,4,10,11] SDS is associated with mutations in the *Shwachman–Bodian Diamond syndrome* (*SBDS*) gene.[12] No patients have been reported with 2 null alleles.[13] We previously reported that loss of the mouse ortholog, *Sbds*, results in early embryonic lethality.[14] These findings indicate that this gene is essential and that mutations that occur with a null mutation are hypomorphic in nature.

Sbds encodes a highly conserved, broadly expressed, 29-kilodalton protein[15] recently shown to function with guanosine triphosphatase elongation factor-like 1 in the release of eukaryotic initiation factor 6 from the 60S ribosomal subunit for translation initiation.[16,17] Tissue culture models have shown that SBDS depletion results in reduced global translation.[18] It remains unclear how mutations in Sbds manifest as disease in particular organs, notably the exocrine pancreas.

The pancreas is a mixed endocrine and exocrine gland arising from common precursors in the primitive foregut.[19,20] Pancreatic transcription factor 1a (*Ptf1a*), which is expressed as early as embryonic day [E]9.5 in both lineages, becomes acinar cell–specific by E14,[21,22] and maintains exocrine function.[23] Acinar cells are specialized secretory cells in which proenzymes are stored in zymogen granules (ZGs) before release into the pancreatic duct.

Acinar cell function is compromised in SDS. Young patients present with low serum amylase and trypsinogen levels. There is some improvement of the latter with age, but amylase levels remain low.[7,8] Imaging studies of pa-

Abbreviations used in this paper: CKO, conditional knock out; E, embryonic day; P, postnatal day; PCR, polymerase chain reaction; Ptf1a, pancreatic transcription factor 1a; SBDS, Shwachman–Bodian Diamond syndrome; SDS, Shwachman–Diamond syndrome; ZG, zymogen granule.

0016-5085/$36.00
http://dx.doi.org/10.1053/j.gastro.2012.04.012

tients have indicated small and fatty pancreata.[24] Furthermore, necropsy has indicated acinar hypoplasia and extensive fat infiltration, but no fibrosis or consistent impairment of ductal and endocrine compartments.[2,3] Endocrine impairment is not commonly reported.[4]

Although SDS has been included in a cluster of ribosomal disorders,[25] it remains unclear why loss of SBDS function results in pancreatic phenotypes that are not typically seen in other conditions within this grouping. The exocrine pancreas needs to be depleted dramatically (<5%) to show overt clinical symptoms of maldigestion, complicating both diagnosis of pancreatic dysfunction and management of SDS.[26] Constitutive homozygosity of previously generated SDS-associated missense (*R126T; c.377G>C*, p.R126T), or the combination of the *R126T* and null alleles in mice supported embryonic development but resulted in perinatal death (our unpublished results). We generated a conditional *Sbds* allele, used in combination with the constitutive null allele,[14] or the missense *R126T* allele, to investigate the function of Sbds in the pancreas. Our findings reveal severe consequences for the pancreas with loss of Sbds and highlight phenotype correlation with residual Sbds function.

Materials and Methods

Mice

R126T and conditional knockout (*CKO*) alleles were generated using knock-in gene targeting methodologies similar to the previously described null *Sbds* allele. The *c.377G>C* (p.R126T) point mutation was generated by site-directed mutagenesis from a mouse bacterial artificial chromosome fragment (QuickChange II XL Site-Directed Mutagenesis Kit; Agilent Technologies, Cedar Creek, TX) and was confirmed by sequencing.[14] Excision of the *CKO* allele was achieved by crossing to mice heterozygous for *Cre*, under the control of a *Ptf1a* promoter.[21] This allele was used in conjunction with either the *R126T* or null allele to generate $Sbds^{CKO/R126T}$; $Ptf1a^{Cre/+}$ or $Sbds^{CKO/KO}$; $Ptf1a^{Cre/+}$ (Figure 1) genotypes, respectively. Littermates with a wild-type *Sbds* allele or no $Ptf1a^{Cre}$ allele ($Sbds^{CKO/+}$; $Ptf1a^{Cre/+}$, $Sbds^{CKO/+}$; $Ptf1a^{+/+}$, $Sbds^{CKO/KO}$; $Ptf1a^{+/+}$, and $Sbds^{CKO/R126T}$; $Ptf1a^{+/+}$) were indistinguishable, consistent with the recessive inheritance pattern of SDS and the constitutive mouse model.[14] Unless otherwise specified, controls with an $Sbds^{CKO/+}$; $Ptf1a^{Cre/+}$ genotype were used for all comparisons to account for *Ptf1a* dosage.

Animals were analyzed at embryonic early, adolescent, and adult stages to investigate the early pancreas, exocrine expansion, and long-term outcomes, respectively. Tail samples were genotyped using the REDExtract-N-Amp Tissue polymerase chain reaction (PCR) Kit (Sigma, St. Louis, MO) (for primers, see Supplementary Figure 1). Mice were euthanized either by CO_2 inhalation or cervical dislocation.

For diet challenges, mice were weaned onto Teklad Custom Research Diet TD.06414 (60% kcal from fat; 34% wt/wt; Harlan Laboratories, Indianapolis, IN).

The Animal Care Committee of The Hospital for Sick Children approved all mouse procedures.

Tissue Preparation

For paraffin embedding, dissected pancreata were fixed in 4% paraformaldehyde, paraffin embedded, and cut into 5 μm sections. H&E and Masson's trichrome staining (Sigma) were used for morphologic analysis.

Dissected pancreata (30 mg) were preserved in RNAlater (Ambion, Austin, TX) for RNA extraction.

Immunostaining

Primary and secondary antibodies are listed in Supplementary Table 1. All images shown are representative of multiple mice from multiple litters.

Paraffin sections were cleared in xylenes and rehydrated. Antigen retrieval was performed by boiling for 20 minutes in citrate buffer (pH 6.0), followed by incubation in 3% H_2O_2 for 10 minutes at room temperature. Sections were blocked for 30 minutes in 5% goat serum and incubated with primary antibody for 1 hour in 1% bovine serum albumin in 1× PBS at room temperature. Secondary antibodies were used at 1:200 for 20 minutes in 1% bovine serum albumin in 1× PBS at room temperature. A biotin conjugate of the duct-binding lectin *Dolichos biflorus* agglutinin (Vector Laboratories, Burlingame, CA) was used to identify pancreatic ducts and was detected with streptavidin-conjugated horseradish-peroxidase (PerkinElmer, Waltham, MA). Proteins were visualized by diaminobenzidine (Sigma) and counterstained with hematoxylin.

Oil Red O Staining

Fresh frozen sections (8 μm) of mouse pancreata were processed for Oil Red O staining at the Toronto Centre for Phenogenomics Pathology Core as previously described.[27]

Quantitative Real-Time PCR

Total RNA was isolated from pancreata using the RNeasy Mini Plus Kit (Qiagen, Valencia, CA) with the addition of 5% β-mercaptoethanol in the homogenizing buffer. Quality control measures and experimental details of the complementary DNA synthesis and real-time PCR are provided in the Supplementary Materials and Methods section and Supplementary Table 2. Test gene transcript levels are presented in relation to β-actin (*Actb*) and *glyceraldehyde-3-phosphate dehydrogenase (Gapdh)* genes, which showed the most stable expression values, determined by GeNORM analysis; available from: http://medgen.ugent.be/~jvdesomp/genorm/.[28]

Blood Biochemistry

Serum from the saphenous vein of mice was analyzed for α-amylase and lipase levels via colorimetric assays and insulin levels via enzyme-linked immunosorbent assay (IDEXX Laboratories, Markham, ON).

Electron Microscopy

Dissected 1-week-old pancreata were fixed in 2% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.3) and processed for toluidine blue staining and electron microscopy by the Advanced Bioimaging Centre at Mount Sinai Hospital in Toronto.

Polysome Analysis

Pancreas tissue (10 ± 3 mg) from P20 animals, before the onset of fat infiltration in mutant models, was homogenized in polysome buffer (100 mmol/L KCl, 5 mmol/L $MgCl_2$, 5 mmol/L Tris-HCl [pH 7.4], 1% deoxycholic acid, 1% Triton X-100 [Sigma] in diethylpyrocarbonate-treated H_2O) followed by centrifugation at 2500 × g (4°C) for 15 minutes. Cyclohexamide (0.1 mg/mL) and heparin (1 mg/mL) were added before loading