ARTICLE

Intellectual disability and abnormal cortical neuron phenotypes in patients with Bloom syndrome

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Bloom syndrome (BS) is a rare autosomal recessive disorder characterized by genomic instability that leads to various complications, including cancer. Given the low prevalence of BS in Japan, we conducted a nationwide survey. We recruited eight patients with BS, three of whom exhibited intellectual disability. The 631delCAA mutation in the *BLM* gene was detected in 9 out of 16 alleles. To investigate neuronal development in patients with BS, we generated induced pluripotent stem cells derived from one of these patients (BS-iPSCs). We examined the phenotypes of the induced cortical neurons derived from the generated BS-iPSCs using a previously reported protocol; the generated BS-iPSCs showed an approximately 10-times higher frequency of sister-chromatid exchange (SCE) than the control iPSCs. Immunocytochemistry revealed shorter axons and higher proliferative potential in BS-iPSC-derived cortical neurons compared with control iPSCs. To our knowledge, our study is the first to clarify the abnormality of the cortical neuron phenotypes derived from patients with BS. Our findings may help identify the pathogenesis of neuronal differentiation in BS and aid in the development of novel therapeutic agents.

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INTRODUCTION

Bloom syndrome (BS) is an autosomal recessive disease caused by loss-of-function mutations in *BLM*, a subfamily of DExH boxes containing DNA and RNA helicases [1]. The absence of a functional BLM protein causes chromosome instability, excessive homologous recombination, and a greatly increased number of sister-chromatid exchanges (SCE), which have been used for diagnosing BS, because SCE was considered to be a characteristic feature of BS [2, 3]. BLM helicases constitute a multiprotein complex called the BTRR (BLM-TOP3A- a- RMI2) complex, which also includes topoisomerase III alpha (TOP3A) and RecQ-mediated genome instability protein 1 (RMI1) and 2 (RMI2) [4–6]. Recently, homozygous frameshift mutations in TOP3A and RMI1 as well as loss of the RMI2 gene as a result of a large homozygous deletion have been associated with a BS phenotype, including the elevated frequency of SCE [7, 8].

BS is characterized by dermatologic complications, growth deficiency, immune deficiency, reduced glucose tolerance, a significantly increased risk of early-onset cancer and the development of multiple cancers, which is a major medical concern for BS [9].

Some individuals with BS exhibit intellectual disabilities [10, 11]. Single-cell transcription profiles in patients with BS reveal significant deregulation of genes that cause inherited forms of primary microcephaly associated with intellectual disability [12]. Although the hypomorphic *BLM* mouse model exhibits tumor formation similar to that associated with BS and may be a preclinical model for BS [13, 14], only one BS-iPSC line has been established [15]; no human cell model of BS for studying cortical neurons has been reported. Owing to the rarity of BS, the actual condition of patients with BS has not been elucidated. In the current study, we initially investigated the clinical features of patients with BS, those with intellectual disabilities, via a nationwide survey in Japan. Finally, we derived induced pluripotent stem cells (iPSCs) from one patient, and thereon differentiated into cortical neurons to analyze their phenotype.

MATERIAL AND METHODS

Investigation of patients with BS in Japan

Following the administration of the first questionnaire in 2010, a second questionnaire was administered. In the survey, 515, 515, and 377 first questionnaires were sent to the relevant specialist departments of training hospitals for pediatrics, dermatology, and hospitals with a cancer center, respectively. The first 15 questionnaires were returned as no destination was found. After the first questionnaire, 12 answers were returned from attending physicians for patients with suspected BS. Eight of the 12 patients in this study were recruited as patients with BS if they exhibited one or more of the following inclusion criteria: short stature; skin lesions such as sun-sensitive erythema, pigmentation or café-au-lait spots; immunodeficiency; together with either a confirmed elevated frequency of SCE or *BLM* gene mutation. We sent a second questionnaire to attending physicians for eight patients with BS. The contents of the questionnaire were as follows: sex; age; dead or alive; family history; consanguineous marriage; height and body weight; initial symptoms; chromosomal abnormality; SCEs; analysis of *BLM* gene mutation; white blood cell count; T cell count; B cell count; serum IgG, IgM, IgA, and IgE levels; antibiotic prophylaxis; gamma globulin replacement; and hematopoietic cell transplantation. In addition, we asked about the presence or absence of the following diseases: (1) Skin disease, (2) Malignancy, (3) Autoimmune disease, (4) Muscle/Bone/Joint disease, (5) Allergy, (6) Neurological disease, (7) Intellectual disability/central nervous

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system malformation: IQ or DQ, (8) Cardiovascular disease, (9) Respiratory disease, (10) Kidney/urinary disease, (11) Gastrointestinal disease, (12) Liver/biliary/pancreatic disease, (13) Eye disease, (14) Otolaryngological disease, and (15) Metabolic and endocrine diseases.

Establishment of BS-iPSCs

Initially, a dermal fibroblast cell line from a patient with BS (Case 3) was generated in our laboratory. Informed consent was obtained from the patient. These fibroblasts were cultured in a growth medium containing Dulbecco's modified Eagle medium (DMEM)/F12 1:1 (Thermo Fisher Scientific, Rockford, IL, United States), 10% fetal bovine serum (Thermo Fisher Scientific), and 500 U/mL penicillin/streptomycin (Thermo Fisher Scientific), and were maintained in 5% CO_2 at 37 °C and passaged every seven days. Thereafter, iPSCs were generated via electroporation with the episomal vector reprogramming factors (OCT3/4, SOX2, KLF4, LIN28, L-MYC, and p53 shRNA) [16, 17]; the procedure was performed using a Nucleofector II Device (Lonza, Basel, Switzerland). We used iPSCs derived from a control individual as wild-type (WT) iPSCs; which was the 201B7 line [18].

Cortical neuron differentiation

In the first stage, we used a serum-free floating culture of embryoid body (EB)-like aggregates via the quick reaggregation (SFEBq) method, as previously described [19]. We treated 5000 single iPSCs with 2 μM dorsomorphin (Sigma-Aldrich, St. Louis, MO) and 10 μM SB431542 (SB; Cayman Chemicals, San Diego, CA) in a differentiation medium, based on a Dulbecco's modified Eagle's medium (DMEM)/F12 (Life Technologies), 5% knockout serum replacement (Life Technologies), and 500 U/ml PS for the first 2 days. After two days the medium was refreshed, using the same media formulation, and this was repeated every 2–3 days. Seven days later, EB aggregates were plated onto Matrigel-coated 96-well plates (Becton, Dickinson and Company, Franklin Lakes, NJ) and treated with 2 μM dorsomorphin and 10 μM SB in the first differentiation medium for an additional 7 days. After induction, the neural precursor cells were cultured in a second differentiation medium containing 10 ng/mL brain-derived neurotrophic factor (BDNF; R&D Systems, Minneapolis, MN), 10 ng/mL glial cell line-derived neurotrophic factor (GDNF; R&D Systems), 1 μM cyclic adenosine monophosphate (cAMP; Wako), and 200 ng/mL ascorbic acid (AA; Sigma-Aldrich). The medium was changed every 2–3 days.

Karyotyping and SCE assays

Karyotyping via chromosomal G-band analysis and SCE assays of BS-iPSCs was performed by the Nihon Gene Research Laboratories (Miyagi-ken, Japan, http://www.ngrl-japan.com) and Chromosome Science Labo Inc. (Sapporo, Japan, http://www.chromoscience.jp).

Immunocytochemistry

After washing the plated cells with PBS, they were fixed in 4% paraformaldehyde (Nacalai Tesque, Kyoto, Japan) for 20 min at 4 °C and then washed again with PBS. The cells were then blocked against nonspecific labeling with 5% donkey serum and 0.1% Triton X-100 (Nacalai Tesque) in PBS for 30 min at 4 °C. Cells were washed with PBS and incubated with primary antibodies overnight at 4 °C, followed by labeling with the appropriate fluorescent dye-labeled secondary antibody. The nuclei were stained with Hoechst 33342 (Life Technologies). The primary antibodies used in this study are listed in Supplementary Table 1. The secondary Alexa Fluor-labeled antibodies included: 594 donkey anti-rabbit, 594 donkey anti-goat, 594 donkey anti-mouse, 488 donkey anti-mouse, 488 donkey anti-rabbit, and 488 donkey anti-goat IgGs (Life Technologies, secondary antibody dilution was 1:1000).

Statistical analysis

SCE data are presented as the mean ± SD. Data related to cortical neurons are presented as the mean ± SEM. Statistical significance was evaluated using a two-tailed Student's *t*-test or two-way analysis of variance, followed by a two-tailed Student's *t*-test using the Statistical Package for the Social Sciences 16.0 J (SPSS Japan, Inc., Tokyo, Japan).

RESULTS

Nationwide survey of BS in Japan

In the survey, 427, 342, and 136 replies were obtained from the relevant specialist departments of training hospitals for pediatrics,

Table 1. Clinical features and gene mutations of BLM in Bloom syndrome investigated by a nationwide survey in Japan

Case No	sex	age	height (age)	BW (age)	skin lesion	intellectual disability	malignancy (age)	miscellaneous	IgG	IgA	IgM	SCE	BLM mutation
1	M	26 (alive)	139 cm (25)	29 Kg (25)	—	—	B cell lymphoma (8)		461	103	21	+	631delCAA/ND
2	F	13 (alive)	125 cm (13)	24 Kg (13)	café au lait spot	+	Burkitt lymphoma (13)	DM (type II)	600	66	27	+	631delCAA/735delACTG
3	F	24 (alive)	140 cm (20)	32 Kg (20)	—	—	—	bronchial asthma	711	110	33	+	631delCAA/631delCAA
4	M	7 (dead)	N.D.	N.D.	depigmentation	+	Wilms tumor (5), myelodysplastic disorder (5)	—	1690	39	27	+	1610insA/ND
5	F	16 (alive)	116 cm (9), 142 cm(16)	20 Kg (9), 42 Kg (16)	sun-sensitive erythema, pigmentation	+	—	ASD 、DM (type II)、urinary stone	700	137	43	+	A2475C/T2979C
6	F	28 (dead)	140 cm (26)	37 Kg (26)	—	ND	lymphoma (13), breast cancer (26)	DM, hepatic disorder	ND	ND	ND	+	631delCAA/1610insA
7	M	23 (dead)	143 cm (21)	43 Kg (21)	sun-sensitive erythema	—	B cell lymphoma (23)	DM (type I)	1166	233	28	+	631delCAA/631delCAA
8	F	40 (alive)	144 cm (19)	45 Kg (19)	sun-sensitive erythema	—	B cell lymphoma (25)	—	770	199	16	+	631delCAA/631delCAA

Case 7 and case 8 were siblings. The unit of IgG, IgA, IgM was mg/dL. Serum Ig levels in Japanese healthy individuals were as follows. Average (±standard deviation), 7 years-old: IgG; 1170 (775-1767), IgA 186 (104-331), IgM 112 (55-227), 13 years-old: IgG; 1319 (846-2009), IgA 266 (180-393), IgM 123 (53-284), over 15 years-old: IgG; 1312 (821-2099), IgA 251 (133-475), IgM 108 (44-264) [29]
DM diabetes mellitus, *ASD* atrial septal defect, -: not affected, SCE+: elevated frequency of sister chromatid exchanges, *ND* not dated

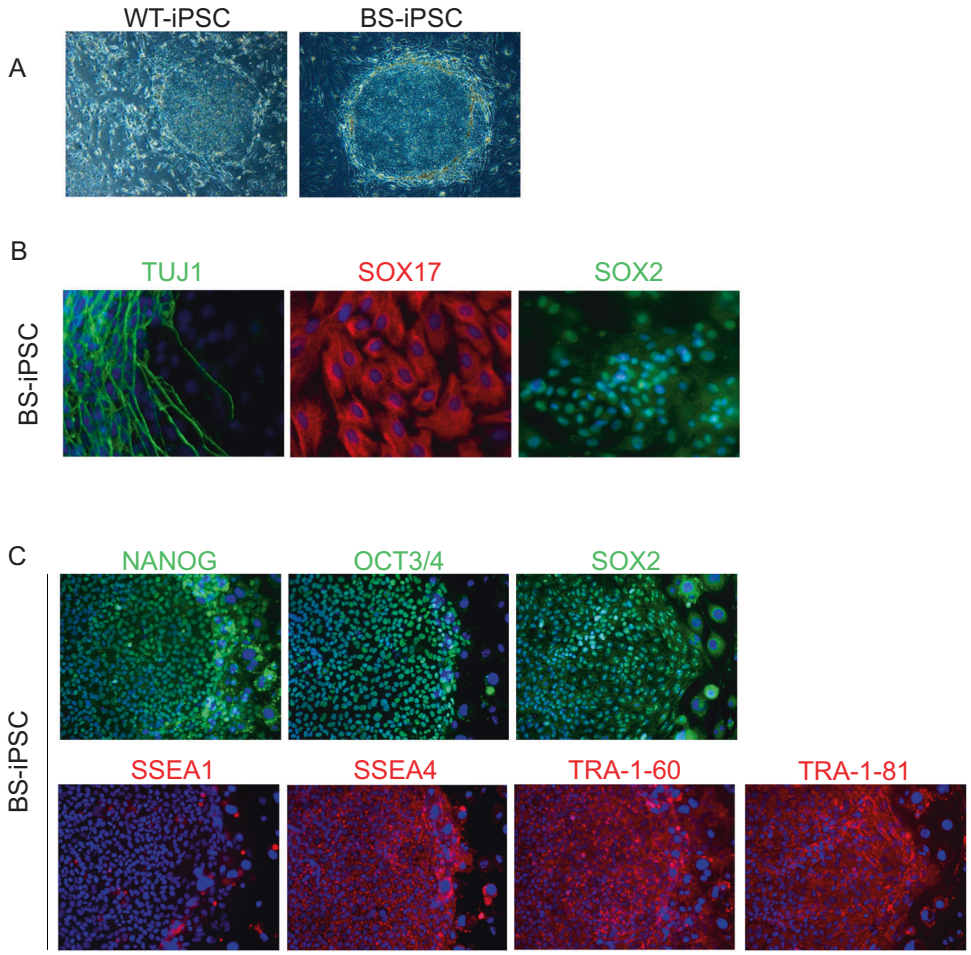

Fig. 1 Establishment of induced pluripotent stem cells (iPSCs) derived from patients with Bloom syndrome (BS). **A** Newly generated iPSCs fully reprogrammed from the fibroblasts of a patient with BS. Embryoid bodies formation of BS-iPSCs. Scale bar = 200 μm. **B** Expression of TUJ1, SOX17, and SOX2 in BS-iPSCs. **C** Expression of ectoderm, endoderm, mesoderm, and pluripotent markers including OCT3/4, NANOG, SOX2, SSEA4, TRA-1-60, and TRA-1-81. SSEA1 was the negative control. Scale bars = 100 μm

dermatology, and hospitals with a cancer center respectively. In the first questionnaire, twelve answers from attending physicians for suspected patients with BS were returned. Eight of the twelve patients in this study were recruited as patients with BS according to the criteria as described in "Material and Methods".

Table 1 presents the clinical features of this study from second questionnaires as documented by the attending physicians. Clinical features of Case 6 were not described in detail. Three patients died. Five of the eight patients had skin lesions, such as sun-sensitive erythema. Three of the seven patients had intellectual disabilities, and there were three cases of malignant B-cell lymphoma. Additionally, diabetes mellitus was observed in three patients.

No obvious susceptibility to infection was reported in any patient. All patients had low serum IgM levels (<50 mg/dL) and elevated frequencies of SCE. The *BLM* gene was investigated in eight patients, and the 631delCAA mutation of the *BLM* gene was detected in 9 of the 16 alleles.

Establishment of iPSCs derived from patients with BS (BS-iPSCs)

We established BS-iPSCs from a patient with homozygous 631delCAA *BLM* (Case 3). Dermal fibroblasts derived from a patient with BS (BS-FbCs) were generated. For the first time, we generated iPSCs derived from BS-FbCs using episomal vector reprogramming factors (*OCT3/4*, *SOX2*, *KLF4*, *LIN28*, *L-MYC*, and *p53 shRNA*) [17]. We confirmed the pluripotency of BS-iPSCs using embryoid body formation (Fig. 1A), which stained the ectoderm, endoderm, and mesoderm with markers, anti-β-III tubulin (TUJ1), SOX17, and SOX2, respectively (Fig. 1B). Thereafter, we examined whether the generated BS-iPSCs had characteristics similar to those of human ES cells. We performed immunostaining using undifferentiated markers and confirmed that OCT3/4, SOX2, and NANOG were expressed in the nuclei of the generated BS-iPSC; in contrast, SSEA4, TRA-1-60, and TRA-1-81 were all expressed in the cytoplasm (Fig. 1C). The generated iPSCs did not express SSEA1, a mouse undifferentiated marker (Fig. 1C). BS-iPSCs may have the potential to acquire additional chromosomal abnormalities due to genomic instability. Therefore, we compared the chromosomal profiles of the cultured BS-iPSCs using karyotype analysis. The chromosomal profiles of BS-iPSCs showed mosaicism with the following karyotypes: 18 of 20 cells had normal karyotype 46, XY, one cell was 46, XY with a deletion in 8q, and one cell was 46, XY with a deletion in 8q together with reciprocal translocations between 1p and 14q, and 4p and 7q.

SCE in peripheral blood and iPSCs derived from a healthy control and BS patient

BS is characterized by genomic instability, while gene translocation events such as SCEs have been observed in patients with BS. Therefore, to confirm the high SCE frequency in BS-iPSCs and peripheral blood, we compared the SCE frequency of peripheral blood and iPSCs of control individuals with that of a patient with

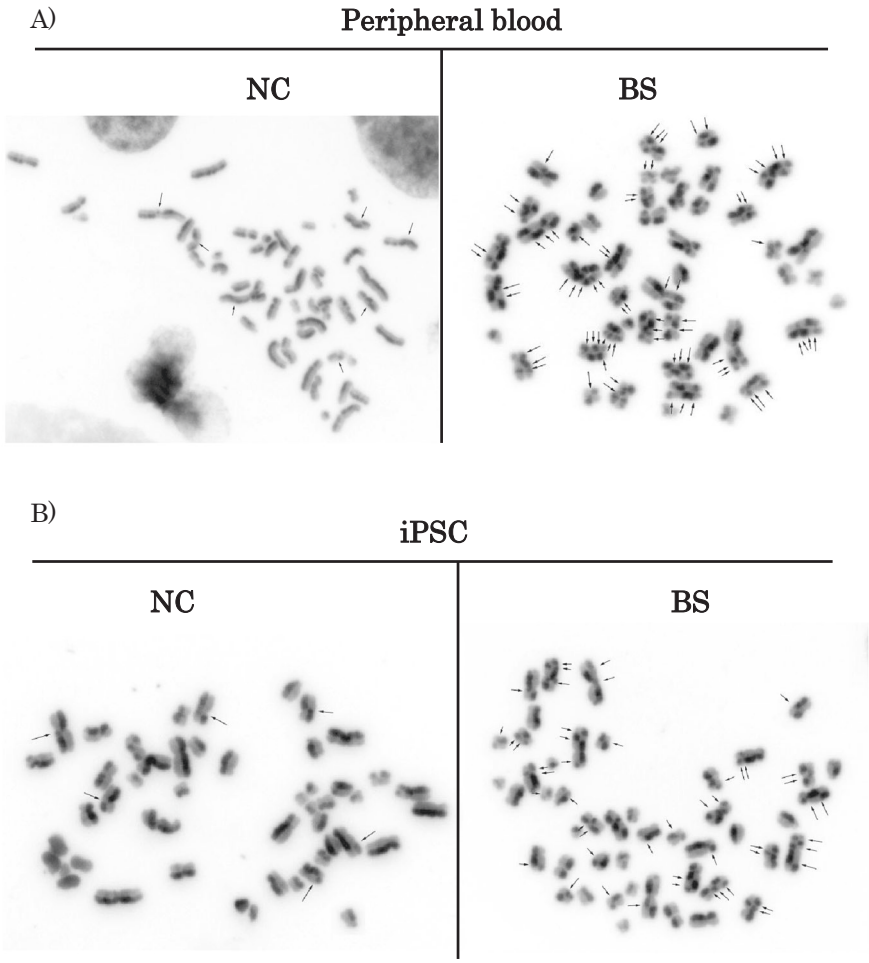

Fig. 2 Sister-chromatid exchange (SCE) of the peripheral blood and induced pluripotent stem cells (iPSCs) derived from patients with Bloom syndrome (BS). The regions of SCE were indicated by arrows. **A** The SCE of BS peripheral blood showed a 10-times higher frequency than that of the normal control. **B** The SCE of BS-iPSC showed a 10-times higher frequency than that of the normal control. The mean frequency and standard deviation of SCE in normal iPSCs and BS-iPSCs were 5.4 ± 2.2 and 57.8 ± 14.2, respectively ($n = 20$)

BS. The mean frequency and standard deviation of SCE in normal iPS and BS-iPS cells were 5.4 ± 2.2 and 57.8 ± 14.2, respectively. Our results show that the SCE frequency of the peripheral blood (Fig. 2A) and BS-iPSCs (Fig. 2B) of patients with BS were ten times higher than that of normal controls, suggesting that the generated BS-iPSCs reflected the genome instability of BS.

Axon elongation and proliferation of cortical neurons derived from WT- or BS-iPSCs

Cortical neurons were induced from BS-iPSCs, using a previously reported protocol (Fig. 3A) [19]. To investigate whether any differences could be observed, such as dendrite and axon development of cortical neurons derived from WT- and BS-iPSCs, we performed co-immunostaining using TUJ1, which is a dendritic marker protein, and anti-TBR1. BS-iPSCs showed no changes in TBR1-positive cortical neuron differentiation in WT- or BS-iPSCs (WT-iPSCs:77.58 ± 6.403%; BS-iPSCs:73.7 ± 3.44%) (Fig. 3B, C). Cortical neurons derived from BS-iPSCs showed shortened axons in our culture system (Fig. 3B, D). Mutations in *BLM* are expected to cause abnormal cell proliferation. Therefore, to investigate whether cortical neurons derived from BS-iPSCs have high proliferation potential, we performed immunostaining using anti-Ki-67 (a marker protein of proliferative cells). The results showed that Ki-67-positive cells were increased in cortical neurons derived from BS-iPSCs at day 72, but there was no change at day 50 (Fig. 3E, F).

DISCUSSION

We conducted a national survey to elucidate the clinical features of patients with BS in Japan. Consistent with a previous report, no obvious susceptibility to infection was reported in every patient [20], but all patients showed a serum IgM level of <50 mg/dL. As we previously reported, the 631delCAA mutation in the *BLM* gene is relatively common in Japan [21]. Four of the twelve patients did not meet the criteria for BS. One patient without SCE elevation was identified as AMeD syndrome (designated as N1037) [22].

One minor observation was that three of the seven patients showed intellectual disabilities. The attending physicians of the recruited patients with BS were asked about the presence or absence of intellectual disability, including IQ or DQ; though these were not documented in any of the three patients. Therefore, we were unable to determine details on intellectual disability. This is a major limitation of this study. Intellectual disability was caused by various etiologies such as mental retardation or autism spectrum disorder. Microcephaly, a complication of BS [23], also caused intellectual disability. We intend to clarify the characteristics of intellectual disability in BS in detail in future studies. To focus on BS-associated intellectual disability, we generated iPSCs derived from a patient with BS recruited in a nationwide survey.

We demonstrated that BS-FbCs could be reprogrammed into iPSCs using Yamanaka factors, and the resulting iPSCs showed undifferentiated states and the potential to differentiate into three germ layers. These results indicate that the generated BS-iPSCs

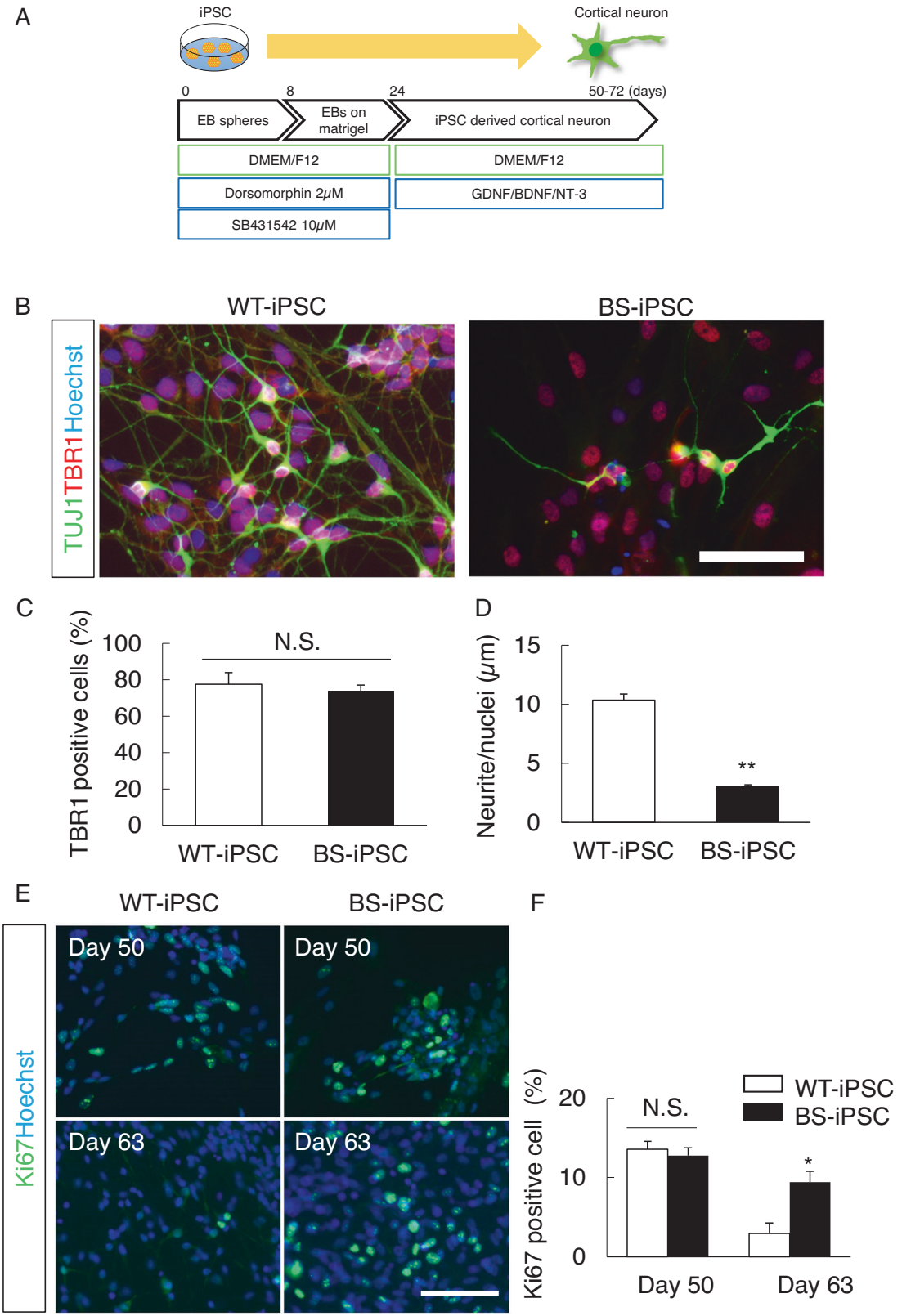

Fig. 3 Cortical neurons from induced pluripotent stem cells derived from patients with Bloom syndrome (BS-iPSCs). **A** Protocol for the induction of cortical neurons from BS-iPSCs (15). **B** Typical image of neurite length in cortical neurons derived from wild type (WT)- or BS-iPSCs. Scale bar = 100 μm. **C** The ratio of TBR1-positive cells in WT- or BS-iPSCs. **D** Quantitative analysis of neurite length and the TBR1 + cell rate. The neurite length of BS-iPSC-derived cortical neurons (TBR1+) was significantly shorter than that of induced iPSCs derived from WT ($n = 3$). **E** Representative image of Ki67+ cells from WT- or BS-iPSC-derived culture systems. **F** Quantitative analysis of Ki67+ cells in WT- or BS-iPSCs. The Ki67+ cell rate increased on day 63, although it did not change on day 50 ($n = 5$–6 or 3). $^{**}P < 0.01$. $^{*}P < 0.05$ versus WT-iPSCs (Student's *t*-test)

had ES-like features. A high frequency of SCE showed the hyperrecombinability of BS-iPSCs, which indicates genomic instability. Finally, we induced cortical neurons from the BS-iPSC. Most induced neuronal cells express TBR1, which is specifically expressed in the olfactory bulb, cerebral cortex, hippocampus, and amygdala [24–27], thus indicating that the generated TBR1-positive cortical neurons were induced with high efficiency. Furthermore, the generated cortical neurons derived from BS-iPSCs showed shorter axons and greater proliferation than those observed in cells derived from WT-iPSCs, suggesting that the *BLM* mutation drives the proliferation of immature neurons which fail to differentiate into mature cortical neurons. These findings provide evidence of intellectual disability found in some patients with BS. However, it is difficult to conclude whether the defects in cortical neurons derived from BS-iPSC, such as shortened axons and upregulated proliferation, are due to *BLM* mutations or chromosomal abnormalities observed in BS-iPSC. Only one iPSC cell line from each of BS and WT have been compared, and therefore gross generalizations cannot be made about the role played by BLM in neuronal differentiation.

It was previously reported that cerebellar Purkinje cells showed positive BLM immunoreactivity at 21 weeks of gestation, which transiently increased at 40 weeks. Further, neurons in the brain pons showed immunoreactivity during the early embryonic stages [28]. Therefore, BLM may be involved in the development of the central nervous system. The present study's data suggests that the BLM mutation at the fetal stage drives the proliferation of immature neurons which fail to differentiate into mature cortical neurons. Further investigation will be needed to determine whether control iPS cell-derived neurons that have a CRISPR-Cas9-induced nonsense mutation in the *BLM* gene, which may minimize chromosomal aberrations induced by the *BLM* gene mutation, show neuronal abnormalities as described above.

In conclusion, we established a human stem cell model system using BS-iPSCs and elucidated the phenotypes of cortical neurons derived from these cells. Consequently, we will be able to identify some aspects of the BS pathomechanism. These in vitro BS models may be useful for identifying novel therapeutic agents for the treatment of BS.

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AUTHOR CONTRIBUTIONS

Research design: MF, CK, YI, AY, and HK. Experiments, data acquisition, and data analysis: CK and HK. Manuscript writing: HK.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL

The generation and pathological analysis (including human gene analysis) of patient-derived iPSCs in this study were approved by the Ethical Review Committee of the National Hospital Organization, Nagara Medical Center (approval no. 26-15). Established human stem cells were handled according to revisions of the guidelines for clinical research using human stem cells by the Ministry of Health, Labour and Welfare of Japan. Written informed consent was obtained from the Bloom syndrome patient for the publication of this report. The 201B7 line was provided by Dr. K. Osafune (Kyoto University).

ADDITIONAL INFORMATION

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