Biliary atresia-specific deciduous pulp stem cells feature biliary deficiency

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Abstract

Background: Biliary atresia (BA) is a severe hepatobiliary disease in infants that ultimately results in hepatic failure; however, its pathological mechanism is poorly elucidated. Current surgical options, including Kasai hepatoportoenterostomy and orthotopic liver organ transplantations, are palliative; thus, innovation in BA therapy is urgent.

Methods: To examine whether BA-specific post-natal stem cells are feasible for autologous cell source for BA treatment, we isolated from human exfoliated deciduous teeth, namely BA-SHED, using a standard colony-forming unit fibroblast (CFU-F) method and compared characteristics as mesenchymal stem cells (MSCs) to healthy donor-derived control SHED, Cont-SHED. BA-SHED and Cont-SHED were intrasplenically transplanted into chronic carbon tetrachloride (CCl4)-induced liver fibrosis model mice, followed by the analysis of bile drainage function and donor integration in vivo. Immunohistochemical assay was examined for the regeneration of intrahepatic bile ducts in the recipient’s liver using anti-human specific keratin 19 (KRT19) antibody.

Results: BA-SHED formed CFU-F, expressed MSC surface markers, and exhibited in vitro mesenchymal multipotency similar to Cont-SHED. BA-SHED showed less in vitro hepatogenic potency than Cont-SHED. Cont-SHED represented in vivo bile drainage function and KRT19-positive biliary regeneration in chronic carbon tetrachloride-induced liver fibrosis model mice. BA-SHED failed to show in vivo bile potency and bile drainage function compared to Cont-SHED.

Conclusion: These findings indicate that BA-SHED are not feasible source for BA treatment, because BA-SHED may epigenetically modify the underlying prenatal and perinatal BA environments. In conclusion, these findings suggest that BA-SHED-based studies may provide a platform for understanding the underlying molecular mechanisms of BA development and innovative novel modalities in BA research and treatment.

Keywords: Biliary atresia, Patient-derived human deciduous pulp stem cells, Bile duct regeneration

Background

Biliary atresia (BA) is a severe hepatobiliary disease in infants with persistent jaundice, alcoholic stools, dark urine, and high levels of serum bilirubin [1]. If left untreated, BA leads to fibrosis, cirrhosis, and ultimately hepatic failure, resulting in rapid deterioration of infant health, failure to thrive, and death by one or two years of life. BA is characterized by obstructive cholestasis associated with progressive fibroinflammatory obliteration of the extrahepatic biliary tree and rapid progression of intrahepatic biliary fibrosis [2]. BA is managed by surgical options, including Kasai hepatopancreaticoenterostomy (KHPE) and orthotopic liver organ transplantations.
KHPE is essential for long-term survival with the native liver to recover bile drainage and liver fibrosis [3]. OLT is the only option for patients who do not respond or have life-threatening complications with or without KHPE. Thus, innovation in BA therapy is urgent.

Human deciduous pulp stem cells were first identified in the dental pulp tissues of exfoliated deciduous teeth, namely stem cells from human exfoliated deciduous teeth (SHED) [4]. SHED express mesenchymal stem cell (MSC) characteristics, including cell proliferation, multipotency, and immunosuppressive function [5]. Considering the recent development of the in vitro hepatic potency and in vivo anti-fibroinflammatory and hepatic regenerative effects [6,7], SHED may offer a novel modality for intractable liver diseases such as BA [8]. Stem cell transplantation has become a more feasible alternative to OLT [9]. Autologous transplantation may be more beneficial to BA than allogenic transplantation due to reduced surgical morbidity, limited immunosuppression-related toxicity, and increased cell engraftment [10].

BA causes deciduous tooth damage, including green pigmentation and dentin hypoplasia, and induces cell death and dentinogenic dysfunction in healthy donor-derived control SHED, referred to as Cont-SHED, via the AKT, extracellular regulated kinase 1/2, and nuclear factor kappa B pathways [11]. However, the stem cell properties and therapeutic potency of BA-patient-derived SHED, referred to as BA-SHED, are not well understood. Thus, this study aimed to demonstrate our hypothesis that BA-SHED is a feasible candidate as an autologous donor for BA research and therapy. We investigated the in vitro stem cell properties and hepatobiliary potency of BA-SHED. We also investigated the therapeutic efficacy of BA-SHED in chronically carbon tetrachloride exposed mice.

Characterization of BA-SHED

BA-SHED and Cont-SHED (P3) were characterized by the criteria of MSCs, including plastic adherent colony formation, surface antigen expression, and multilineage adipogenic, chondrogenic, and odontogenic/osteogenic differentiation, as described previously [13]. Isolated mononuclear cells (1.0 × 10⁴ cells per flask) were seeded in T-75 flasks and cultured in a complete growth medium for 16 days. The colonies formed were stained with a solution of 4% paraformaldehyde and 0.1% toluidine blue in PBS (pH 7.4). The expressions of CD146, CD105, CD34, CD19, CD14, CD11b, and HLA-DR were examined in P3 BA-SHED by flow cytometric (FCM) analysis. The cells were maintained under adipogenic, chondrogenic, and odontogenic/osteogenic culture conditions. The expressions of adipocyte-, chondrocyte-, and odontoblast/osteoblast-specific genes (peroxisome proliferator-activated receptor gamma 2 [PPARG2] and lipoprotein lipase [LPL] for adipocytes, SRY-box 9 transcription factor [SOX9] and collagen type X alpha 1 [COL10A1] for chondrocytes, and runt-related transcription factor 2 [RUNX2] and bone gamma-carboxylate protein [BGLAP] for odontoblasts/osteoblasts) were analyzed by quantitative reverse transcription polymerase chain reaction (RT-qPCR). Specific staining for adipogenesis, chondrogenesis, and odontogenesis/osteogenesis was analyzed by Oil Red O, Alcian blue, and Alizarin Red S staining, respectively. Cell proliferation was analyzed by cell viability, bromodeoxyuridine uptake, and population doubling assays. Telomerase activity was evaluated using a qPCR-based telomeric repeat amplification protocol using a TeloTAGGG Telomerase PCR ELISA kit (Merck, Darmstadt, Germany) according to the manufacturer’s instructions. Heat-inactivated samples were used as the negative controls.
Induction of BA-SHED into hepatocyte-like cells, BA-SHED-Heps
BA-SHED and Cont-SHED (P3; 2.5 × 10^5 cells per dish) were seeded on human fibronectin-coated 100-mm culture dishes (Corning, Corning, NY, USA) and maintained in complete growth medium until they reached confluence. The cultured cells were treated in a sequential hepatogenic medium based on Iscove’s modified Dulbecco’s medium (IMDM; Thermo Fisher Scientific) containing premixed P/S (Nacalai Tesque). The BA-SHED cells were preincubated with human epidermal growth factor (20 ng/mL; PeproTech, Rocky Hill, NJ, USA) and human fibroblast growth factor 2 (FGF2; 10 ng/mL; PeproTech) for 2 days. They were then stimulated with FGF2 (10 ng/mL; PeproTech), hepatocyte growth factor (HGF; 20 ng/mL; PeproTech), and nicotinamide (5 mmol/L; Merck) for 7 days and then cultured with oncostatin M (20 ng/mL; PeproTech), dexamethasone (1 µmol/L; Merck), and ITS+premix (50 mg/mL; Thermo Fisher Scientific) for 21 days as previously described [7]. The medium was changed twice a week. The expressions of hepatocyte-specific genes and hepatic function were assessed.

Hepatocyte-specific gene expression analysis in BA-SHED-Heps
Total RNA was extracted from cultured BA-SHED-Heps and Cont-SHED-Heps. Target genes were analyzed by RT-qPCR. Cont-SHED and BA-SHED were used as controls.

Immunofluorescence of BA-SHED-Heps
Cultured cells were blocked with 5% normal donkey serum (Thermo Fisher Scientific) and incubated with the primary antibodies, followed by treatment with Alexa Fluor 488- or Alexa Fluor 568-conjugated secondary antibodies (1:200; Thermo Fisher Scientific) and observed under an Axiovert light microscope (Carl Zeiss Microscopy, Jena, Germany). The primary antibodies used are summarized in Additional file 1: Table S1.

Secretion and hepatic metabolic assays of BA-SHED-Heps
BA-SHED-Heps and Cont-SHED-Heps were washed with Hanks’ balanced salt solution (HBSS) without phenol red (Nacalai Tesque) and incubated for 48 h in a freshly prepared hepatogenic culture medium. The hepatogenic culture medium contained FGF2 (10 ng/mL; PeproTech), HGF (20 ng/mL; PeproTech), and nicotinamide (0.61 g/L; Merck) in IMDM (Thermo Fisher Scientific). Some cultures were incubated with indirect bilirubin (25 nM; Merck) in HBSS for 60 min. The conditioned medium (CM) was collected from cultures. The contents of ALB, glucose, triglycerides, urea, and bilirubin in the CMs were measured by ELISA and colorimetric analysis.

Hepatic kinetic assay in BA-SHED-Heps
BA-SHED-Heps and Cont-SHED-Heps were incubated with indocyanine green (ICG; 1 mg/mL; Merck) in IMDM (Thermo Fisher Scientific) for 1 h. After washing with HBSS (Nacalai Tesque), the cells were cultured for 6 h. The cells were incubated overnight with 0.1% bovine serum albumin (BSA; Merck) in IMDM (Thermo Fisher Scientific) and treated for 5 h with Dil-conjugated acetylated low-density lipoprotein (Dil-Ac-LDL; 10 µg/mL; Cell Applications, San Diego, CA, USA) and 0.1% BSA (Merck) in IMDM (Thermo Fisher Scientific). BA-SHED-Heps and Cont-SHED-Heps were also incubated with choly-lysyl-fluorescein (CLF; 5 µM; BD Bioscience, Franklin Lake, NJ, USA) in HBSS (Nacalai Tesque) at 37 °C for 15 min. All samples were fixed with 4% paraformaldehyde and stained with DAPI (1 µg/mL; Thermo Fisher Scientific). All samples were imaged with an Axio Imager M.2 fluorescent microscope equipped with an Apotome 2 (Carl Zeiss Microscopy). Cont-SHED and BA-SHED were used as controls.

CYP3A4 activity test in BA-SHED-Heps
BA-SHED-Heps and Cont-SHED-Heps were incubated with or without dexamethasone (50 µM; Merck) for 24 h. CYP3A4 activity in BA-SHED-Heps and Cont-SHED-Heps was analyzed by a spectrometric assay using a P450-Glo CYP3A4 Kit with Luciferin-IPA (Promega, Madison, WI, USA). Results were measured with a GloMax Navigator luminometer (Promega) and shown as normalized relative light unit values to the untreated well in each culture condition according to the manufacturer’s instructions.

Transplantation of BA-SHED into CCl4-treated chronic liver fibrosis model mice
CCl4 was freshly mixed with olive oil (CCl4:olive oil = 1:4 volume/volume; FUJIFILM Wako Pure Chemicals) immediately before administration. The CCl4 solution (1.0 mL/kg body weight) or olive oil (1.0 mL/kg body weight; FUJIFILM Wako Pure Chemicals) was intraperitoneally injected into mice twice a week for eight weeks. BA-SHED and Cont-SHED (1 × 10^6 in 100 µL PBS per mouse) or PBS (100 µL) were infused into 4-week-CCl4-treated mice via the spleen. All mice were maintained...
under immunosuppressant-free conditions throughout the experiment.

In vivo assays for hepatic fibroinflammatory biomarkers in BA-SHED transplanted mice

Serum was harvested from mice 8 weeks after CCl₄ administration. Serum AST and ALT levels were measured by colorimetric assay using a Transaminase CII-Test Kit (FUJIFILM Wako Pure Chemicals) according to the manufacturer’s instructions. The expressions of alpha-smooth muscle actin 2, smooth muscle, aorta (Act2a), type 1 collagen alpha 1 chain (Col1a1), matrix metalloprotease (Mmp9), Timp2, tissue inhibitor of metalloproteinase 1 (Timp1), Timp2, interleave 6 (Il6), transforming growth factor-beta (Tgfβ), and tumor necrosis factor-alpha (Tnfa) were examined in mouse liver tissues by RT-qPCR. Serum human albumin (ALB) and bilirubin levels were measured by enzyme linked immunosorbent assay (ELISA) and colorimetric assay.

In vivo monitoring of transplanted donor cells in CCl4 treated mice

BA-SHED and Cont-SHED labeled with XenoLight DiR NIR fluorescent dye (DiR; 10 μg/mL; Perkin Elmer, Waltham, MA; 1 × 10⁶ in 100 μL PBS) or PBS (100 μL PBS) were intrasplenically infused into 4-week-CCl₄ treated mice. Ventral images of the mice were obtained 24 h after infusion with an optical in vivo imaging system IVIS Lumina III (Perkin Elmer) using living image software (Perkin Elmer).

Histological and immunohistochemical analyses in BA-SHED transplanted mice

Mouse livers were harvested 8 weeks after CCl₄ administration. The livers were fixed with 4% paraformaldehyde in PBS and processed for paraffin sectioning. The sections were stained with hematoxylin and eosin (H&E) and processed for paraffin sectioning. The paraffin sections were stained with hematoxylin and eosin (H&E) and examined in mouse liver tissues by RT-qPCR. Serum human albumin (ALB) and bilirubin levels were measured by enzyme linked immunosorbent assay (ELISA) and colorimetric assay.

Double immunofluorescent analysis in BA-SHED transplanted mice

Sections were treated with primary antibodies using an Opal 3-plex kit (Perkin Elmer, Waltham, MA, US) according to the manufacturer’s instructions and stained with DAPI (1 μg/mL; Thermo Fisher Scientific). All samples were imaged with an Axio Imager M.2 fluorescent microscope equipped with an Apotome 2 (Carl Zeiss Microscopy).

FCM analysis

Cultured cells (0.1 × 10⁶ cells per 100 μL HBSS) were suspended in ice-cold FCM buffer. The FCM buffer consisted of 2% heat-inactivated FBS (Merck) in HBSS (Nacalai Tesque). The cell suspension was incubated with R-phycocerythrin (R-PE)-conjugated primary antibodies (1 μg per 100 μL HBSS; Additional file 1: Table S2) at 4 °C for 45 min and measured on a FACSVersus flow cytometer (BD Biosciences, Franklin Lake, NJ, USA). As controls, isotype-matched antibodies conjugated with R-PE were used instead of primary antibodies. The percentage of positive cells was determined using FACSuite software (BD Biosciences) compared to control cells stained with corresponding isotype-matched antibodies in which a false-positive rate of less than 1% was accepted.

RT-qPCR assay

RNA samples were extracted from the cell and tissue samples using TRIzol reagent (Thermo Fisher
Fig. 1 (See legend on previous page.)
scientific), digested with DNase I (Promega), and purified using an RNase Mini Kit (Qiagen). cDNA was prepared from the purified total RNA by reverse transcription using a ReverTra Ace qPCR kit (TOYO-OBO, Tokyo, Japan) according to the manufacturer’s instructions and used for RT-qPCR assays. Gene expression was analyzed by RT-qPCR using the cDNA mixed with EagleTaq Master Mix (Roche Applied Science, Babaria, Germany) and target TaqMan probes (Thermo Fisher Scientific; Additional file 1: Tables S3 and S4) with a Light Cycler 96 system real-time PCR cycler (Roche Applied Science). The PCR steps were as follows: preincubation 1 at 50 °C for 120 s, preincubation 2 at 95 °C for 600 s, two-step amplification at 95 °C for 15 s, and 60 °C for 60 s (45 cycles). Human and mouse 18S ribosomal RNA were used for normalization.

**ELISA and colorimetric assay**

The total protein concentration of samples was quantified using commercially available kits (Additional file 1: Table S5), according to the manufacturer’s instructions. The results were measured using a Multiskan GO microplate reader (Thermo Fisher Scientific).

**Statistical analysis**

All tests were carried out in triplicates or more. Statistical results are expressed as the mean ± standard error of mean (SEM). Comparisons between two groups were performed using independent two-tailed Student’s t-tests. Multiple group comparisons were analyzed by one-way repeated measures analysis of variance followed by Tukey’s post hoc test. Differences were considered statistically significant at *P* < 0.05. All statistical analyses were performed using PRISM 6 software (GraphPad, Software, La Jolla, CA, USA).

**Results**

**BA-SHED displays MSC characteristics in vitro**

BA-SHED were isolated from three patients with BA (5–7 years old) using the CFU-F method. BA-SHED formed plastic-adherent colonies but showed a reduced colony frequency compared to Cont-SHED (Fig. 1a–c). BA-SHED exhibited reduced proliferation potential compared to Cont-SHED by bromodeoxyuridine uptake and population doubling assays (Fig. 1d, e). Telomerase activity was lower in BA-SHED than in Cont-SHED (Fig. 1f).

BA-SHED displayed a similar immunophenotype to Cont-SHED by FCM analysis (Fig. 1g, Additional file 1: Fig. S1). The primitive MSC marker CD146 was lower in BA-SHED than in Cont-SHED (Fig. 1g, Additional file 1: Fig. S1). BA-SHED and Cont-SHED expressed low levels of antigenic markers by FCM analysis and exhibited low immunogenicity by lymphocyte-mixed reaction test (Additional file 1: Fig. S2). BA-SHED displayed in vitro multipotency into adipocytes, chondrocytes, and odontoblasts/osteoblasts under lineage-specific induction conditions, as indicated by the expression of lineage-specific genes, including PPARG2, LPL, SOX9, COL10A1, RUNX2, and BGLAP, by RT-qPCR analysis and formation of lineage-specific matrix by Oil-Red O, Alcian blue, and Alizarin-Red S staining (Fig. 1h, i). The odontogenic/osteogenic capacity of BA-SHED was lower than that of Cont-SHED (Fig. 1h, i). These findings indicate that BA-SHED fulfilled the criteria for MSCs.

**BA-SHED exhibit a hepaticogenic potency in vitro**

Considering the in vitro hepaticogenic capacity of SHED into immature hepatocyte-like cells under hepaticogenic induction conditions with the stimulation of hepaticogenic cytokines [7], BA-SHED were induced into hepatocyte-like cells, referred to as BA-SHED-Heps, and the in vitro hepatic characteristics were analyzed. BA-SHED-Heps expressed higher levels of hepatocyte-specific genes, including ALB, HGF, keratin 18, and glycogen synthase kinase 3 beta (GSK3B), fatty acid synthase (FASN), and other hepatocyte-related genes, such as hepatocyte growth factor (HGF), CD146, and keratin 18 (KRT18). BA-SHED displayed low immunogenicity by lymphocyte-mixed reaction test (Additional file 1: Fig. S2). BA-SHED displayed a similar immunophenotype to Cont-SHED by FCM analysis (Fig. 1g, Additional file 1: Fig. S1). The primitive MSC marker CD146 was lower in BA-SHED than in Cont-SHED (Fig. 1g, Additional file 1: Fig. S1). BA-SHED and Cont-SHED expressed low levels of antigenic markers by FCM analysis and exhibited low immunogenicity by lymphocyte-mixed reaction test (Additional file 1: Fig. S2).

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Fig. 2 (See legend on previous page.)
sterol regulatory element-binding transcriptional factor 1 (SREBF1), arginase 2 (ARG2), argininosuccinate lyase (ASL), argininosuccinate synthase 1 (ASS1), carbamoylphosphate synthase 1 (CPS1), ornithine transcarbamylase (OTC), fumarylacetoacetate hydrolase (FAH), N-acetyl glutamate synthase (NAGS), and tyrosine aminotransferase (TAT), in comparison with non-induced intact BA-SHED by RT-qPCR (Fig. 2a). BA-SHED-Heps exhibited higher expression levels of KRT18, CYP3A7, GSK3B, FASN, SREBF1, ARG2, ASL, OTC, NAGS, and TAT, but a similar expression of HGF, ASS1, and CPS1, compared to Cont-SHED-converted immature hepatocyte-like cells, Cont-SHED-Heps (Fig. 2a). BA-SHED did not express CYP3A4 but exhibited a similar expression of CYP3A7 to Cont-SHED-Heps (Fig. 2a). Immunohistochemical analysis detected ALB, KRT18, and cadherin 1 (CDH1) in both BA-SHED-Heps and Cont-SHED-Heps (Fig. 2b).

BA-SHED-Heps and Cont-SHED-Heps exhibited the endocytotic and exocytotic activities of ICG (Additional file 1: Fig. S3). BA-SHED-Heps showed the secreting capacity of ALB, glucose, and triglycerides into the CM (Fig. 2c). BA-SHED-Heps showed no xenobiotic activity of CYP3A4, less production of urea, and similar secretion of direct and total bilirubin compared to Cont-SHED-Heps (Fig. 2d). BA-SHED-Heps, but not BA-SHED or Cont-SHED, showed intracellular accumulation of LDL and bile acid by Dil-Ac-LDL and CLF treatments (Fig. 2e, f). These findings indicated that BA-SHED exhibited less hepatogenic potency than Cont-SHED.

BA-SHED transplantation ameliorates liver fibrosis in chronically CCl₄-treated mice

BA-SHED and Cont-SHED (1 x 10⁶ per mouse) were intrasplenically infused into 4-week-CCl₄-treated mice, referred to as BA-SHED and Cont-SHED mice, respectively. All mice were analyzed 8 weeks after CCl₄ treatment. Biochemical assays showed that the serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the CCl₄ mice were reduced in both the BA-SHED and Cont-SHED mice (Fig. 3a). The livers of BA-SHED and Cont-SHED mice, referred to as BA-SHED and Cont-SHED livers, respectively, showed decreased gene expression of Acta2 and Colla1 compared to the livers of CCl₄ mice, referred to as CCl₄ livers, by RT-qPCR (Fig. 3b). Immunohistochemical analysis and Sirus Red staining revealed decreased ACTA2-positive cells and fibrous tissue deposition in the BA-SHED and Cont-SHED livers (Fig. 3c, d). The BA-SHED and Cont-SHED livers showed reduced levels of fibroinflammation-related marker genes, including Mmp, Mmp2, Timp1, Timp2, Il6, Tgfb, and Tnfa, compared to the CCl₄ livers by RT-qPCR (Fig. 3e, f).

Transplanted donor BA-SHED integrates into the liver tissue of chronically CCl₄-treated mice

Using in vivo imaging, the fluorescence intensity of DiR-labeled donor cells was detected in the right upper quadrant of the abdomen corresponding to the BA-SHED and Cont-SHED livers, but not in the CCl₄ mice, 1 day after transplantation (Fig. 4a). Immunohistochemical analysis using human-specific antibodies showed that human leukocyte antigens A, B, and C, human hepatocyte-specific paraffin 1 (HepPar1), and human ALB-positive cells were detected in the parenchymal periphery of the BA-SHED and Cont-SHED livers, but not in the non-treated and CCl₄ livers (Fig. 4b, c). The HepPar1 positive area was lower in the BA-SHED livers than in the Cont-SHED livers (Fig. 4c). Serum human ALB levels were lower in the BA-SHED mice than in the Cont-SHED mice, but not in the non-treated and CCl₄ mice, as determined by ELISA (Fig. 4d).

BA-SHED transplantation does not rescue biliary excretion in chronically CCl₄-treated mice

Histological analysis showed that the intrahepatic bile ducts in the periporal region of mouse livers disappeared in the CCl₄ livers (Fig. 4e). The reconstruction of intrahepatic bile ducts was observed in the periporal region of the Cont-SHED livers, but not in that of the BA-SHED livers (Fig. 4e). Immunohistochemical analysis using
Fig. 3 (See legend on previous page.)
To understand the treatment discrepancy between Cont-SHED and BA-SHED, we suggested that healthy donor-derived SHED are a feasible autologous cell source for BA treatment when biliary deficiency is rescued.

This study demonstrated for the first time that SHED may orchestreally contribute to de novo bile duct formation in chronically injured livers. Further studies are necessary to clarify the cellular and molecular mechanisms of SHED-mediated cholangiogenesis.

The present study demonstrates the first generation of patient-derived BA-SHED. BA-SHED exhibited similar in vivo anti-fibroinflammatory effects and less in situ hepatic regeneration in chronically CCl₄-treated mice compared to Cont-SHED; moreover, BA-SHED failed to induce in vivo biliary regeneration. Autologous induced pluripotent stem cell (iPSC)-derived neural cells clearly show the therapeutic advantages to both cell survival and immune response rather than allogenic iPSC-derived neural cells in the primate brains [20, 21]. Although the potential low immunogenicity of allogenic SHED reduces the immune response [13], allogenic rejection in recipients has never been eliminated. Autologous SHED transplantation is a novel regenerative approach for treating pulp diseases [22]. These findings suggest that BA-SHED are a feasible autologous cell source for BA treatment when biliary deficiency is rescued.
Diverse factors, such as genetic alternation, viral infections, and immune dysregulation, are implicated in the pathogenesis of BA at the prenatal and perinatal stages [23, 24]. However, the etiology of BA has not yet been fully elucidated. Given that the present BA-SHED transplant study demonstrated the deficiency of in vivo bile duct regeneration and the less reduction of hyperbilirubinemia in chronically CCl₄-treated mice, BA-SHED
are not feasible in the present animal models but are supposed to be a practical source for BA research and treatment. Basic helix-loop-helix family member A15 (BHLHA15) is involved in the commitment of HPCs into hepatocytes with high gene expression and metabolic activity of CYP3A4 accompanied by the suppression of cholangiogenic differentiation [25]. Bhlha15 knockout mice promote pancreatitis by epigenetic reprogramming of genes via histone modification [26]. The gene expression and activity of CYP3A4 were silenced in BA-SHED-Heps. BA-SHED-Heps failed to express similar profile to CONT-SHED-Heps. These findings indicate that BA-SHED may exhibit hepatobiliary deficiency through epigenetic modification under prenatal and perinatal BA environments. The present in vitro odontogenic/osteogenic deficiency in BA-SHED may support the epigenetic regulation of BA-SHED under the BA environments.

The abnormal development of BA has been explained by developmental errors in the differentiation and morphogenesis of the bili duct system. Epigenetic modifications modify cell type-specific gene expression and regulate the development of various tissues and organs in the body [27]. Previous studies have shown that DNA hypomethylation alters interferon-gamma and CD11a expression in CD4-positive T cells of BA infants [28, 29] and causes biliary defects and interferon-gamma overexpression in zebrafish [30], suggesting that abnormal DNA methylation participates in the development of BA disorders. BA-specific iPSCs from peripheral blood cells show suppressed in vitro biliary potency but exhibit normal potency in HPCs [31, 32]. The present study evaluated that BA-SHED exhibited more demethylated DNA status than Cont-SHED. Thus, these findings suggest that the modified DNA methylation patterns of imprinted genes could change the present in vivo potency of biliary-specific deficiency and hepatic sufficiency in BA-SHED. However, genome-wide DNA methylation patterns and targeted DNA methylation in cholangiocyte-specific genes have not been analyzed in BA-SHED. Future DNA methylation analyses in BA-SHED could reveal the disease mechanism underlying BA and provide epigenetically engineered BA-SHED as a feasible autologous modality for BA treatment.

In conclusion, the present study demonstrated that SHED induced in vivo biliary duct and hepatocyte regeneration in chronically CCl₄-induced liver fibrosis mice. On the contrary, BA-SHED showed biliary deficiency and reduced hepatic regeneration in mice with liver fibrosis, indicating that BA-SHED are not feasible source for BA treatment. The therapeutic discrepancy may be due to the epigenetic modification in BA-SHED underlying the prenatal and perinatal BA environment. Thus, these findings suggest that BA-SHED-based studies may provide an innovative platform for establishing disease models, revealing disease mechanisms, and developing novel modalities, such as disease treatment and drug screening, in BA. This disease-specific SHED-based approach may be a feasible tool for the innovation of disease mechanisms and effective therapies for various disorders.
Available data and materials
All data generated and analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate
Human deciduous teeth samples were collected as discarded biological/clinical samples from BA and healthy donors (n = 3, 5–7 years old) in the Department of Pediatric Dentistry and Special Needs Dentistry of Kyushu University Hospital. Procedures for handling the human samples were approved by the Kyushu University Institutional Review Board for Human Genome/Gene Research (Protocol Number: 738-01, 02, 03, and 04). Written informed consent was obtained from the guardians on behalf of each child donor. All animal experiments in this study were approved by the Institutional Animal Care and Use of Laboratory Animals of Kyushu University (protocol number: A20-041-0 and A21-222-0). All methods were performed in accordance with the relevant guidelines and regulations.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing financial and non-financial interests.

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