Tolloid-Like 1 Negatively Regulates Hepatic Differentiation of Human Induced Pluripotent Stem Cells Through Transforming Growth Factor Beta Signaling

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Single nucleotide polymorphisms in Tolloid-like 1 (TLL1) and the expression of *TLL1* are known to be closely related to hepatocarcinogenesis after hepatitis C virus elimination or liver fibrosis in patients with nonalcoholic fatty liver disease. TLL1 is a type of matrix metalloprotease and has two isoforms in humans, with the short isoform showing higher activity. However, the functional role of TLL1 in human liver development is unknown. Here, we attempted to elucidate the function of human TLL1 using hepatocyte-like cells generated from human pluripotent stem cells. First, we generated TLL1-knockout human induced pluripotent stem (iPS) cells and found that hepatic differentiation was promoted by TLL1 knockout. Next, we explored TLL1-secreting cells using a model of liver development and identified that kinase insert domain receptor (FLK1)-positive cells (mesodermal cells) highly express TLL1. Finally, to elucidate the mechanism by which TLL1 knockout promotes hepatic differentiation, the expression profiles of transforming growth factor beta (*TGF*β), a main target gene of TLL1, and its related genes were analyzed in hepatic differentiation. Both the amount of active TGFβ and the expression of TGFβ target genes were decreased by TLL1 knockout. It is known that TGFβ negatively regulates hepatic differentiation. *Conclusion:* TLL1 appears to negatively regulate hepatic differentiation of human iPS cells by up-regulating TGFβ signaling. Our findings will provide new insight into the function of TLL1 in human liver development. (*Hepatology Communications* 2020;4:255-267).

hronic hepatitis is the main cause of liver cancer and cirrhosis. It is estimated that the number of deaths caused by viral hepatitis is 1.45 million on a global scale.[1] Furthermore, a meta-analysis has estimated that 25% of the global adult population suffers from nonalcoholic fatty liver disease (NAFLD).[2] Recently, a single nucleotide polymorphism (SNP) in the Tolloid-like 1 (*TLL1*) gene, which is one of the matrix metalloproteinases, has been found to be closely related with liver cancer development after elimination of hepatitis C virus[3,4] and with progression of hepatic fibrosis in patients with NAFLD.[5] However, the function of TLL1 in the liver is poorly understood.

TLL1 is one of the zinc-dependent matrix metalloproteases and belongs to a subfamily known as the bone morphogenetic protein 1 (BMP1)/tolloid-like proteinases (BTPs). The BTPs include TLL1 and BMP1, which is a homolog of TLL1, and their structures are similar to each other.[6,7] BTPs have an astacin-like catalytic domain at the N-terminus and multiple complement C1r/C1s, Uegf, Bmp1 (CUB) domains and epidermal growth factor (EGF) domains at the C-terminus. The multiple domains at the C-terminus of BTPs are involved in regulating the enzyme activity of BTPs.[8] Human TLL1 is reported to have two isoforms. TLL1 isoform 1 is a longer isoform that is transcribed from exon 1 to exon 21, whereas TLL1 isoform 2 is transcribed from a shorter messenger RNA species that contains exons 1-9 and is terminated with an additional exon located between exons 9 and 10. For those reasons, enzyme activity is consider to differ between TLL1 isoform 1 and isoform 2. On the other hand, mouse TLL1 is reported to have only one type of isoform (https://www.ncbi.nlm.nih.gov/gene/21892). There is thus a species difference between humans and mice in the TLL1 isoform number.

Procollagen, cytokines, and growth factors have been reported as substrates for BTPs.[9] BTPs have protein-cleaving ability and contribute to the formation of extracellular matrix and activation and inactivation of cytokines. TLL1-knockout (KO) mice have been created to elucidate the function of TLL1. TLL1-KO mice have interventricular septal defect and heart and aortic positioning abnormalities, resulting in embryonic lethality at 14.5 to 16.5 days postcoitum.[10] Although TLL1-KO has been shown to cause cardiac abnormalities, the function of TLL1 in the liver has not been reported. Elucidation of the function of TLL1 in human liver is essential to clarify the association between TLL1 and liver disease. However, because TLL1 in mice differs from that in humans, research using human models is essential.

In this study, we attempted to elucidate the function of the *TLL1* gene in human liver using a hepatic differentiation model of human pluripotent stem cells. Human pluripotent stem cells are useful as a model of liver development because they differentiate into hepatocytes by mimicking early liver development. We optimized the growth factors and small molecular compounds added in hepatic differentiation and

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succeeded in developing an efficient hepatic differentiation protocol of human induced pluripotent stem (iPS) cells.[11-13] In addition, we searched for genes and compounds that can improve the homologous recombination efficiency of human iPS cells using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 system. We found that RAD51 recombinase (RAD51) overexpression and valproic acid (VA) treatment could enhance homologous recombination efficiency,[14] which is essential for efficient CRISPR-Cas9-mediated gene knockin.

In order to elucidate the function of TLL1 in liver development, we attempted to establish TLL1-KO human iPS cells using the CRISPR-Cas9 system. Then, by performing hepatic differentiation of TLL1-KO human iPS cells, we elucidated the function of TLL1 in human liver development. We also attempted to identify TLL1-producing cells and to elucidate the mechanism by which TLL1 mediates the control of hepatic differentiation.

Materials and Methods

HUMAN iPS CELLS

The human iPS cell lines YOW-iPS cells and FCL-iPS cells[11] were maintained on 1 μg/cm^2 recombinant human laminin 511 E8 fragments (iMatrix-511, Nippi, Tokyo, Japan) with StemFit AK02N medium (Ajinomoto). To passage human iPS cells, near-confluent human iPS cell colonies were treated with TrypLE Select Enzyme (Thermo Fisher Scientific) for 3 minutes at 37°C. After centrifugation, human iPS cells were seeded at an appropriate cell density (5 × 10^4 cells/cm^2) onto iMatrix-511 and were then subcultured every 6 days. The genotype of *TLL1* in the two human iPS cell lines was rs17047200 AA (low risk SNP for hepatocellular carcinoma).[3]

ELECTROPORATION

The *TLL1* locus was targeted using donor plasmids and CRISPR-Cas9 plasmids. Efficient targeting experiments of human iPS cells were performed as described in our previous study.[14] Briefly, human iPS cells were treated with 10 μM VA for 24 hours. Human iPS cells (1.0 × 10^6 cells) were dissociated into single cells by using TrypLE Select Enzyme and were resuspended in prewarmed Nucleofector

Solution (Lonza). Electroporation was performed by using a four-dimensional (4D)-Nucleofector System and 4D-Nucleofector Kit (P3) (both from Lonza) according to the manufacturer's instructions. The ratio of Nucleofector Solution to the plasmid solution was 90 μL:10 μL (total 100 μL). The plasmid solution consisted of 5 μg donor plasmids, 5 μg CRISPR-Cas9 plasmids, and 1 μg RAD51-expressing plasmids. After electroporation, the cells were seeded onto 1 μg/cm^2 iMatrix-511-coated dishes and cultured with StemFit AK02N medium containing 10 μM Rho-associated protein kinase (ROCK) inhibitor. After culturing for 2 days, the medium was replaced with 10 μM puromycin-containing medium, which was removed 48 hours after its addition at which time the original medium was added. At 10 days after electroporation, 24 individual colonies were selected and seeded onto a 1-μg/cm^2 iMatrix-511-coated 24-well plate. After most of the wells became nearly confluent, polymerase chain reaction (PCR) was performed to examine whether the clones were correctly targeted.

CRISPR-Cas9 PLASMID

Plasmids expressing human codon-optimized *Streptococcus pyogenes* (hSp)Cas9 and single guide RNA (sgRNA) were generated by ligating double-stranded oligonucleotides into the *Bbs*I site of pX330 (Addgene no. 42230; http://www.addgene.org/42230/). The sgRNA sequences are shown in Table 1.

DONOR PLASMID

For knockin of the elongation factor 1 alpha (EF1α)-puromycin resistant protein (PuroR)-polyadenylation sequence (pA) cassette into the *TLL1* locus, a donor template plasmid was generated by

TABLE 1. SEQUENCES OF PRIMERS USED IN TARGETING EXPERIMENTS

	Primer Sequence
CRISPR-Cas9 plasmid	sgRNA: caccgTCGGCGAGGAAATGGACCTC
Donor plasmid	5 arm forward: atttctagaCCTTCCAAGGGCTCTGCCTT
	5 arm reverse: attgcatgcGAAAGTGGAGATGGTGGTGG
	3 arm forward: attgcggccgcCTCTATCGGCAAGAACTGTG
	3 arm reverse: attggatccTCAACGCCTAACATTGGCTC
Genotyping	forward: AGTTCTGGAGACGCGGCACT
	reverse: GCCTTGCTTGGACTCTATGG

conjugating the following four fragments: two homology arms (1.09 kb for the 5′ arm and 1.00 kb for the 3′ arm), an EF1α-PuroR-pA cassette, and linearized backbone plasmids (pENTR donor plasmids). The backbone plasmids were the kind gift of Dr. Akitsu Hotta (Center for iPS Cell Research and Application, Kyoto University).

HEPATIC DIFFERENTIATION

Before the initiation of hepatic differentiation, human iPS cells were dissociated into single cells by using TrypLE Select Enzyme and plated onto Matrigel-coated dishes. The cells were then cultured in StemFit AK02N medium for 24 hours. The differentiation protocol for the induction of definitive endoderm cells, hepatoblast-like cells, and hepatocyte-like cells was based on our previous reports with some modifications.[11] Briefly, in the definitive endoderm differentiation, human iPS cells were cultured for 4 days in Roswell Park Memorial Institute 1640 (RPMI1640) medium (Sigma-Aldrich), which contained 100 ng/mL Activin A (R&D Systems), 2× GlutaMAX, and 0.5× B27 Supplement Minus Vitamin A (Thermo Fisher Scientific). For the induction of hepatoblast-like cells, the definitive endoderm cells were cultured for 5 days in RPMI1640 medium containing 20 ng/mL BMP4 (R&D Systems), 20 ng/mL fibroblast growth factor 4 (FGF4; R&D Systems), 2× GlutaMAX, and 0.5× B27 Supplement Minus Vitamin A. To perform hepatic differentiation, the hepatoblast-like cells were cultured for 5 days in RPMI1640 medium containing 20 ng/mL hepatocyte growth factor, 2× GlutaMAX, and 0.5× B27 Supplement Minus Vitamin A. Finally, the cells were cultured for 11 days in hepatocyte culture medium (Lonza) without EGF but with 20 ng/mL oncostatin M and 3× GlutaMAX.

Results

ESTABLISHMENT OF TLL1-KNOCKOUT iPS CELLS

In order to generate TLL1-KO human iPS cells, genome editing was performed targeting exon 6 of the *TLL1* gene (Fig. 1A) because the enzyme active site of TLL1 is encoded by exon 6. Genotyping used the primer indicated by a red arrow in Fig. 1A to examine whether homologous recombination occurred. We confirmed that exon 6 of *TLL1* was deleted in both alleles (Fig. 1B), which suggests that we succeeded in establishing TLL1-KO iPS cells. The acquisition efficiency of the TLL1-KO iPS cells was 5.8% (data not shown).

Next, we evaluated whether deletion of the *TLL1* gene affects the pluripotent state of human iPS cells. There was no morphologic difference between wild-type (WT) iPS cells and TLL1-KO iPS cells (Fig. 1C). Expression levels of pluripotent markers (octamer-binding transcription factor 3/4 [*OCT3/4*] and SRY-box transcription factor 2 [*SOX2*]) in WT iPS cells and TLL1-KO iPS cells were analyzed by real-time reverse-transcription (RT)-PCR. There was no difference in the expression levels of pluripotent markers between WT iPS cells and TLL1-KO iPS cells (Fig. 1D). Immunostaining analysis showed that WT iPS cells and TLL1-KO iPS cells were positive for *OCT3/4* and *SOX2* (Fig. 1E). These results indicate that deletion of the *TLL1* gene does not affect the pluripotent state of human iPS cells.

TLL1 EXPRESSION ANALYSIS DURING HEPATIC DIFFERENTIATION OF HUMAN iPS CELLS

We investigated the expression profile of endogenous TLL1 during hepatic differentiation of human iPS cells. Expression of *TLL1* isoforms 1 and 2 in undifferentiated iPS cells (day 0), definitive endoderm cells (day 4), hepatoblast-like cells (day 9), and hepatocyte-like cells (day 25) was evaluated by western blot analysis (Fig. 2A). Results showed that expression levels of both *TLL1* isoform 1 and isoform 2 increased gradually during hepatic differentiation. Because the expression levels of hepatocyte markers (hepatocyte nuclear factor 4 alpha [HNF4α] and aspartate aminotransferase [AAT]) in hepatocyte-like cells (day 25) were sufficiently high, it was confirmed that human iPS cells were indeed differentiated into hepatocyte-like cells (Fig. 2B). In addition, the expression level of *TLL1* in TLL1-KO iPS cells was evaluated by western blot analysis. Both *TLL1* isoform 1 and isoform 2 were strongly expressed in WT iPS cell-derived hepatocyte-like cells, while they
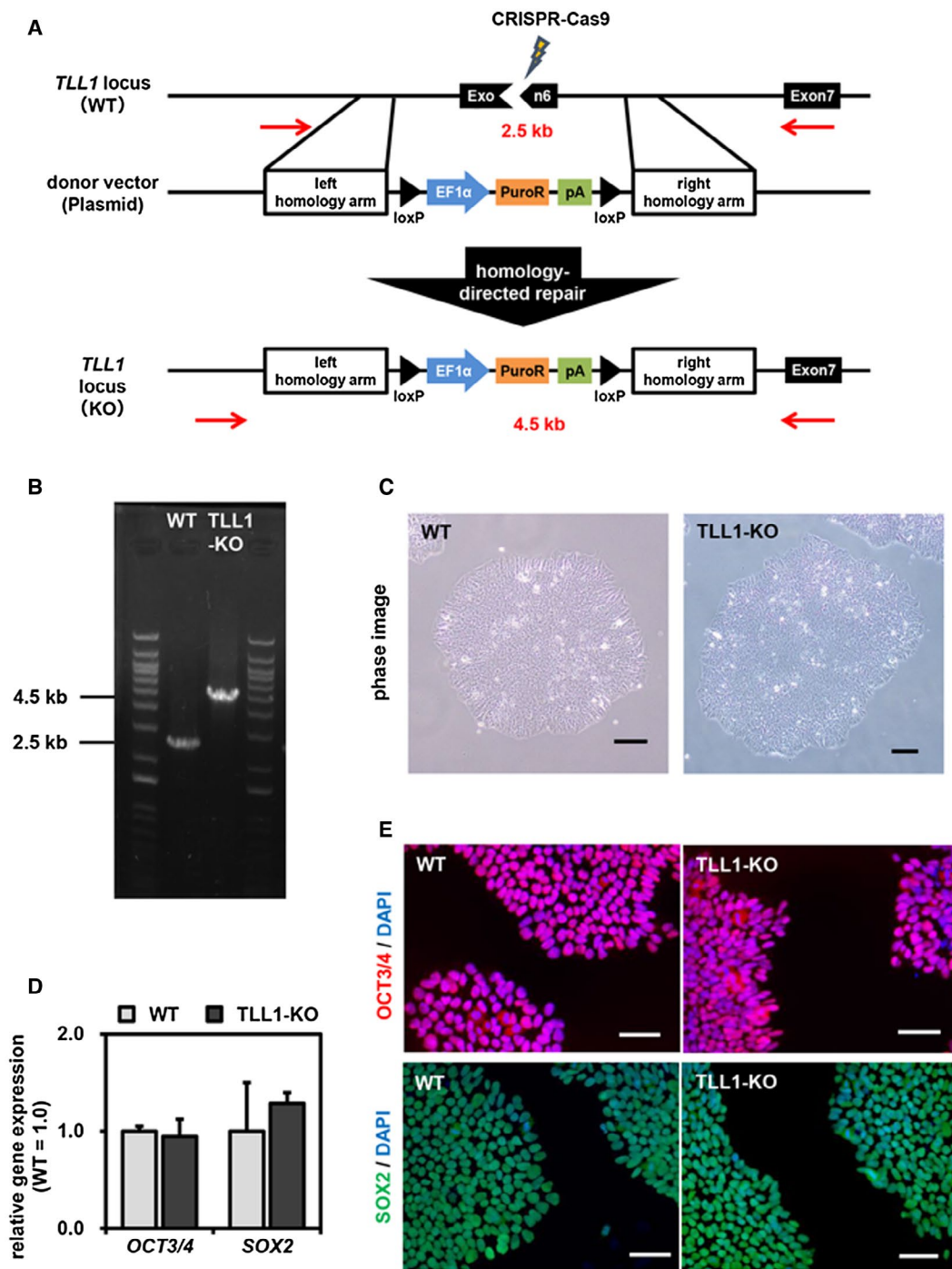
FIG. 1. Establishment of TLL1-knockout iPS cells. (A) Schematic overview of the targeting strategy for *TLL1* is shown. PCR primers that can distinguish the wild type and mutant alleles are shown with red arrows. The following donor plasmids were used to target the *TLL1* locus. Donor plasmids: EF1α, PuroR, pA. The CRISPR-Cas9 system was used to produce *TLL1* sequence-specific double strand breaks. (B) Genotyping was performed to examine whether the human iPS cell clones were correctly targeted. (C) Phase-contrast images of WT iPS cells and TLL1-KO iPS cells are shown. Scale bars, 100 μm. (D) Gene expression levels of pluripotent markers (*OCT3/4* and *SOX2*) were measured by real-time RT-PCR analysis in WT iPS cells and TLL1-KO iPS cells. Gene expression levels in undifferentiated WT iPS cells were taken as 1.0. (E) WT and TLL1-KO iPS cells were subjected to immunostaining with anti-OCT3/4 (red) and anti-SOX2 (green) antibodies. Nuclei were counterstained with DAPI (blue). Scale bars, 50 μm. All data are represented as means ± SE (n = 4). Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; loxP, Cre-protein site sequence.
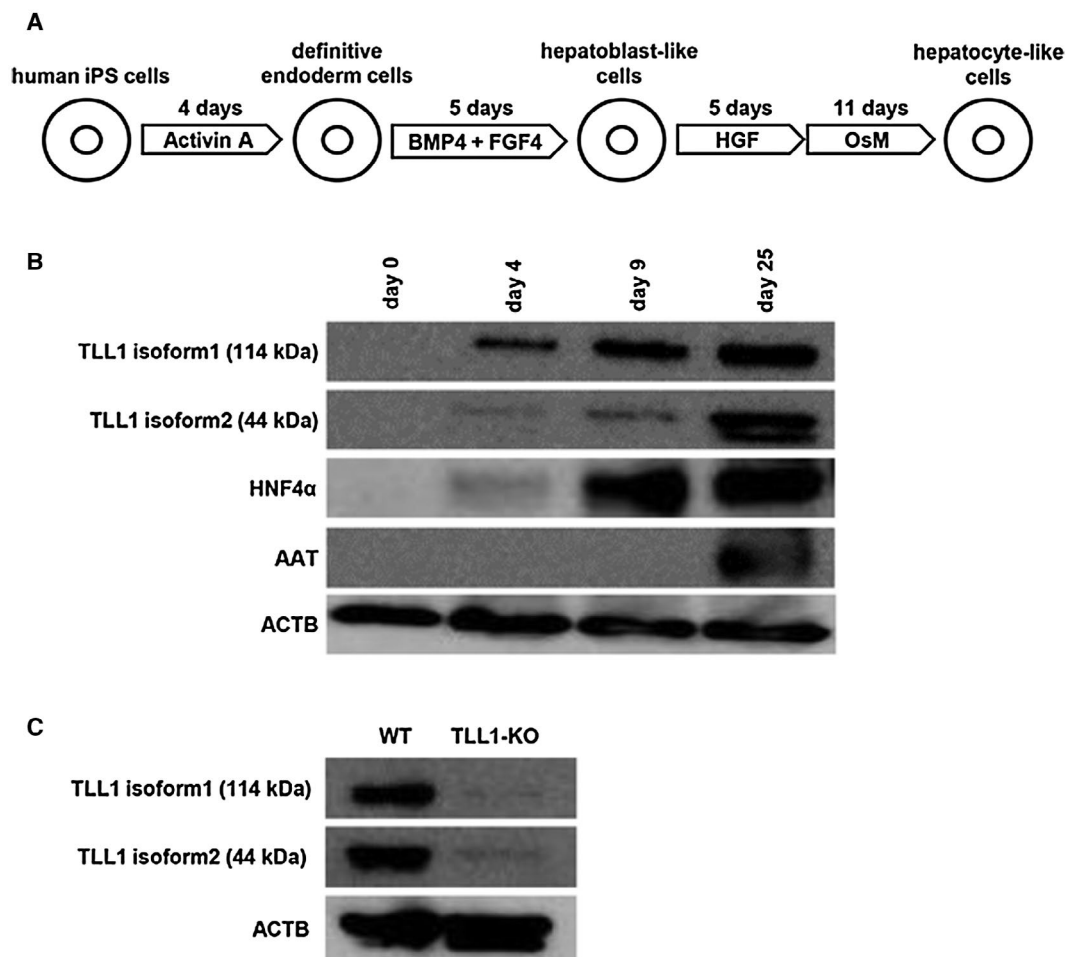
FIG. 2. TLL1 expression analysis during hepatic differentiation of human iPS cells. Human iPS cells (YOW-iPS) were differentiated into hepatocyte-like cells as described in Materials and Methods. (A) Schematic overview shows the protocol for hepatic differentiation. (B) Protein expressions of TLL1 isoform 1 (114 kDa), TLL1 isoform 2 (44 kDa), HNF4α, AAT, and ACTB in undifferentiated iPS cells (day 0), definitive endoderm cells (day 4), hepatoblast-like cells (day 9), and hepatocyte-like cells (day 25) were examined by western blotting. (C) Protein expressions of TLL1 isoform 1, TLL1 isoform 2, and ACTB in the WT iPS cell- and TLL1-KO iPS cell-derived hepatocyte-like cells were examined by western blotting. Abbreviations: ACTB, actin beta; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; OsM, oncostatin M.

were not expressed in TLL1-KO iPS cell-derived hepatocyte-like cells (Fig. 2C). Therefore, it was confirmed that TLL1 was not translated as a protein in TLL1-KO iPS cell-derived hepatocyte-like cells.

DEFINITIVE ENDODERM AND HEPATOBLAST DIFFERENTIATIONS WERE PROMOTED BY TLL1 KNOCKOUT

We next examined whether deletion of the *TLL1* gene affects the definitive endoderm differentiation of human iPS cells. For this purpose, WT iPS cells and TLL1-KO iPS cells were differentiated into definitive endoderm cells, and gene expression levels of definitive endoderm markers (*FOXA2* and *SOX17*) were analyzed by real-time RT-PCR. These endoderm markers were significantly increased in TLL1-KO iPS cell-derived definitive endoderm cells compared to WT iPS cell-derived definitive endoderm cells (Supporting Fig. S1A). In addition, immunostaining analyses revealed that *FOXA2* and *SOX17* expression levels in TLL1-KO iPS cell-derived definitive endoderm cells were higher than those in WT iPS cell-derived definitive endoderm cells (Supporting Fig. S1B). These results suggest that deletion of the *TLL1*

gene promotes definitive endoderm differentiation of human iPS cells.

We also examined whether deletion of the *TLL1* gene affects hepatoblast differentiation of human iPS cells. WT iPS cells and TLL1-KO iPS cells were differentiated into hepatoblast-like cells, and the gene expression levels of hepatoblast markers (alpha fetoprotein [*AFP*], keratin 19 [*CK19*], and *HNF4α*) were analyzed by real-time RT-PCR. Gene expression levels of *AFP*, *CK19*, and *HNF4α* were significantly increased in TLL1-KO iPS cell-derived hepatoblast-like cells as compared to WT iPS cell-derived hepatoblast-like cells (Fig. 3A). In addition, immunostaining analyses revealed that the *HNF4α* and *CK19* expression levels in TLL1-KO iPS cell-derived hepatoblast-like cells were higher than those in WT iPS cell-derived hepatoblast-like cells (Fig. 3B), suggesting that deletion of the *TLL1* gene promotes hepatoblast differentiation of human iPS cells.

To elucidate the function of *TLL1* isoform 1 and isoform 2, we examined whether overexpression of *TLL1* isoform 1 and isoform 2 affects hepatoblast differentiation in TLL1-KO iPS cells (Supporting Fig. S2). Gene expression levels of hepatoblast markers (*AFP*, *CK19*, and *HNF4α*) in TLL1-KO iPS cell-derived hepatoblast-like cells were decreased by the overexpression of *TLL1*

isoform 1 and isoform 2. Interestingly, gene expression levels of hepatoblast markers in Adenovirus-TLL1 isoform 2-transduced cells were slightly lower than those in Adenovirus-TLL1 isoform 1-transduced cells, suggesting that *TLL1* isoform 2 has a stronger negative effect on hepatoblast differentiation than *TLL1* isoform 1. These results suggest that the function of *TLL1* isoform 1 would differ from that of isoform 2 in hepatoblast differentiation.

HEPATIC DIFFERENTIATION IS PROMOTED BY TLL1 KNOCKOUT

To examine the effect of *TLL1* gene deletion on hepatic differentiation of human iPS cells, WT iPS cells and TLL1-KO iPS cells were differentiated into hepatocyte-like cells and gene expression levels of hepatocyte markers (albumin [*ALB*] and cytochrome P450 family 3 subfamily A member 4 [*CYP3A4*]) were analyzed by real-time RT-PCR. Gene expression levels of *ALB* and *CYP3A4* were significantly increased in TLL1-KO iPS cell-derived hepatocyte-like cells compared to WT iPS cell-derived hepatocyte-like cells (Supporting Fig. S3A). Immunostaining analyses showed that the AAT expression level in TLL1-KO iPS cell-derived hepatocyte-like cells was higher than
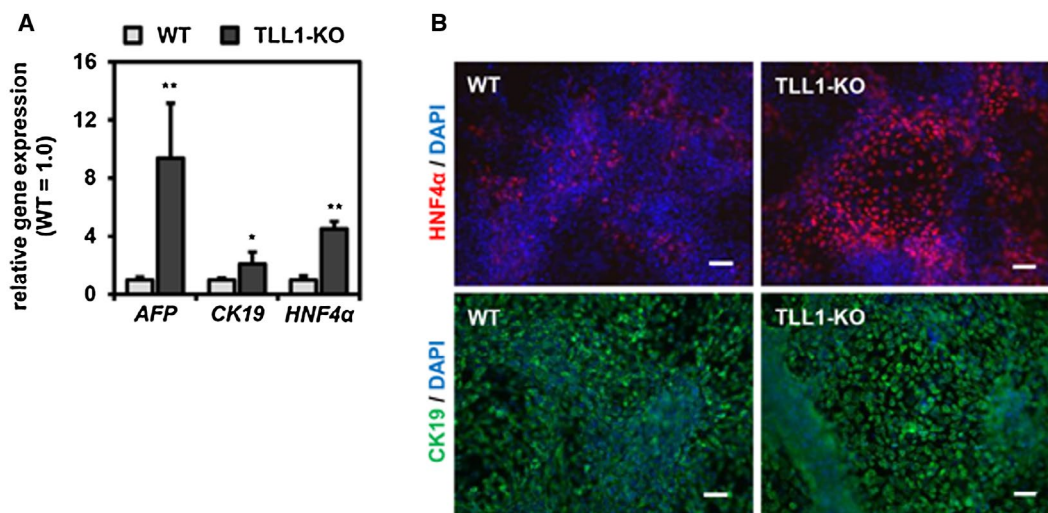

FIG. 3. Hepatoblast differentiations were promoted by TLL1 knockout. (A,B) Human iPS cells (YOW-iPS) were differentiated into hepatoblast-like cells as described in Materials and Methods. (A) Gene expression levels of hepatoblast markers (*AFP*, *CK19*, and *HNF4α*) were examined in WT iPS cell-derived and TLL1-KO iPS cell-derived hepatoblast-like cells by real-time RT-PCR. Gene expression levels in WT iPS cell-derived hepatoblast-like cells were taken as 1.0. (B) Expression of hepatoblast markers (HNF4α and CK19) in human iPS cell-derived hepatoblast-like cells was examined by immunohistochemistry. Nuclei were counterstained with DAPI (blue). Scale bars, 50 μm. All data are represented as means ± SE (n = 4). Statistical significance was evaluated by unpaired two-tail Student *t* test (*$P < 0.05$, **$P < 0.01$). Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

that in WT iPS cell-derived hepatocyte-like cells (Supporting Fig. S3B). In addition, we investigated the effect of *TLL1* gene deletion on hepatic function of human iPS cell-derived hepatocyte-like cells. At day 25 of differentiation, we examined ALB production and urea production capacities. Compared to WT iPS cell-derived hepatocyte-like cells, ALB production and urea production capacities were significantly increased in TLL1-KO iPS cell-derived hepatocyte-like cells (Supporting Fig. S3C,D). We also measured *CYP3A4* activity and found it to be significantly higher in TLL1-KO iPS cell-derived hepatocyte-like cells compared to WT iPS cell-derived hepatocyte-like cells (Supporting Fig. S3E). These results indicate that deletion of the *TLL1* gene promotes hepatic differentiation of human iPS cells and may further improve hepatic function of hepatocyte-like cells. Finally, this suggests that TLL1 might have a suppressive function on hepatic differentiation of human iPS cells.

TLL1 IS PRODUCED BY FETAL LIVER KINASE-1-POSITIVE MESODERMAL CELLS

TLL1 is known to be expressed at low levels in hepatocytes (https://www.ncbi.nlm.nih.gov/gene/7092). Therefore, we examined which cell types were producing TLL1 during hepatic differentiation of human iPS cells. It is known that TLL1 is strongly expressed in mesodermal tissues, such as fetal heart tissue (https://www.ncbi.nlm.nih.gov/gene/7092). It has also been reported that cells expressing a mesoderm marker (fetal liver kinase-1 [FLK1]) are observed during hepatic differentiation of human embryonic stem cells.[15] From these facts, we considered that TLL1 might be produced from FLK1-positive cells during hepatic differentiation of human iPS cells.

First, the percentage of FLK1-positive cells was analyzed in hepatic differentiation of human iPS cells. Percentages of FLK1-positive cells in undifferentiated cells (day 0), definitive endoderm cells (day 4), hepatoblast-like cells (day 9), and hepatocyte-like cells (day 25) were quantified by fluorescence-activated cell sorting (FACS) (Fig. 4A). At day 9, the percentage of FLK1-positive cells was 23%, which was the highest value during hepatic differentiation. Subsequently, we examined whether TLL1 was

produced from FLK1-positive cells. Gene expression levels of hepatoblast markers, mesodermal markers, and TLL1 in FLK1-positive cells, FLK1-negative cells, and cells before sorting were investigated by real-time RT-PCR. Gene expression levels of hepatoblast markers (*ALB*, *HNF4α*, *AFP*, *HNF1α*, and transthyretin [*TTR*]) were similar between cells before sorting and FLK1-negative cells. In the FLK1-positive cells, gene expression levels of hepatoblast markers were significantly lower than those in the cells before sorting or in FLK1-negative cells (Fig. 4B; Supporting Fig. S4). Taken together, these findings suggest that the fraction of FLK1-negative cells contained hepatoblast-like cells. Next, in order to examine whether mesodermal cells were present in the FLK1-positive fraction, we examined the gene expression levels of the mesodermal markers (*FLK1*, T-box transcription factor T [*TBXT*], and cluster of differentiation [*CD*]*31*) by real-time RT-PCR. Gene expression levels of the mesodermal markers were similar between the cells before sorting and the FLK1-negative cells, while gene expression levels of mesodermal markers were significantly higher in FLK1-positive cells compared to the other two groups (Supporting Fig. S5). This suggests that the fraction of FLK1-positive cells contained mesodermal cells. Finally, we used real-time RT-PCR to investigate whether the *TLL1* genes, including *TLL1* isoform 1, *TLL1* isoform 2, and total *TLL1*, were expressed in the FLK1-positive or FLK1-negative cells. Results showed that gene expression levels of *TLL1* isoform 1, *TLL1* isoform 2, and total *TLL1* were all significantly higher in FLK1-positive cells compared to the cells before sorting and FLK1-negative cells (Fig. 4C). In addition, gene expression levels of *TLL1* isoform 1 and isoform 2 were compared in FLK1-positive cells at day 9 of differentiation (Supporting Fig. S6). Gene expression level of *TLL1* isoform 1 was higher than that of *TLL1* isoform 2. These results indicate that the main producer of TLL1 was the FLK1-positive cells. The above results suggest that TLL1 (mainly TLL1 isoform 1) was produced by FLK1-positive cells, which are observed during hepatoblast differentiation of human iPS cells.

We expected that hepatic differentiation would be promoted by removal of FLK1-positive cells because the main producer of TLL1 was FLK1-positive cells. We removed FLK1-positive cells from the WT-iPS
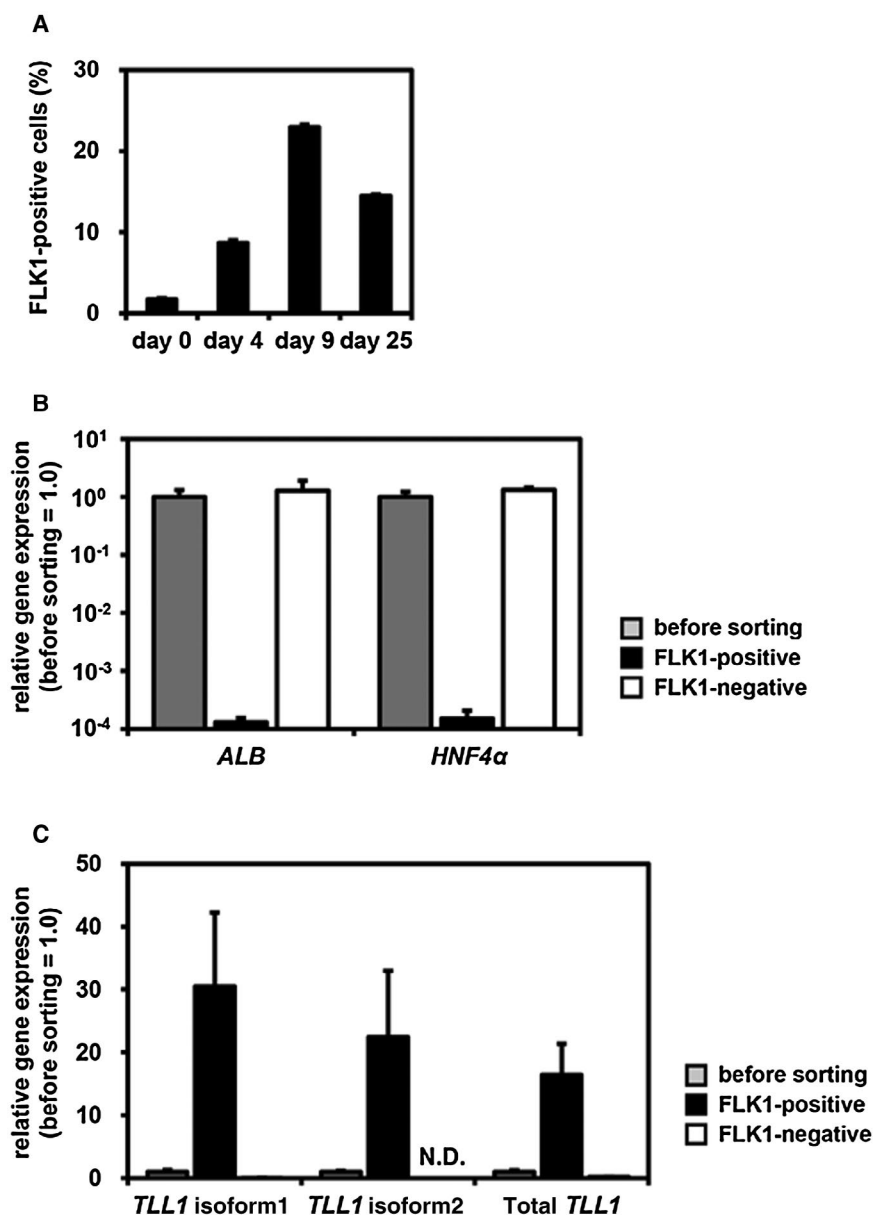
FIG. 4. TLL1 was secreted from FLK1-positive mesodermal cells. (A) Percentage of FLK1-positive cells during hepatic differentiation was examined by FACS analysis (day 0, undifferentiated iPS cells; day 4, definitive endoderm cells; day 9, hepatoblast-like cells; day 25, hepatocyte-like cells). (B,C) At day 9 of differentiation, FLK1-positive cells were sorted. (B) Gene expression levels of hepatoblast markers (*ALB* and *HNF4α*) in FLK1-negative and FLK1-positive cells were examined by real-time RT-PCR. Gene expression levels in the hepatoblast-like cells (before sorting) were taken as 1.0. (C) Gene expression levels of *TLL1* isoform 1, isoform 2, and total *TLL1* in FLK1-negative and FLK1-positive cells were examined by real-time RT-PCR. Gene expression levels in the hepatoblast-like cells (before sorting) were taken as 1.0. All data are represented as means ± SE (n = 4). Abbreviation: ND, not determined.

cell-derived hepatoblast-like cells by cell sorting and then performed hepatic differentiation (Supporting Fig. S7). Unexpectedly, hepatic differentiation was not promoted by removal of FLK1-positive cells (Supporting Fig. S7A). To investigate this result, the

percentage of FLK1-positive cells was examined by FACS analysis (Supporting Fig. S7B), which showed that FLK1-positive cells were in hepatocyte-like cells differentiated from FLK1-negative hepatoblast-like cells. These results suggest that FLK1-positive cells

differentiated from FLK1-negative hepatoblast-like cells would exhibit negative effects on hepatic differentiation.

GENETIC DEPLETION OF TLL1 PROMOTES HEPATIC DIFFERENTIATION IN ASSOCIATION WITH ATTENUATION OF TRANSFORMING GROWTH FACTOR BETA SIGNALING

Next, we investigated the mechanisms by which TLL1 suppresses hepatic differentiation. It is known that BMP1, a homolog of TLL1, cleaves latent transforming growth factor beta (TGFβ)-binding proteins.[16] Following cleavage, the TGFβ precursor becomes active TGFβ. It has also been reported that TGFβ suppresses hepatic differentiation of hepatoblast-like cells.[17] From these reports, we predict that TLL1 might suppress hepatic differentiation by increasing the amount of active TGFβ in the culture supernatant.

In order to investigate the involvement of TGFβ signaling, we first examined gene expression levels of various TGFβ ligands in human iPS cell-derived hepatoblast-like cells and found *TGFβ1* to be the most highly expressed (Fig. 5A). Subsequently, we examined whether there is a difference in the sensitivity of TGFβ1 between FLK1-positive cells (mesodermal cells) and FLK1-negative cells (hepatoblast-like cells). TGFβ1 is known to bind to TGFβ receptor 2 (TGFBR2) and transmit signals into cells.[18] The level of *TGFBR2* expression was quantified by real-time RT-PCR and was not significantly different between FLK-negative cells and FLK1-positive cells (Supporting Fig. S8). It was therefore suggested that both FLK1-positive cells (mesodermal cells) and FLK1-negative cells (hepatoblast-like cells) have similar sensitivities to TGFβ1 stimulation.

Next, we examined the relationship between TLL1 and TGFβ signaling. First, the amount of active TGFβ1 contained in the culture supernatant of hepatoblast-like cells was quantified by enzyme-linked immunosorbent assay. Compared to WT iPS cell-derived hepatoblast-like cells (32.2 ng/mL per 24 hours per milligram protein), TLL1-KO iPS cell-derived hepatoblast-like cells contained a significantly lower amount of TGFβ1 in the supernatant (21.9 ng/mL

per 24 hours per milligram protein) (Fig. 5B). In addition, we examined whether the amount of TGFβ1 contained in the supernatant affects intracellular TGFβ signaling. TGFβ1 has been shown to activate SMAD family member 2/3 (SMAD2/3) (phosphorylation).[18] The amount of phosphorylated SMAD3 was reduced by the deletion of TLL1 (Fig. 5C). In addition, gene expression levels of TGFβ target genes (cyclin dependent kinase inhibitor 2B [*P15*], cyclin dependent kinase inhibitor 1A [*P21*], and matrix metallopeptidase 9 [*MMP9*]) were quantified by real-time RT-PCR. Gene expression levels of the TGFβ target genes were significantly reduced by the deletion of TLL1 (Fig. 5D). These results suggest that TLL1-KO iPS cell-derived hepatoblast-like cells had a reduced amount of TGFβ1 released in the supernatant and attenuated TGFβ signaling compared to WT iPS cell-derived hepatoblast-like cells.

TGFβ is known to promote epithelial-mesenchymal transition (EMT).[18] We examined whether the activation of TGFβ signaling would affect the EMT of hepatoblast-like cells. For this purpose, we analyzed gene expression levels of EMT-related markers in WT iPS cell-derived hepatoblast-like cells and TLL1-KO iPS cell-derived hepatoblast-like cells by real-time RT-PCR. We found that gene expression levels of epithelial markers (claudin [*CLDN1*] and occludin [*OCLN*]) were significantly increased by TLL1 deletion (Fig. 5E), while gene expression levels of mesenchymal markers (actin A2 [*ACTA2*] and collagen type I alpha 1 chain [*COL1A1*]) were significantly reduced by TLL1 deletion (Supporting Fig. S9A). Moreover, gene expression levels of the EMT-promoting transcription factors (Snail family transcriptional repressor [*SNAI*]*1*, [*SNAI*]*2*, and Twist family basic helix-loop-helix transcription factor 1 [*TWIST1*]) were significantly reduced by the deletion of TLL1 (Supporting Fig. S9B). These results suggest that EMT is promoted in WT iPS cell-derived hepatoblast-like cells compared to TLL1-KO iPS cell-derived hepatoblast-like cells. From the above, it is possible that TLL1 produced by mesodermal cells exerts a suppressive effect on hepatic differentiation by releasing TGFβ1, activating TGFβ signaling, and promoting EMT. Although most data in this study were obtained using the human iPS cell line (YOW-iPS cells), similar results could be obtained using the other human iPS cell line (FCL-iPS cells) (Supporting Fig. S10).
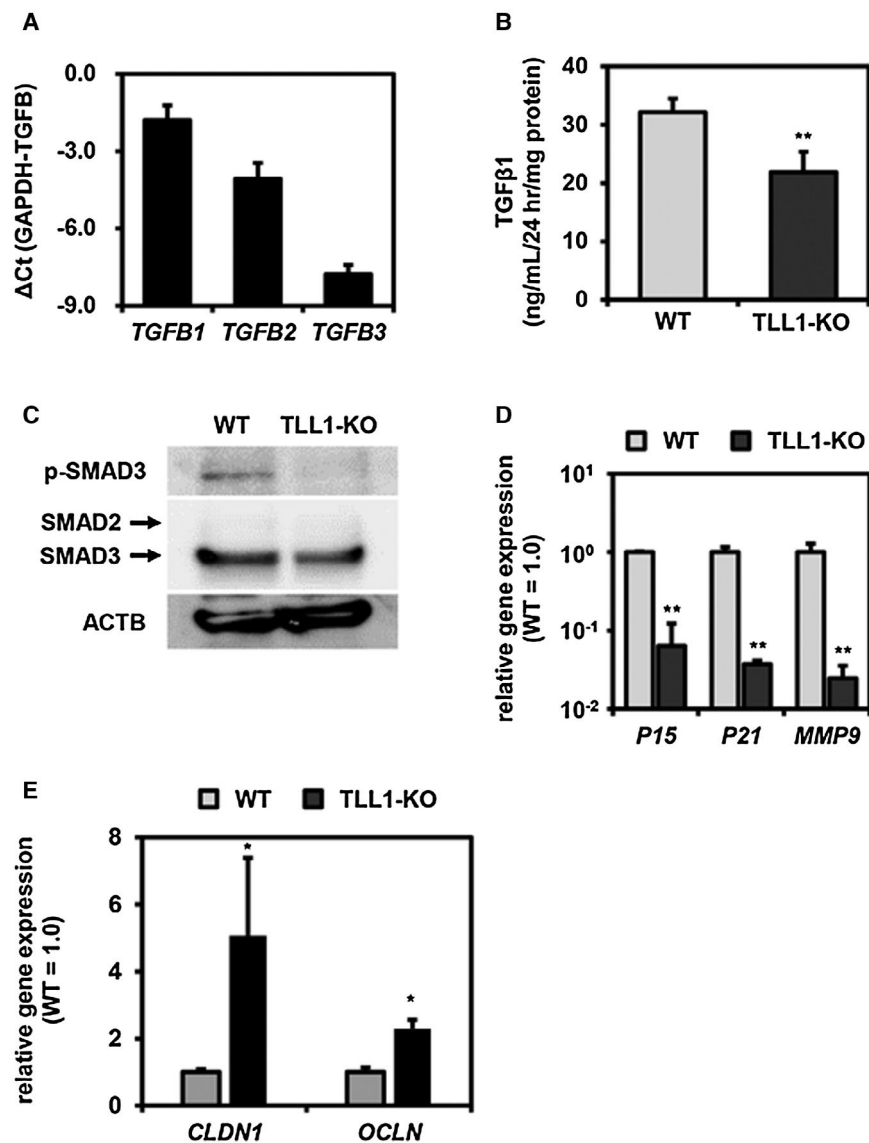
FIG. 5. TLL1 negatively regulated hepatic differentiation through the activation of TGFβ signaling. Human iPS cells (YOW-iPS) were differentiated into hepatoblast-like cells as described in Materials and Methods. (A) Gene expression levels of *TGFB*1, *TGFB2*, and *TGFB3* were examined by real-time RT-PCR. (B) The amount of active TGFB1 in the culture supernatant was examined by enzyme-linked immunosorbent assay. (C) Protein expressions of p-SMAD3, SMAD2, SMAD3, and ACTB were examined by western blotting analysis. (D) Gene expression levels of TGFβ-target genes (*P15*, *P21*, and *MMP9*) in WT iPS cell-derived and TLL1-KO iPS cell-derived hepatoblast-like cells were examined by real-time RT-PCR. Gene expression levels in WT iPS cell-derived hepatoblast-like cells were taken as 1.0. (E) Gene expression levels of EMT-related genes (*CLDN1* and *OCLN*) in WT iPS cell-derived and TLL1-KO iPS cell-derived hepatoblast-like cells were examined by real-time RT-PCR. Gene expression levels in WT iPS cell-derived hepatoblast-like cells were taken as 1.0. All data are represented as means ± SE (n = 4). Statistical significance was evaluated by unpaired two-tail Student *t* test (*$P < 0.05$, **$P < 0.01$). Abbreviations: ACTB, actin beta; *CLDN1*, claudin; Ct, cycle threshold; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; hr, hour; *MMP9*, matrix metallopeptidase 9; *OCLN*, occludin; pSMAD3, phosphorylated SMAD3.

Discussion

The *TLL1* gene has been suggested to be closely related to liver diseases.[3,5] To investigate the function of this gene in the human liver, we established a TLL1-deficient human iPS cell line. We found that TLL1 deficiency promoted the hepatic differentiation of human iPS cells. We also showed that the

suppressive effect of TLL1 on hepatic differentiation was mediated by TGFβ signaling. In addition, we identified that FLK1-positive cells were the source of TLL1 produced during hepatic differentiation of human iPS cells.

This study is the first to show that TLL1 suppresses hepatic differentiation of human iPS cells (Fig. 3). A previous study using mice suggested that the functions of BMP1 and TLL1 overlap.[10] To clarify the functions of BTPs in hepatic differentiation in the future, it may be helpful to create a TLL1 and BMP1 double-knockout human iPS cell line. Methods for using BTP-specific inhibitors[19] may also be effective.

We showed that TLL1 inhibited hepatic differentiation by positively regulating TGFβ signaling (Fig. 5). It is known that EMT occurs at initiation of liver development[20] and that TGFβ1 is generally considered to be one of the master positive regulators of EMT.[21] However, TLL1 also inhibited initiation of hepatic differentiation (Fig. 3). Therefore, TLL1 may also inhibit EMT using other than TGFβ signaling at initiation of hepatic differentiation.

We showed that deletion of the *TLL1* gene promotes hepatoblast differentiation (Fig. 3). However, the functions of TLL1 in hepatic maturation and hepatocyte maintenance still have room for clarification. It will thus be essential to establish a TLL1-conditional KO human iPS cell line and to use it to analyze the function of TLL1 in various hepatic differentiation steps. In addition, the effect of TLL1 on differentiation into nonparenchymal cells, such as hepatic stellate cells and cholangiocytes, is still unknown. Recent methods for the differentiation of nonparenchymal cells, including hepatic stellate cells[22] and cholangiocytes,[23] might clarify the function of TLL1 in nonparenchymal cells.

This study revealed that *TLL1* is highly expressed in mesodermal cells (FLK1-positive cells) during hepatic differentiation (Fig. 4C). In the future, it will be necessary to examine what type of cells the fraction of FLK1 and TLL1 double-positive cells differentiates into, including hepatic stellate cells, mesenchymal cells around blood vessels, and submesothelial cells.[24,25] If we could construct liver tissue containing nonparenchymal cells, such as hepatic stellate cells and sinusoidal endothelial cells, such liver tissue would be a valuable model for evaluating liver fibrosis.

Goldman et al.[15] have reported that FLK1-positive cells are generated during hepatic differentiation of human embryonic stem cells and that contamination of FLK1-positive cells promotes hepatic differentiation. This seems to contradict our finding that the *TLL1* highly expressed in FLK1-positive cells suppresses hepatic differentiation of human iPS cells. However, the effect of FLK1-positive cells on hepatic differentiation was not clarified in the present study. Coculturing TLL1-deficient FLK1-positive cells or TLL1-expressing FLK1-positive cells with hepatoblast-like cells would provide more detailed information.

We expect that the function of TLL1 in human liver will be fully clarified in the future using the TLL1-KO iPS cells established in this study. We also expect that TLL1-KO iPS cells will be used to clarify the role of TLL1 in liver diseases, such as liver fibrosis. We eagerly anticipate that drug screenings for liver diseases using TLL1-KO iPS cell-derived cells will lead to the development of therapeutic agents for liver fibrosis and other liver diseases.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1466/suppinfo.