is necessary to include a treatment protocol for NAFLD alongside with diabetes in these patients. Though therapies that focus on normalizing the abnormal metabolic system are in practice, satisfactory therapeutic outcomes have not been obtained. At present, research to analyze the morphological development of the liver is underway; however, several aspects remain to be elucidated.

Regardless of obesity, diabetes patients show high incidence of three major complications, namely, retinopathy, kidney disease, and peripheral neuropathic pain \[4\]. Angiopathy is the major cause of these complications resulting from a disrupted capillary system \[5, 6\], as seen from the analysis of tight junctions that act as barrier mechanisms. The expression of tight junction-forming factors was significantly reduced in the retina of diabetic mice \[7\]. Similarly, decreased expression of zonula occludens-1 (ZO1, encoded by the \text{Tjp1} gene) in the small intestine of diabetic mice is associated with inflammation and endotoxin invasion \[8\]. The binding disorders of hepatic tight junction are also one of the risk factors for causing liver disease. Functionally, tight junctions in the liver prevent the leakage of bile

---

**Abstract**

Diabetes patients are at a high risk of developing complications related to angiopathy and disruption of the signal transduction system. The liver is one of the multiple organs damaged during diabetes. Few studies have evaluated the morphological effects of adhesion factors in diabetic liver. The influence of diurnal variation has been observed in the expression and functioning of adhesion molecules to maintain tissue homeostasis associated with nutrient uptake. The present study demonstrated that the rhythm-influenced functioning of tight junction was impaired in the liver of \text{ob/ob} mice. The tight junctions of hepatocytes were loosened during the dark period in control mice compared to those in \text{ob/ob} mice, where the hepatocyte gaps remained open throughout the day. The time-dependent expression of zonula occludens 1 (ZO1, encoded by \text{Tjp1} gene) in the liver plays a vital role in the functioning of the tight junction. The time-dependent expression of ZO1 was nullified and its expression was attenuated in the liver of \text{ob/ob} mice. ZO1 expression was inhibited at the mRNA and protein levels. The expression rhythm of ZO1 was found to be regulated by heat shock factor (HSF)1/2, the expression of which was reduced in the liver of \text{ob/ob} mice. The DNA-binding ability of HSF1/2 was decreased in the liver of \text{ob/ob} mice compared to that in control mice. These findings suggest the involvement of impaired expression and functioning of adhesion factors in diabetic liver complications.

**Keywords** Diabetes · Liver · ZO1 · Tight junction proteins · Diurnal variations

---

**Introduction**

Obesity associated with binge eating leads to the development of metabolic diseases, such as diabetes and dyslipidemia. Diabetes and obesity are strongly linked to the onset and progression of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) \[1\]. Although NAFLD and NASH progress to fatty liver, there exists an underlying relationship between the alleviation of diabetes and NAFLD that could be exploited to establish liver-focused treatments \[2\]. Since diabetes patients are at a high risk of developing hepatitis and liver cancer \[3\], it
from the bile duct and facilitate the uptake of nutrients [9]. However, the relationship between the alteration of liver-associated tight junctions and the associated pathological conditions remains unclear.

In the living system, factors linked to the defense and maintenance of homeostasis are controlled by the biological clock system. In type 2 diabetes, the expression amplitude of the clock gene, which is the core of the biological clock system, is attenuated in the liver, adipocyte, and pancreas [10, 11]. Conversely, the onset of type 2 diabetes may cause an irregular rhythm in the membrane protein activity [12]. Therefore, elucidating the pathophysiology of diabetes warrants biochemical analysis based on the biological clock system. Since alteration in the expression of tight junction-related factors was dependent on the time difference [13], it was assumed that disrupted physiological time-dependency in diabetes would result in the abnormal expression of liver tight junction-related factors, leading to the pathological manifestation of hepatitis. This study aimed to evaluate and explore the mechanism associated with the transformation of altered liver tight junction function in diabetes using ob/ob mice.

Materials and methods

Animal experiments

Six-week-old male C57BL/6J Ham Slc-ob/ob mice and age/sex-matched C57BL/6J Ham Slc-+/+ mice (control mice) were purchased from the Japan SLC Inc. (Shizuoka, Japan). Mice were housed in a light-controlled room at a temperature of 24±1°C and 60±10% humidity, with food and water available ad libitum. In the light/dark cycle, zeitgeber time (ZT) with ZT0 and ZT12 were defined as time of lights on and off, respectively. During the dark period, a dim red light was used to aid in animal treatment. Mice were reared in the above environment for two weeks to let them synchronized with the light/dark environment. After synchronization, 8-week-old mice were used in each experiment. Nutrients are taken up into hepatocytes mainly during the light period in mice. First, we selected ZT6, suspecting when the nutrient uptake into the liver raising higher [14, 15]. Second, we selected ZT18, when the time point of anti-phase from ZT6. Thus, Evans blue administration and collecting tissues were performed at ZT6, middle of the light period, and ZT18, middle of the dark period, in this study.

Vascular permeability was assessed by intravenous (i.v) administration of Evans blue dye (30mg/kg) at ZT6 and ZT18, according to the previous report [16]. After 1h post-injection, the mice were sacrificed by cervical dislocation under an anesthesia condition with isoflurane inhalation and thoroughly perfused with 0.9% saline to clear the circulation of any residual dye. The perfused liver was collected after fixation with 4% paraformaldehyde. Frozen liver sections were processed for fluorescent microscopic evaluation and photographed with a BZ-9000 microscope. For the assessment of Evans blue infiltration, 100mg of the liver was soaked in 500 µL of 4% paraformaldehyde, incubated at 55°C for a day (to extract Evans-blue), and centrifuged; the absorbance of the supernatant was measured at 610nm using an Infinite® 200 PRO plate reader (Tecan Group Ltd., Männedorf, CH). All the experiments were conducted under a protocol approved by the internal committee for animal experiment in Sanyo Onoda City University (Ethical approval protocol IC: #A-2021-13-A).

Biological variables

Plasma glucose and insulin levels were measured using LabAssay™ Glucose (Fujifilm Wako Chemical, Miyazaki, Japan) and mouse Insulin ELISA Kit (Merckodia AB, Uppsala, Sweden) according to the manufacturer’s protocol. Hepatic total lipid was extracted by Folch method, as described previously [17]. Hepatic triglyceride (LabAssay™ Triglyceride, Fujifilm Wako Chemical) and cholesterol (LabAssay™ Cholesterol, Fujifilm Wako Chemical) levels were measured using commercial assay kits following manufacturer’s instructions. The absorbance in each assay was measured using Infinite® 200 PRO plate reader (Tecan Group Ltd.).

Cell culture

Hepa1-6 cells were purchased from the Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan). Cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco BRL, Paisley, UK) supplemented with 10% fetal bovine serum (AFC Biosciences, Lenexa, KS, USA) and 50 U/mL penicillin/50µg/mL streptomycin (Gibco) at 37°C in a humidified 5% CO2 atmosphere.

The transcription reporter assay was performed by modifying the method reported in the past. [13, 14]. Mouse heat shock factor 1 (HSF1) and HSF2 expression vectors were purchased from OriGene (HSF1: MR208087, HSF2: MR208286, Guangzhou, China). Mouse tight junction protein 1 (mTjp1)-reporter vectors containing the mTjp1 promoter region spanning from −650 to +50, −248 to +50, and −50 to +50 (relative to the transcription start site, +1) were constructed using the VectorBuilder services (VectorBuilder Inc, Chicago, IL, USA). Hepa1-6 cells were seeded at a density of 2×10⁴ cells/well in 24-well culture plates. Cells were transfected with 50 ng of the green fluorescent protein (GFP) reporter construct and 500 ng (total) of the respective
expression vector using Lipofectamine® 3000 Reagent (Thermo Fisher Scientific, CA, USA). The pCMV-6 empty vector was added to obtain a constant final DNA concentration in all transfections. After 24h post-transfection, the fluorescence intensity was analyzed using an Infinite® 200 PRO plate reader (Tecan Group Ltd.). The ratio of GFP intensity to protein concentration in each sample served as a measure of normalization.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the mouse liver using the PureLink® RNA Mini Kit (Thermo Fisher Scientific) as per the manufacturer's instructions. Reverse transcription was performed with the PrimeScript™ RT reagent Kit (Takara Bio, Otsu, Japan). The cDNA equivalent of 10 ng of RNA was amplified by PCR using a StepOnePlus™ Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) with TB Green® Premix Ex Taq™ II (Takara Bio). The reaction was first incubated at 95°C for 30s, followed by 40 cycles at 95°C for 5s and 60°C for 30s. All experiments that used kits were performed as per the manufacturer’s instructions. The Comparative Ct method was applied to calculate the relative mRNA expression levels. The data were normalized to 18S ribosomal RNA gene (Rn18s) used as the internal control.

Protein extraction and western blotting

Hepatic membrane proteins were extracted from the mice liver using the FractionPREP™ cell fractionation kit (K270; Biovision, Mountain View, CA, USA) as per the manufacturer’s instructions [18]. Briefly, each tissue sample was minced using a scalpel and washed with ice-cold PBS twice. Samples were homogenized using Potter-Elekhjem Tissue Grinder in the 400 µL cytosol extraction buffer with a protease inhibitor cocktail (Nacalai tesque, Kyoto, Japan). The samples were then incubated on ice for 20min with gentle tapping 3–4 times every 5min after pipetting several times to mix well. The homogenate was centrifuged at 700×g for 10min at 4°C. The pellet was added 400 µL membrane extraction buffer with a protease inhibitor cocktail, mixed after adding 22 µL of membrane extraction buffer B, and then incubated on ice for 1min. The solution was centrifuged at 1000×g at 4°C for 5min. The supernatant was used as hepatic membrane fraction.

Western blots were performed as previously described [19]. Hepatic membrane fractions were denatured at 60°C for 30min. Denatured samples containing 20µg of each protein fraction were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride membrane. Separated proteins were stained with Coomassie Brilliant Blue (CBB) as a control for equal loading of the membrane fraction. The membranes were blocked with 1% skim milk (#9999; Cell Signaling Technology, Beverly, MA) in Tween20-TBS at room temperature of 1h with constant agitation. The membranes were incubated with primary antibodies against ZO1 (1:1000; ab15602; Abcam, Cambridge, UK) and β-Actin (1:1000; ab6276; Abcam, Cambridge, UK). After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, and the signal was detected using the Chemiluminescent substrate (ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Table 1 Primer sets for qPCR analysis of gene expression

| Gene name | Accession ID | Primer Sequence |
|-----------|-------------|-----------------|
| Tjp1      | NM_001163574.1 | F: CTCCGATCATTCCAC-GCA GT<br> R: TCGGTTCG-GAA GAGTTG |
|           |             | Tjp2 NM_001198985.2 | F: GGAGCCACAGATCT-GAAGGTGAACAC<br> R: ACGTTGGAATTC-TAGCAAGTGAGAC |
| Occludin  | NM_001360536.1 | F: GATTCGCGCGC-CAAGGT<br> R: TGCCCAGGATAGCGCT-GAC |
| Claudin 1 | NM_016674.4   | F: CAGCGGCAGATA-CAGTG<br> R: ATGCATTCATGC-CAATGGTGGA |
| Claudin 3 | NM_009902.4   | F: CCGCTTATCTGC-CGGAT<br> R: CGACTCTCGTAGT-GGTACG |
| Claudin 5 | NM_013805.4   | F: AGGATGGGCTGGCTT-GATCCT<br> R: GTACTCTGCACACC-GACGA |
| Clock     | NM_007715     | F: AACCGTACAGGTT-TATGGCAAT<br> R: TTGGTGCCAAC-CAATGGCAAG |
| Bmal1     | NM_007489     | F: AGCAGATAGGA-CACCTCGCAGA<br> R: CGGGTTCATGAAACT-GAACATC |
| Per2      | NM_011066     | F: ATCAGCCATGTGC-CGTC<br> R: CGTGCTCAGTGCT-GCTTTC |
| Dbp       | NM_016974     | F: AAGCATTCCAGG CatGAGAC<br> R: TTCTTGATCTCCG-GCTCCAG |
| Nr1d1     | NM_145434     | F: TGCTAAGTCG-GCATT<br> R: GTAGTTGTCGGCT-CAGGAA |
| Rn18s     | NR_003278.3   | F: CGCTACCACTC-CAAGGA<br> R: GCTGGAATTACCCCGCT-T
UK), ZO2 (1:1000; 18900-1-AP, Proteintech, Rosemont, IL), Oc1n (1:1000; 13409-1-AP, Proteintech), Cldn1 (1:2000; ab15098, Abcam) Cldn3 (1:1000; 16456-1-AP, Proteintech), and Cldn5 (1:1000; ab131259, Abcam) diluted with Can Get Signal Solution 1 (Toyobo, Osaka, Japan). Specific antigen-antibody complexes were visualized using HRP-conjugated anti-rabbit IgG (1:10000; sc-2032; Santa Cruz Biotechnology, Santa Cruz, CA) diluted with Can Get Signal Solution 2 (Toyobo) and ImmunoStar LD (Wako Chemicals, Osaka, Japan). Visualized images were scanned by a BIO-RAD ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, CA, USA).

Chromatin immunoprecipitation (ChIP) analysis

ChIP assay was performed with reference to past papers [13, 14]. Cross-linked chromatin from liver was sonicated on ice and nuclear fractions were obtained by centrifugation at 10,000 x g for 5 min. Supernatants were incubated with the following antibodies: anti-HSF1 (1:200; ab16502; Abcam), anti-HSF2 (1:200; ab32360; Abcam), or rabbit anti-IgG (1:200; sc66931; Santa Cruz Biotechnology). DNA was purified using the DNA purification kit (Promega, Madison, WI, USA) as per the manufacturer’s instructions and amplified by PCR for the surrounding HSF response element (HSE) in the 5’-flanking region of the mouse Tjp1 gene. Primer sequences used for amplification were as follows: forward, 5’- AATGGTATGGCATAGGAGTG-3’; and reverse, 5’- TTACGCTTGACCAGAGGAAG-3’. TB green premix Ex Taq™ II (Takara Bio) with the StepOnePlus™ Real-Time PCR System (Life Technologies) was used to quantify the products. The cDNA equivalent of 10 ng of RNA was amplified by PCR using a StepOnePlus™ Real-Time PCR System (Life Technologies) with TB Green® Premix Ex Taq™ II (Takara Bio). The reaction was first incubated at 95°C for 30s, followed by 45 cycles at 95°C for 5s and 60°C for 30s. All data were normalized to the PCR products of input DNA. The quantitative reliability of PCR was evaluated by kinetic analysis of the amplified products to ensure that signals were derived exclusively from the exponential phase of amplification. ChIP was performed either in the absence of antibodies or in the presence rabbit IgG as a negative control.

Statistical analyses

All data are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using the GraphPad Prism software (ver. 8; GraphPad Software, San Diego, CA, USA). Differences among the groups were analyzed by two-way ANOVA, followed by Tukey’s (when group and time interacted) or Sidkey’s test (no interaction).

P < 0.05 was considered statistically significant. Details of the statistical analysis were described in the Supplementary Tables. Although no statistical methods were used to predetermine the sample size, the sample sizes used in the present study are similar to those reported in previous studies [8, 12, 20]. The experiments were not randomized.

Results

Attenuation of time dependency of tight junctions in the mouse diabetic liver

Eight-week-old ob/ob mice used in this study exhibited diabetes-like biological abnormalities with elevated plasma glucose and insulin concentrations (Supplementary Fig.1a-e). To examine possible alterations in the diurnal variation of tight junctions in the diabetic liver, vascular leakage was analyzed using Evans blue in wild-type and ob/ob mice. Fluorescent microscopic analysis revealed dark period-dependent permeation of Evans blue in the hepatic parenchymal cells of wild-type and time independent increase of Evans blue permeation in the liver of ob/ob mice (Fig.1a, b). These results indicate attenuated time dependency and tight junction functions in the diabetic liver, in vivo.

Biological clock system composed by clock genes affects transcriptions of many downstream genes [10, 14]. In this study, the expressions of Per2, Dbp, and Nr1d1 mRNAs in the liver of ob/ob mice were significantly decreased compared to those of wild-type mice, and the time-dependent change of mRNA expressions was disappeared in Bmal1 and Dbp mRNAs of ob/ob mice (Supplementary Fig.2a-e). These observations let us to speculate that disrupted biological clock system in ob/ob mice would result in abnormal expression of liver tight junction-related factors leading to loss of time dependent permeation of Evans blue.

Influence of diabetes on the mRNA expression of tight junction-related genes in the mouse liver

Tight junctions are adhesion sites on the cell membrane that are densely packed with multiple interacting proteins. Evans blue more permeates in the liver of ob/ob mice without the time dependency, however, a mechanism underlying this alteration is unclear. To identify the attenuated genes associated with loss of diurnal variation in diabetes, the mRNA expression levels of significant tight junction-related genes were evaluated. Accordingly, the expression levels of Tjp1, Tjp2, occludin (Ocln), claudin 1 (Cldn1), Cldn3, and Cldn5 were measured (Fig.2a-f). The expression of Tjp1 mRNA was trend to decreased in the light periods in ob/ob mice (P = 0.098). Since the time dependency of Tjp1 mRNA
Fig. 1 Tight junction dysfunction in the liver of ob/ob mice
(a) Representative image showing Evans blue fluorescence that evaluates the tight junction ability of liver tissue. Nuclei were also stained with 4′6-diamidino-2-phenylindole (blue). Scale bars indicate 50μm. Data were collected for more than three mice in each group. (b) Quantification of Evans blue infiltrated into liver tissue of wild-type and ob/ob mice. The data are expressed as µg of Evans-blue per liver weight (g). The data represent the mean ± S.E.M. of 5 mice. *, P < 0.05; significantly different between the two groups; #, P < 0.05, ##, P < 0.01; significantly different between wild-type and ob/ob mice at the corresponding time point (two-way ANOVA with Tukey’s post hoc test).

Fig. 2 Diurnal expression of hepatic tight junction related gene in ob/ob mice
Temporal expression profiles of Tjp1 (a), Tjp2 (b), Ocln (c), Cldn1 (d), Cldn3 (e), and Cldn5 (f) mRNA in the liver of wild-type and ob/ob mice. Values are shown as means with S.E.M. (n = 3). The mean value of the wild type mice at ZT6 was set at 1. *P < 0.05, significantly different between the two groups (two-way ANOVA with post hoc test; Tjp1 mRNA: Tukey’s test, Cldn1 mRNA: Sidak’s test).
Transcriptional regulation of \textit{Tjp1} by HSFs

Sequence analysis of the promoter region of \textit{Tjp1} genes revealed a highly conserved HSE located between $-468$ and $-448$ base pairs (bp) upstream from the transcription start site (relative to the transcription start site, $+1$) (Fig. 4a). Further, the repression of \textit{Tjp1} transcription by HSFs was explored. The activity of \textit{Tjp1} reporters was diminished by the elimination of their promoter sequences (Fig. 4b). Although the reporter assay revealed that cells cotransfected with \textit{Tjp1}:(-650/+50) reporter vector or \textit{Tjp1}:(-248/+50) reporter vector and HSF1/2 had high fluorescence, cells cotransfected with \textit{Tjp1}:(-50/+50) reporter vector and HSF1/2 had slightly increased fluorescence compared with cells cotransfected with \textit{Tjp1}:(-50/+50) reporter vector and pCMV-6. These results suggest that the HSFs element within $-468$ to $-448$bp was important for \textit{Tjp1} transcriptional regulation (Fig. 4b). The \textit{Tjp1}:(-248/+50) reporter also showed 5 times the transcriptional activity of the control due to the presence of single HSFs response element. Since the HSFs transcription mechanism requires multiple HSFs response

**Fig. 3** Influence of diabetes on the expression of tight junction related protein in the liver of mouse

(a) Upper: Representative images of western blot of ZO1, Ocln, Cldn1, and Cldn3 protein in the liver of wild-type and \textit{ob/ob} mice. Lower: CBB stain indicates the equal loading of proteins from the membrane fraction. (b-e) Temporal expression profiles of ZO1 (b), Ocln (c), Cldn1 (d), and Cldn3 (e) protein in the liver of wild-type and \textit{ob/ob} mice. Values are shown as means with S.E.M. (n=3). The mean value of the wild-type mice at ZT6 was set at 1. *, $P<0.05$; significantly different between the two groups; #, $P<0.05$, **, $P<0.01$; significantly different between wild-type and \textit{ob/ob} mice at the corresponding time point (two-way ANOVA with post hoc test; ZO1: Tukey’s test, Cldn1: Sidak’s test).

**Influence of diabetes mellitus on the expression of tight junction-related proteins in mouse liver**

\textit{Tjp1} mRNA expression in the light phase decreased in the liver of \textit{ob/ob} mice which leading to loss of time dependent expression (Fig.2a). We also measured the plasmalemmal expression levels of tight junction-associated proteins encoded by the mRNAs measured in Fig.2. As a result, ZO1 protein expression in the liver of wild-type mice showed a significant time dependent change (Fig.3a, b). No significant differences were observed in protein expression patterns of Ocln, Cldn1, and Cldn3 between wild-type and \textit{ob/ob} mice (Fig.3c-e). In this study, ZO2 and Cldn5 proteins were not detectable. These results indicate that decreased of ZO1 would be the most responsible protein for slacking tight junction function off in \textit{ob/ob} mice.

Expression was attenuated in \textit{ob/ob} mice, we speculated that ZO1/Tjp1 might play a central role in the time independency of tight junctions in diabetes.

Transcriptional regulation of \textit{Tjp1} by HSFs

Sequence analysis of the promoter region of \textit{Tjp1} genes revealed a highly conserved HSE located between $-468$ and $-448$ base pairs (bp) upstream from the transcription start site (relative to the transcription start site, $+1$) (Fig. 4a). Further, the repression of \textit{Tjp1} transcription by HSFs was explored. The activity of \textit{Tjp1} reporters was diminished by the elimination of their promoter sequences (Fig. 4b). Although the reporter assay revealed that cells cotransfected with \textit{Tjp1}:(-650/+50) reporter vector or \textit{Tjp1}:(-248/+50) reporter vector and HSF1/2 had high fluorescence, cells cotransfected with \textit{Tjp1}:(-50/+50) reporter vector and HSF1/2 had slightly increased fluorescence compared with cells cotransfected with \textit{Tjp1}:(-50/+50) reporter vector and pCMV-6. These results suggest that the HSFs element within $-468$ to $-448$bp was important for \textit{Tjp1} transcriptional regulation (Fig. 4b). The \textit{Tjp1}:(-248/+50) reporter also showed 5 times the transcriptional activity of the control due to the presence of single HSFs response element. Since the HSFs transcription mechanism requires multiple HSFs response
In contrast, the amount of HSF1/2 binding to the Tjp1 promoter in the liver of ob/ob mice was decreased in both light and dark phases (Fig. 4d). These results indicate that the decrease in HSF1/2 expression during diabetes mellitus eliminates the time-dependent expression fluctuation of ZO1 and disrupts the tight junction mechanism of the liver.

**Discussion**

Morphological wear or physiological effects, including angiopathy and modulation of insulin signals, are associated with the complications linked with tissue damage in diabetes.
In this study, we found that ZO1 protein expression showed time-dependency in the liver of wild-type mice. Furthermore, we found that the time-dependent ZO1 protein expression in the liver of diabetic mice was disappeared and then tight junction function was also disturbed. These results indicate a loss of function of the defense mechanism in healthy hepatocytes due to attenuated diurnal variability in diabetic liver.

Vascular permeability associated with angiopathy is evaluated using Evans-blue because of its high binding affinity to albumin and retention in the blood [23]. The perisinusoidal space or space of Disse in the liver is a region around the sinusoid where plasma collects, and hence with the highest retention of Evans blue compared to all other tissues [24]. The space of Disse is liver-specific and serves as a site that expresses several transporters and facilitates the uptake of nutrients, such as fatty and amino acids [25]. Since the intercellular space widened at night, it was presumed that the nocturnal mice would match the timing of ingestion of nutrients in the dark period. The expansion of the space of Disse causes damage to the hepatic morphology and leads to the development of liver damage [26]. Since vascular permeability was enhanced, both in the resting and active period in diabetic mice, it was considered as an indication of possible liver damage.

Tight junctions in the liver are essential for maintaining the physiological function and hence, abnormal functioning of the same is associated with liver diseases [27]. Few studies have defined tight junction-related factors that are altered in liver diseases. The expression of ZO1, a protein that forms tight junctions, is downregulated in liver diseases, such as NASH and hepatic cancer [28, 29]. The expression of ZO1 was decreased in ob/ob mice, suggesting the possibility of NASH-related liver damage in diabetes, as proven clinically.

ZO1 is a functional membrane protein expressed on liver parenchymal and endothelial cells. Proteins associated with cell adhesion in the liver are found on the apical surface of vascular endothelial cells and liver parenchymal cells [30]. ZO1 also expressed on the apical surface as a component of tight junctions [7, 8]. Not only in tight junction formation, ZO1 is also involved in cell differentiation and proliferation. In addition, ZO1 possesses scaffold protein function, and then stabilizes the activity of transporters [31, 32]. Therefore, we could not exclude the possibility that such functions of ZO1 might be disturbed in the liver of ob/ob mice, leading to enhancement of vascular permeability.

Reduction of HSF1 and HSF2 expression leads to liver injury. In HSF1 knockout mice, increased cytokine production and decreased clearance of reactive oxygen species, exacerbates the progression of hepatitis [33]. HSF2 positively regulates Psmb5 expression constituting 20S core proteasome complex; which suggests that Decreased proteasome activity is associated with liver fat accumulation and liver disease [34, 35]. Decreased expression of HSF suppresses transcription of heat shock protein genes, thus weakening the protective effect from environmental stress and oxidative stress caused by diabetes [35, 36]. Attenuated expression of HSFs increased the signals involved in promoting hepatitis in ob/ob mice. HSF1 drives the cascade of heat shock proteins which affects the expression of genes with downstream physiological functions. HSF1 binds to clock genes and controls the circadian clock mechanism in living organisms [37]. HSF1 functions as resetting the biological clock mechanism affected by changes in temperature and UV stimulation [38]. The amplitude of the clock gene expression reduces in ob/ob mice [10], but its association with HSF1 remains unclear. The expression of the clock gene may be related to the decrease in HSF1 expression (Fig.4c, d), which causes liver injury in ob/ob mice.

Complications associated with systemic organs have been reported in people with diabetes. Several studies have focused on the signaling mechanism associated with the suggested risk of development of hepatitis. However, morphological evaluation and differential expression profiles of the liver during the course of diabetes have not been elucidated. The expression amplitude of the clock gene in the subcutaneous adipose tissue is attenuated in diabetic patients compared with healthy [39]. HSF1 expression is reduced in diabetic human muscle specimens [40]. Since HSF1 regulates the expression of ZO1, it is considered that the decrease in HSF expression causes the decrease in tight junction function associated with the decrease in ZO1. The present study characterizes the morphological changes induced by diabetes in the liver and elucidates a novel mechanism involving cell-binding disorders in diabetes.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-022-07940-x.

Author contributions All authors (Y.T., N.M., M.H., and K.U.) participated in designing the study, interpreted the data and contributed to the writing and/or revising the manuscript. Y.T., N.M., and K.U. performed research, and Y.T. and K.U. analyzed data.

Funding This work was supported by KAKENHI Grant-in-Aid for Young Scientists (20K19715) to Y.T., KAKENHI Grant-in-Aid for Research Activity Start-up (19K23832) to Y.T., and KAKENHI Grant-in-Aid for Fundamental Research(C) (19K07327) to K.U.

Data availability The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors state that there are no conflicts of
interest to disclose.

Ethics approval Animals in this study were treated according to the guidelines stipulated by the Animal Care and Use Committee of the Sanyo-Onoda City University. All experiments were conducted under the protocol approved by the Internal Committee for Animal Experiments at Sanyo-Onoda City University (approved protocol ID #A-2020-13-A).

References

1. Tilg H, Moschen AR, Roden M (2017) NAFLD and diabetes mellitus. Nat Rev Gastroenterol Hepatol 14:32–42. DOI: https://doi.org/10.1038/nn.4265

2. Targher G, Carey KE, Byrne CD, Roden M (2021) The complex link between NAFLD and type 2 diabetes mellitus - mechanisms and treatments. Nat Rev Gastroenterol Hepatol 18:599–612. DOI: https://doi.org/10.1038/s41575-021-00448-y

3. Koh WP, Wang R, Yu MC, Yuan JM (2013) Diabetes mellitus and risk of hepatocellular carcinoma: findings from the Singapore Chinese Health Study. Br J Cancer 108:1182–1188. DOI: https://doi.org/10.1038/BJC.2013.25

4. Feldman EL, Callaghan BC, Pop-Busui R, Zochodne DW, Wright BA, Bennett DL, Bril V, Russell JW, Viswanathan V (2019) Diabetic neuropathy. Nat Rev Dis Primers 5:42. DOI: https://doi.org/10.1038/s41571-019-00977-x

5. Rask-Madsen C, King GL (2013) Vascular complications of diabetes: mechanisms of injury and protective factors. Cell Metab 17:20–33. DOI: https://doi.org/10.1016/j.cmet.2012.11.012

6. Santos GPS, Prazeres PHDM, Mintz A, Birbrair A (2017) Role of pericytes in the retina. Eye 32:483–486. DOI: https://doi.org/10.1038/eye.2017.220

7. Song HB, Jun HO, Kim JJ, Yu SY, Kim KW, Kim JH (2014) Suppression of protein kinase C-ζ attenuates vascular leakage via prevention of tight junction protein decrease in diabetic retinopathy. Biochem Biophys Res Commun 444:63–68. DOI: https://doi.org/10.1016/j.bbrc.2014.01.002

8. Xu J, Liang R, Zhang W, Tian K, Li J, Chen X, Yu T, Chen Q (2020) Faecalibacterium prausnitzii-derived microbial anti-inflammatory molecule regulates intestinal integrity in diabetes mellitus mice via modulating tight junction protein expression. J Diabetes 12:224–236. DOI: https://doi.org/10.1111/jdi.13075

9. Roehlen N, Roza Suarez AA, El Saghire H, Saviano A, Schuster C, Lupberger J, Baumert TF (2020) Tight junction proteins occludin and Claudin-1 are under the circadian control in the mouse large intestine: implications in intestinal permeability and susceptibility to colitis. PLoS ONE 9:e98016. DOI: https://doi.org/10.1371/journal.pone.0098016

10. Akamine T, Kusunose N, Matsunaga N, Koyanagi S, Ohdo S (2014) Clock-controlled output gene Dbp is a regulator of Arnt/HIF-1β diurnal expression of Glycoprotein 2 (Gp2) gene is controlled by a molecular clock in mouse Peyer’s patches. Genes Cells 25:270–278. DOI: https://doi.org/10.1111/gtc.12758

11. Santos RAS, Ferreira AJ, Simões e Silva AC (2008) Recent advances in the angiotensin-converting enzyme 2-angiotensin(1–7)-Mas axis. Exp Physiol 93:519–527. DOI: https://doi.org/10.1113/EXPPHYSIOL.2008.042002

12. Tian S, Haney RA, Feder ME (2010) Phylogeny disambiguates the evolution of heat-shock cis-regulatory elements in Drosophila. PLoS ONE 5:e10669. DOI: https://doi.org/10.1371/journal.pone.0010669

13. Tan GK, Ng JK, Trasti SL, Schul W, Yip G, Alonso S (2010) A pivotal role of liver sinusoidal endothelial cells in NAFLD/NASH progression. Lab Invest 2015:1130–1144. DOI: https://doi.org/10.1038/labinvest.2015.95
27. Zeisel MB, Dhawan P, Baumert TF (2019) Tight junction proteins in gastrointestinal and liver disease. Gut 68:547–561. https://doi.org/10.1136/GUTJNL-2018-316906
28. Zhang X, Wang L, Zhang H, Tu F, Qiang Y, Nie C (2019) Decreased expression of ZO1 is associated with tumor metastases in liver cancer. Oncol Lett 17:1859–1864. https://doi.org/10.3892/OL.2018.9765
29. Winkler S, Hempel M, Hsu MJ, Gericke M, Kühne H, Brückner S, Erler S, Burkhardt R, Christ B (2019) Immune-Deficient Ptp/Rag2/-/- mice featured higher adipose tissue mass and liver lipid accumulation with growing age than wildtype C57BL/6N mice. Cells 8:775. https://doi.org/10.3390/CELLS8080775
30. Nagaoka K, Udagawa T, Richter JD (2012) CPEB-mediated ZO-1 mRNA localization is required for epithelial tight-junction assembly and cell polarity. Nat Commun 3:675. doi: https://doi.org/10.1038/ncomms1678
31. Roehlen N, Roca Suarez AA, El Saghire H, Saviano A, Schuster C, Lupberger J, Baumert TF (2020) Tight Junction Proteins and the Biology of Hepatobiliary Disease. Int J Mol Sci 21:825. doi: https://doi.org/10.3390/ijms21030825
32. Jurkiewicz D, Michalek K, Skowronek K, Nałęcz KA (2017) Tight junction protein ZO-1 controls organic cation/carnitine transporter OCTN2 (SLC22A5) in a protein kinase C-dependent way. Biochim Biophys Acta Mol Cell Res 1864:797–805. doi: https://doi.org/10.1016/j.bbamcr.2017.02.014
33. Yue S, Zhu J, Zhang M, Li C, Zhou X, Zhou M, Ke M, Busuttil RW, Ying QL, Kupiec-Weglinski JW, Xia Q, Ke B (2016) The myeloid heat shock transcription factor 1/β-catenin axis regulates NLR family, pyrin domain-containing 3 inflammasome activation in mouse liver ischemia/reperfusion injury. Hepatology 64:1683–1698. https://doi.org/10.1002/hep.28739
34. Lecomte S, Desmots F, Masson FL, Goff PL, Michel D, Christians ES, Drean YL (2010) Roles of heat shock factor 1 and 2 in response to proteasome inhibition: consequence on p53 stability. Oncogene 29:4216–4224. doi: https://doi.org/10.1038/onc.2010.171
35. Das D, Paul A, Lahiri A, Adak M, Maity SK, Sarkar A, Paul S, Chakrabarti P (2021) Proteasome dysfunction under compromised redox metabolism dictates liver injury in NASH through ASK1/PPARγ binodal complementary modules. Redox Biol 45:102043. doi: https://doi.org/10.1016/j.redox.2021.102043
36. Emery SM, Dobrowsky RT (2016) Promoting Neuronal Tolerance of Diabetic Stress: Modulating Molecular Chaperones. Int Rev Neurobiol 127:181–210. DOI: https://doi.org/10.1016/bs.irn.2016.03.001
37. Tamaru T, Hattori M, Honda K, Benjamin I, Ozawa T, Takamatsu K (2011) Synchronization of circadian Per2 rhythms and HSF1-BMAL1:CLOCK interaction in mouse fibroblasts after short-term heat shock pulse. PLoS ONE 6:e24521. https://doi.org/10.1371/JOURNAL.PONE.0024521
38. Kawamura G, Hattori M, Takamatsu K, Tsukada T, Ninomiya Y, Benjamin I, Corsi-Sassone P, Ozawa T, Tamaru T (2018) Cooperative interaction among BMAL1, HSF1, and p53 protects mammalian cells from UV stress. Commun Biology 1:204. doi: https://doi.org/10.1038/s42003-018-0209-1
39. Stenvers DJ, Jongejan A, Atiqi S, Vreijling JP, Limonard EJ, Endert E, Baas F, Moerland PD, Fliers E, Kalsbeek A, Bisschop PH (2019) Diabetologia 62:704–716. DOI: https://doi.org/10.1007/s00125-019-4813-5
40. Rodrigues-Krause J, Krause M, O’Hagan C, De Vito G, Boreham C, Murphy C, Newsholme P, Colleran G (2012) Cell Stress Chaperones 17:293–302. DOI: https://doi.org/10.1007/s12192-011-0319-x

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.