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The Evaluation of Lipid Analysis for PXB-Cells LA as a Human Non-Alcoholic Fatty Liver Disease Model

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Fatty liver can progress into serious conditions, and the number of patients with fatty liver disease has risen globally in recent years. Various lipid metabolism disorders can cause fatty liver, and *in vitro* models, such as hepatoma cell lines, have been utilized in research related to lipid metabolism disorders, including the development of treatment strategies. We previously demonstrated that fresh hepatocytes (PXB-cells®) from chimeric mice with humanized livers display lipid metabolism similar to that of normal human hepatocytes. Additionally, we developed PXB-cells Lipid Analysis (PXB-cells LA) as a model of non-alcoholic fatty liver disease (NAFLD). PXB-cells LA exhibited increased levels of intracellular lipid droplets and lipids, especially triglycerides, compared to PXB-cells. Additionally, albumin secretion, drug metabolism, bile excretion transporters, mitochondria-derived oxidative phosphorylation, and intracellular adenosine triphosphate levels were attenuated in PXB-cells LA, while inflammatory marker levels were elevated. Collectively, these findings indicate hepatic dysfunction. Additionally, PXB-cells LA showed a fractional profile with a peak in very low-density lipoproteins, similar to PXB-cells. PXB-cells LA also secreted lipoproteins with a higher triglyceride content, associated with NAFLD. Taken together, these results suggest that PXB-cells LA is a useful cellular model of human NAFLD.

Key words NAFLD, human hepatocytes, humanized liver Mouse, lipid, *in vitro* model

INTRODUCTION

Metabolic syndrome (MS) is a leading cause of reduced life expectancy, and its prevalence has surged globally in recent years. Patients with MS face a higher incidence of non-alcoholic fatty liver disease (NAFLD), which is closely linked to diabetes,[1] lipid metabolism abnormalities,[2] and cardiovascular events.[3] Various *in vitro* models, cultured in media enriched with free fatty acids that induce lipid accumulation, have been employed for the study and treatment of NAFLD.[4] Although hepatoma cell lines are cost-effective and readily available, they present limitations, including discrepancies in drug metabolism and lipoprotein profiles compared to normal human hepatocytes.[5]

PXB-cells are fresh human hepatocytes derived from chimeric mice with humanized livers, known as PXB-mice®. As transplanted human hepatocytes proliferate over 500-fold in the chimeric mouse liver, the hepatocytes from same donor remain stably available for multiple years. Additionally, PXB-cells are available in a fresh (non-frozen) state; they retain major hepatic metabolic functions and the expression of human transporters in *in vitro* culture. Therefore, PXB-cells have been used in various studies, such as those on drug metabolism,[6] toxicity,[7] and hepatitis B virus infection.[8] We previously demonstrated that PXB-cells maintain the secretory function of very low-density lipoprotein (VLDL), which is not observed in hepatoma cell lines, such as HepG2 and HuH7, and have also demonstrated reduced triglyceride secretion following treatment with the antilipidemic agent fenofibrate.[9]

PXB-mice spontaneously accumulate lipid droplets (LDs) in hepatocytes as steatosis occurs after transplantation.[10] Since infusion of PXB-mice with human growth hormone (GH) decreases steatosis, this phenomenon is due to differences in GH amino acid sequences between mice and humans.[11] Similarly, PXB-cells contain large LDs just after perfusion and at the start of cell culture. As using fresh hepatocytes from the same fatty liver donor multiple times is not feasible, PXB-cells is a potential platform to provide the freshly isolated steatotic human hepatocytes with reproducibility. However, LDs in PXB-cells gradually disappear, and the characteristics of fatty liver cells cannot be maintained for longer than one week after seeding. In addition, PXB-cells can replicate hepat-

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ic functions, such as the formation of bile canaliculi and albumin production, within a week of seeding. Hence, we developed the PXB-cells Lipid Analysis (PXB-cells LA) method as a model for the long-term persistence of lipid accumulation after isolation.

In the present study, we compared the hepatic function and lipid metabolism of PXB-cells LA and PXB-cells to clarify the differences in the characteristics of these two cell types and to evaluate the utility of PXB-cells LA as a cellular model of NAFLD.

MATERIALS AND METHODS

Culture of Human Hepatocytes Human hepatocytes (PXB-cells) were isolated from humanized murine livers 14–16 weeks after transplantation of cryopreservation human hepatocytes (IVT-JFC, 1years old, Male, Caucasian, BioIVT) according to a previously described procedure.[12] PXB-cells were seeded at a density of 4×10^5 cells per well in collagen-coated 24-well microplates (denoted as day 0) and maintained in HCGM[13] until day 8. Meanwhile, PXB-cells LA were cultured in lipid-maintained medium (PhoenixBio, Higashi-hiroshima, Hiroshima, Japan, preparing for distribution) after seeding until day 6, and then maintained in HCGM for 2 days (Fig. 1).

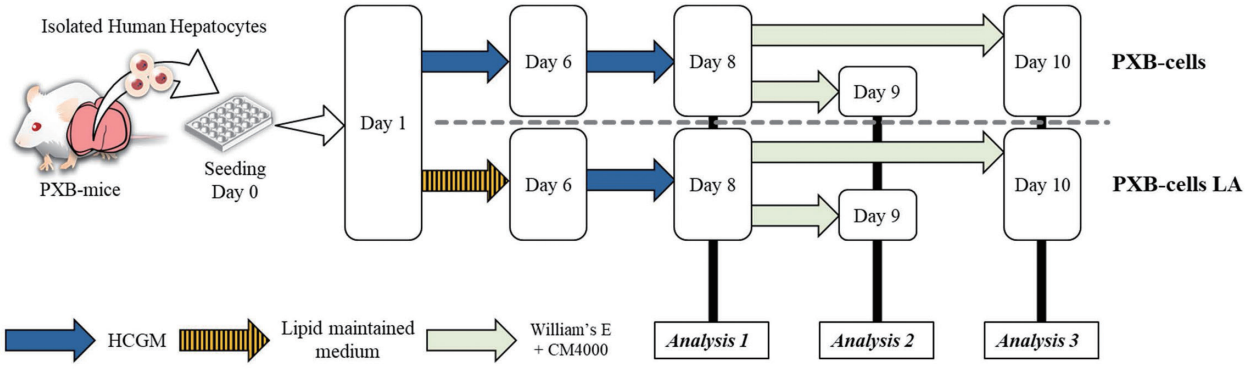
Oil Red O Staining PXB-cells and PXB-cells LA were fixed in 4% paraformaldehyde (Fujifilm Wako Pure Chemical Corporation, Osaka, Osaka, Japan) for 10 min. After rinsing with 60% isopropyl alcohol (Fujifilm Wako Pure Chemical Corporation), the cells were stained with Oil Red O (Fujifilm Wako Pure Chemical Corporation) for 10 min. After rinsing with 60% isopropyl alcohol, the medium was replaced with PBS(-) and observed.

Lipid Assay PXB-cells and PXB-cells LA were incubated in 500 µL of William's E medium supplemented with CM-4000 (Thermo Fisher Scientific, Waltham, MA, USA) for 2 days (days 8–10). The culture medium was assessed for lipoprotein content using using LipoSEARCH® (Immuno-Biological Laboratories, Fujioka, Gunma, Japan) according to the manufacturer's instructions.[14] Intracellular triglyceride and cholesterol levels on day 10 were determined using the Cholestest Cho and TG Kits (Sekisui Medical, Chuo, Tokyo, Japan), respectively, according to the manufacturer's instructions.

Real-Time Polymerase Chain Reaction (RT-PCR) In a fluorescent temperature cycler (CFX Connect™ RT-PCR Detection System; Bio-Rad Laboratories, Hercules, CA, USA), 2.5% of RT reaction solution was amplified in 25 µL of 1 × SYBR Premix Ex Taq (Takara Bio, Kusatsu, Shiga, Japan) containing 0.2 µM of each primer. The samples were incubated in a thermal cycler for initial denaturation at 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The oligonucleotide primers used in this study are listed in Table 1. To confirm the amplification of specific transcripts, melting curve profiles were produced at the end of each PCR by cooling the sample to 60°C and then heating slowly to 95°C with continuous fluorescence measurements. The relative expression of the target mRNAs was normalized to that of glyceraldehyde-3-phosphate dehydrogenase mRNA.

Mitochondrial Dysfunction Assay Lactate dehydrogenase (LDH) levels in the supernatant were measured as a biomarker of cytotoxicity using the Cytotoxicity LDH Assay Kit-WST (Dojindo Laboratories, Kamimashiki, Kumamoto, Japan). Cells lysed with 1% Triton X were used as positive controls, and cytotoxicity was evaluated based on the relative levels of extracellular LDH. Intracellular adenosine triphosphate (ATP) levels were measured by the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA). Mitochondrial dysfunction was assessed based on the amount of ATP. All measurements were performed according to the manufacturer's protocol.

Western Blotting Cell lysates were prepared using RIPA buffer (Nacalai Tesque, Nakagyou, Kyoto, Japan). Cell lysates (10 µg/lane) were separated on 4–15% gradient sodium dodecyl sulfate-polyacrylamide gels and transferred onto activated polyvinylidene fluoride membranes. After blocking with a blocking buffer (Nacalai Tesque), membranes were incubated with anti-fat-specific protein 27 (Novus Biologicals, Centennial, CO, USA), anti-Perilipin 2 (Abcam, Cambridge, Cambridge, UK), or anti-β-actin antibodies (IMGENEX, Bhubaneswar, Odisha, India). Membranes were



Analysis 1: Oil red O staining and Mitochondrial dysfunction assay
Analysis 2: Intracellular gene expression (RT-PCR)
Analysis 3: Intracellular; protein (western blot), and lipid
Extracellular; protein (Enzyme-linked immunosorbent assay), lipid, and lipoprotein profile

Fig. 1. Summary of PXB-Cells, PXB-Cells LA and Study Details

Table 1. Primer Sequence Used in This Study

Gene name	Forward	Reverse
ACC	5’-TGATGTCAATCTCCCCGCAGC-3’	5’-TTGCTTCTTCTCTGTTTTCTCCCC-3’
ACSL1	5’-AGCGGCGCCTTAAATAGCA-3’	5’-CATGGGCTTGCATTGTCCT-3’
Albumin	5’-ACTGCATTGCCGAAGTGGA-3’	5’-GCAGCACGACAGAGTAATCA GGA -3’
APOA1	5’-TGTGTACGTGGATGTGCTCAAAGA-3’	5’-TGTGTACGTGGATGTGCTCAAAGA-3’
APOA5	5’-ACGCACGCATCCAGCAGAAC-3’	5’-TCGGAGAGCATCTGGGGGTC-3’
APOB100	5’-TCGCCTGCCAAACTGCTTC-3’	5’-CATTGGTGCCTGTGTTCCATTC-3’
ApoC3	5’-TACATGAAGCACGCCACCAAG-3’	5’-AGCCACGGCTGAAGTTTGGTC-3’
CYP1A2	5’-AACAAGGGACACAACGCTGAAT-3’	5’-GGAAGAGAAACAAGGGCTGAGT-3’
CYP3A4	5’-CCAAGCTATGCTCTTCACCG-3’	5’-TCAGGCTCCACTTACGGTGC-3’
BSEP	5’-AGGGAGCTACCAGGATAGTTTAAGG-3’	5’-TCGTGCACCAGGTAAGAAAGC-3’
MRP2	5’-TGAGCAAGTTTGAAACGCACAT-3’	5’-AGCTCTTCTCCTGCCGTCTTCT-3’
DGAT1	5’-GGCCTTCTTCCACGAGTACC-3’	5’-GGCCTCATAGTTGAGCACG-3’
DGAT2	5’-AGTGGCAATGCTATCATCAT-3’	5’-GAGGCCTCGACCATGGAAGAT-3’
FAS	5’-CAGCAGTTCACGGACATGGAG-3’	5’-CGGCACGCAGCTTGTAGTAGA-3’
GAPDH	5’-GCACCGTCAAGGCTGAGAAC-3’	5’-TGGTGAAGACGCCAGTGGA-3’
MGAT1	5’-CAGGGCCGATGTCCATTGG-3’	5’-GGGTATGCCAGTCAAAGTAAAGC-3’
MGAT2	5’-CCTTCGGGGAGAATGACCTAT-3’	5’-GAGGGAGATGCCCATGATCTT-3’
MTTP	5’-TCTCTACTCGGGTTCTGGCATTCTA-3’	5’-GCTGCGATTAAGGCTTCCAGTC-3’
FSP27	5’-ATTGATGTGGCCCGTGTAACG-3’	5’-CAGCAGTGCAGATCATAGGAAA-3’
PLIN2	5’-TTGCAGTTGCCAATACCTATGC-3’	5’-CCAGTCACAGTAGTCGTCACA-3’
LRG1	5’-TGCTGGATCTAACCCGAAAC-3’	5’-AGAGCTTTCAGGCCGTGTAG-3’
NDUFA9	5’-GATTGTGGCCACTGTGTTTGG-3’	5’-CTCCAGCTTCCTTGGACAGT-3’
MT-ND1	5’-CTTAGCTCTCACCATCGCTCT-3’	5’-AGATTGTTTGGGCTACTGCTC-3’
SDHA	5’-GGCTTGCGAGCTGCATTTGG-3’	5’-GTTCTGCTAAACGGCATGCCA-3’
UQCRC1	5’-GTTAGCCTGCTGGACAACG-3’	5’-CTTGATGTAGTAAGCTGTGTGC-3’
MT-CYB	5’-GATCCTCCAAATCACCACAGGAC-3’	5’-GGAGGATAATGCCGATGTTTCAG-3’
ATP5FB	5’-GAGACCAAGAAGGTCAAGATG-3’	5’-GAAGGGATTCGGCCCAATAATGCAG-3’
MT-ATP6	5’-TAGCCATACACAACACTAAAGGACGA-3’	5’-GGGCATTTTAATCTTAGAGCGAAA-3’

ACC: acetyl-CoA carboxylase, ACSL: long-chain acyl-CoA synthetases, APO: apolipoprotein, CYP: cytochrome P450, BSEP: bile salt export pump, MRP: multidrug resistance-associated protein, DGAT: diacylglycerol acyltransferase, FAS: fatty acid synthase, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, MGAT; monoacylglycerol acyltransferase, MTTP: microsomal triglyceride transfer protein, FSP: fat-specific protein, PLIN: perilipin, LRG: leucine-rich alpha-2-glycoprotein, NDUF: NADH ubiquinone oxidoreductase subunit, MT-ND; mitochondrially encoded NADH ubiquinone oxidoreductase core subunit, SDHA: succinate dehydrogenase A, UQCR: ubiquinol cytochrome c reductase, MT-CYB: mitochondrial cytochrome b, ATP5FB; ATP synthase F1 subunit beta, MT-ATP6: mitochondrially encoded ATP synthase membrane subunit 6

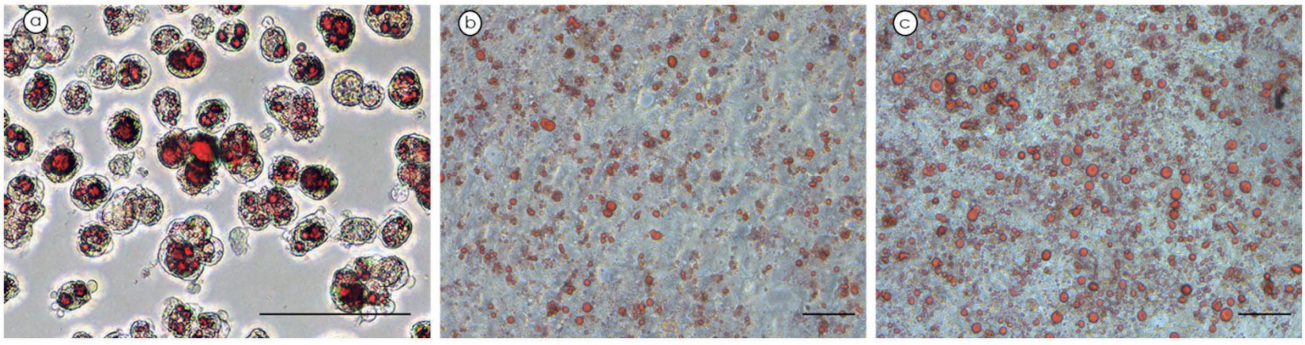


Fig. 2. Lipid Droplets (LDs) Formation on Day 0 and 8

a: PXB-cells (Day 0), b: PXB-cells (Day 8), c: PXB-cells LA (Day 8), scale bar: 200 μm

then incubated with horseradish peroxidase-conjugated anti-mouse IgG (Promega) or anti-rabbit IgG (Promega). Immunoreactive bands were developed using the ECL Prime Western Blotting Reagent (GE Healthcare Bio-Sciences, Piscataway, NJ, USA), detected using a Light-Capture II imaging system (AE-6982/C/FC; ATTO, Taito, Tokyo, Japan), and analyzed with a CS Analyzer (ver. 3.0 software; ATTO). Furthermore, β-actin was used as a normalization control.

Enzyme-Linked Immunosorbent Assay Levels of albumin (ALB), leucine rich alpha 2 glycoprotein 1 (LRG), apolipoprotein AI, apolipoprotein B-100 (ApoB-100), apolipoprotein CIII, and apolipoprotein A5 (ApoA5) in the cultured medium were measured using the LZ-test “Eiken” U-ALB (Eiken Chemical, Taito, Tokyo, Japan), human LRG assay kit (Immuno-Biological Laboratories), human apolipoprotein AI ELISA kit (Abcam), human ApoB-100 assay kit (Immuno-Biological Laboratories), human apolipoprotein CIII ELISA kit (Abcam), and human ApoA5 assay kit (27191; Immuno-Biological Laboratories) respectively, according to each manufacturer’s procedure manual.

Statistical Analysis Data are presented as the mean ± standard deviation (SD). The significance of differences between groups was analyzed using the Kruskal–Wallis test, followed by the Steel–Dwass multiple comparison test, Mann–Whitney U-test, and Student's *t*-test. Statistical significance was set at $P < 0.05$. Statistical analyses were performed using Bell Curve for Excel (Social Survey Research Information).

RESULTS AND DISCUSSION

Evaluation of Lipid Accumulation in PXB-Cells LA As demonstrated by Oil Red O staining, human hepatocytes contain LDs at immediately after isolation, indicating that PXB mice were under the steatosis condition (Fig. 2a). After culturing 8 days, PXB-cells LA displayed numerous large intracellular LDs (Figs. 2b and 2c). Regarding intracellular lipid levels, PXB-cells LA had 1.2-fold higher cholesterol and 1.5-fold higher triglyceride levels than PXB-cells (Fig. 3). Additionally, the expression of fat-specific protein 27 and perilipin 2, regulatory proteins involved in LD formation and size expansion, was observed (Figs. 4a and 4b). These results collectively indicate that PXB-cells LA exhibit lipid accumulation, a hallmark of steatosis.

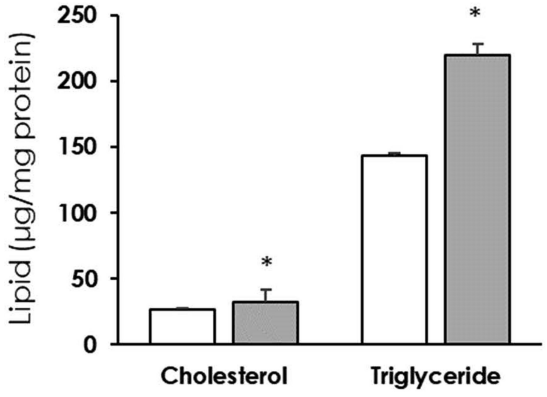


Fig. 3. Intracellular Lipid of the PXB-Cells and the PXB-Cells LA

White: PXB-cells, Gray: PXB-cells LA, * $P < 0.05$ vs PXB-cells

Evaluation of Hepatic Dysfunction in PXB-Cells LA PXB-cells LA exhibited decreased expression of genes related to liver function, such as albumin synthesis (albumin), drug-metabolizing enzymes (CYP1A2 and CYP3A4), and bile excretion transporters (BSEP and MRP2) (Fig. 5).[15,16,17] Decreased albumin synthesis and drug metabolism are associated with increased lipid accumulation in hepatocytes and the progression of NAFLD.[18,19] Bile acid excretion transporters are negatively correlated with fat accumulation in the liver tissues of patients with NAFLD, particularly in areas of high-fat accumulation.[20] Therefore, increased lipid accumulation in PXB-cells LA may have led to the inhibition of these hepatic functions.

In addition to structural and molecular changes, mitochondria in patients with NAFLD exhibit dysfunction due to abnormal lipid metabolism.[21,22] We hypothesize that lipid accumulation in PXB-cells LA leads to abnormal lipid metabolism and affects mitochondrial oxidative phosphorylation (OXPHOS) activity. Our evaluation of OXPHOS in PXB-cells LA revealed that the expression of complexes I, III, and V in the electron transfer system was significantly downregulated ($P < 0.05$). Notably, these results were specific to complexes derived from the mitochondrial genome (Fig. 6a). The mitochondrial toxicity assessment results also indicated that intracellular ATP levels were significantly reduced in PXB-cells LA, and LDH in the supernatant was lower than that in positive subjects, suggesting mitochondrial function suppression rather than cytotoxicity (Figs. 6b and 6c). This is supported by the observed alterations in OXPHOS gene expression.

LRG1, an adipokine primarily produced in the liver, mediates obesity-induced hepatic steatosis and insulin resistance.[23] The expression of LRG1 was higher in PXB-cells LA than in PXB-cells at both the gene and protein levels (Fig. 7).

Collectively, these findings suggest that PXB-cells LA serve as a cellular model of NAFLD, as they displayed a significant increase in lipid accumulation, along with a clear impairment of primary liver function and mitochondrial activity, and increased expression of inflammatory markers compared to PXB-cells, which represent normal human hepatocytes.

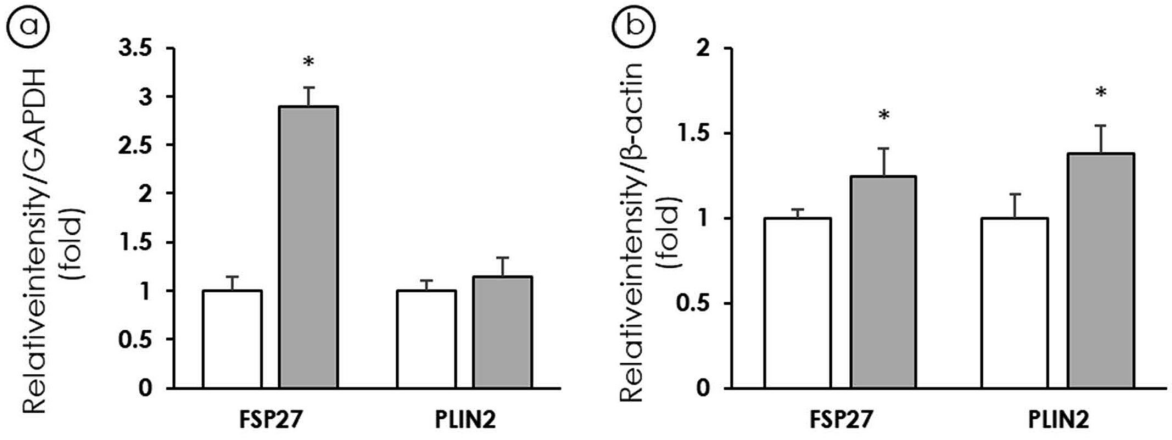


Fig. 4. Gene Expression and Intracellular Protein Related LDs Formation

a. LDs formation related gene expression, b: LDs formation related intracellular protein, White: PXB-cells, Gray: PXB-cells LA, FSP27: fat-specific protein 27, PLIN2: perilipin 2, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, * $P < 0.05$ vs PXB-cells

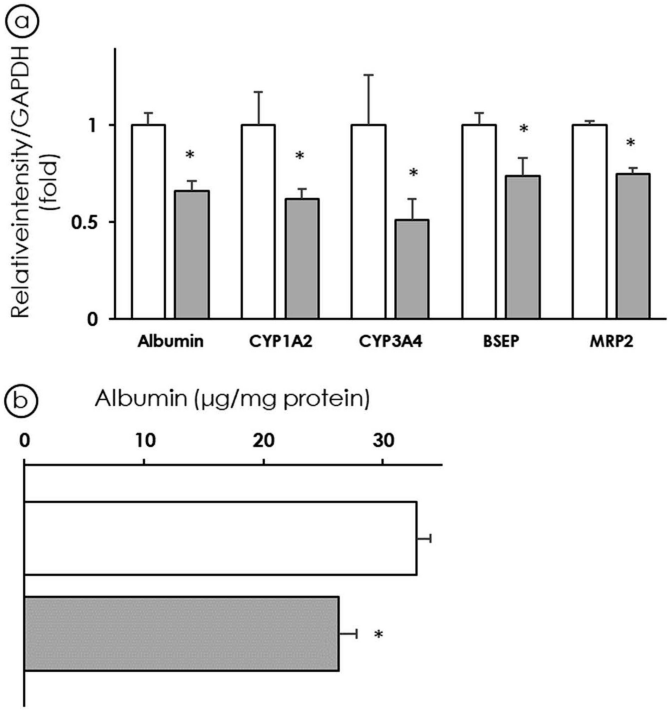


Fig. 5. Gene Expression and Human Albumin Secretion of PXB-Cells and PXB-Cells LA

a. Hepatic marker genes expression, b: albumin concentration in culture supernatant. White: PXB-cells, Gray: PXB-cells LA, CYP: cytochrome P450, BSEP: bile salt export pump, MRP2: multidrug resistance-associated protein 2, * $P < 0.05$ vs PXB-cells

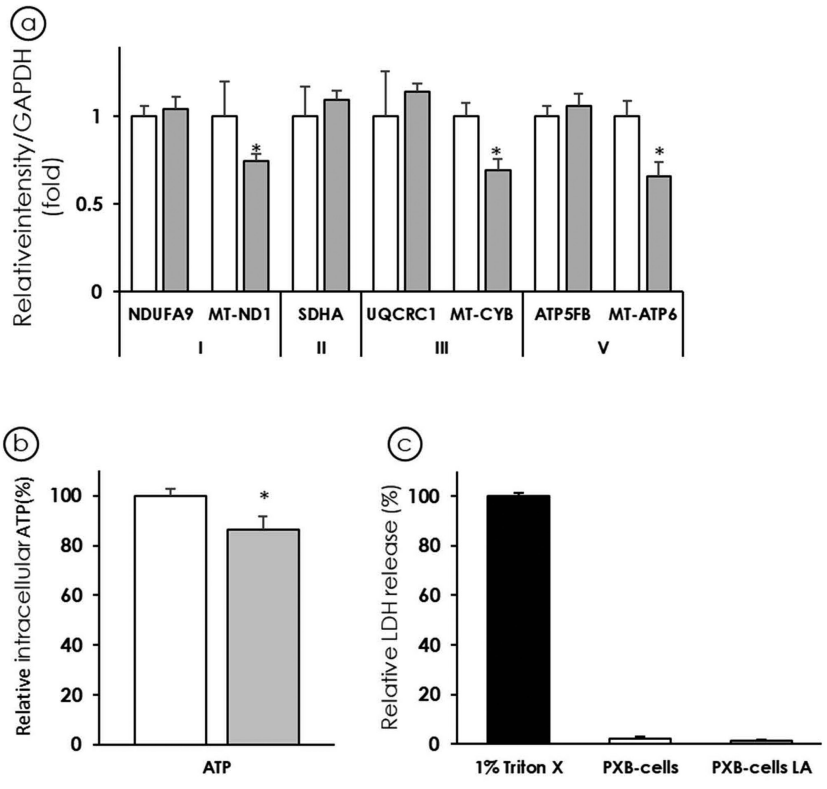


Fig. 6. Expression of OXPHOS Gene and Mitochondrial Toxicity Evaluation

a. OXPHOS related genes expression, PXB-cells (White), PXB-cells LA (Gray), b: Relative intracellular adenosine triphosphate (ATP: PXB-cells as 100), PXB-cells (White), PXB-cells LA (Gray), c: Relative lactate dehydrogenase (LDH) release (1% Triton X as 100), NDUFA9: NADH ubiquinone oxidoreductase subunit A9, MT-ND1: mitochondrially encoded NADH ubiquinone oxidoreductase core subunit 1, SDHA: succinate dehydrogenase A, UQCRC1: ubiquinol cytochrome c reductase core protein 1, MT-CYB: mitochondrial cytochrome b, ATP5FB: ATP synthase F1 subunit beta, MT-ATP6: mitochondrially encoded ATP synthase membrane subunit 6, * $P < 0.05$ vs PXB-cells

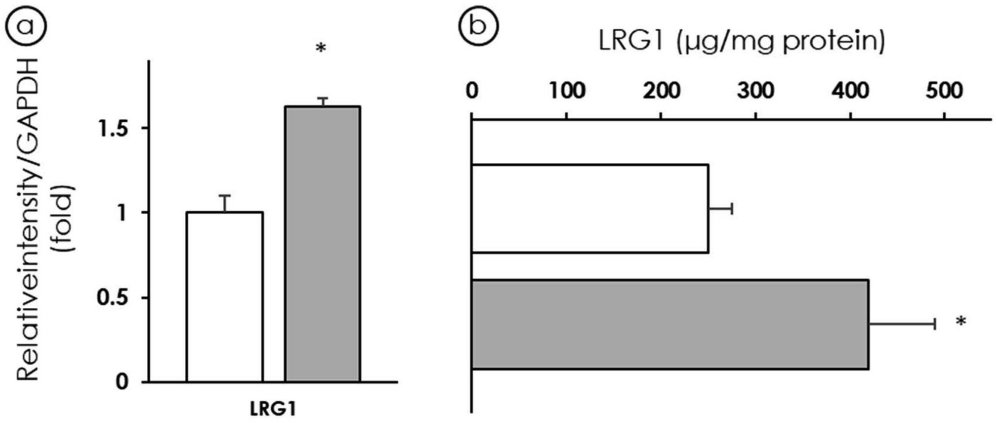


Fig. 7. Genetic and Protein Analysis of Inflammation Marker

a. Gene expression, b: Protein concentration in supernatant, White: PXB-cells, Gray: PXB-cells LA, LRG1: leucine-rich alpha-2-glycoprotein 1, * $P < 0.05$ vs PXB-cells

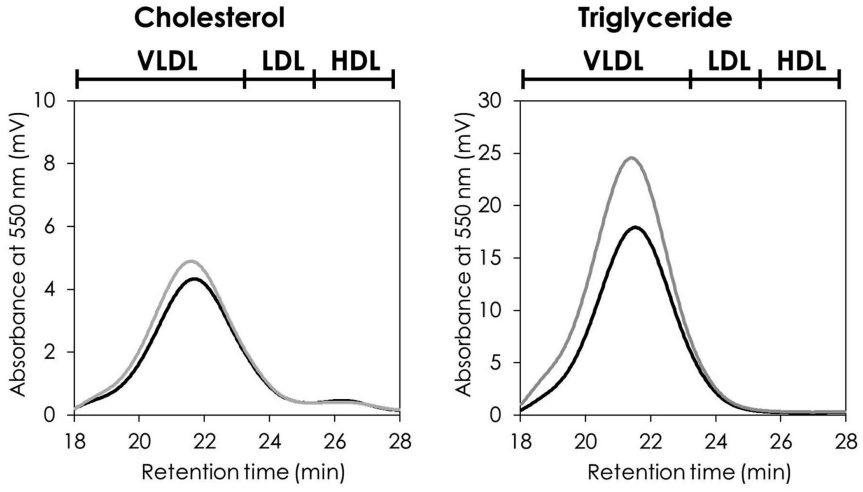


Fig. 8. Lipoprotein Profile in Supernatant

Black line: PXB-cells, Gray line: PXB-cells LA

Table 2. The Quantity of Cholesterol and Triglyceride in Each Lipoprotein Fraction of Supernatant

	Cholesterol (μg/mL protein)		Triglycerides (μg/mL protein)	
	PXB-cells	PXB-cells LA	PXB-cells	PXB-cells LA
Total	2.9 ± 0.1	3.7 ± 0.2*	28.1 ± 1.4	43.8 ± 2.0*
VLDL				
all	2.4 ± 0.1	3.1 ± 0.2*	26.2 ± 1.3	41.1 ± 1.9*
large	1.9 ± 0.1	2.6 ± 0.1*	22.1 ± 1.1	35.9 ± 1.6*
medium	0.4	0.4	3.4 ± 0.2	4.4 ± 0.3*
small	0.1	0.1	0.7	1.0 *
LDL				
all	0.3	0.3	1.5 ± 0.1	2.1 ± 0.1*
large	0.1	0.1	0.7	0.9 ± 0.1
medium	0.1	0.1	0.4	0.5
small	0.1	0.1	0.2	0.3
very small	0.1	0.1	0.2	0.3
HDL				
all	0.2	0.2	0.4	0.6
very large	0.1	0.1	0.1	0.1
Large	0	0.1	0.1	0.1
medium	0	0	0	0.1 *
small	0	0	0.1	0.1
very small	0	0.1	0.2	0.2

VLDL: Very low-density lipoprotein, LDL: Low-density lipoprotein, HDL: high-density lipoprotein, * $P < 0.05$ vs PXB-cells

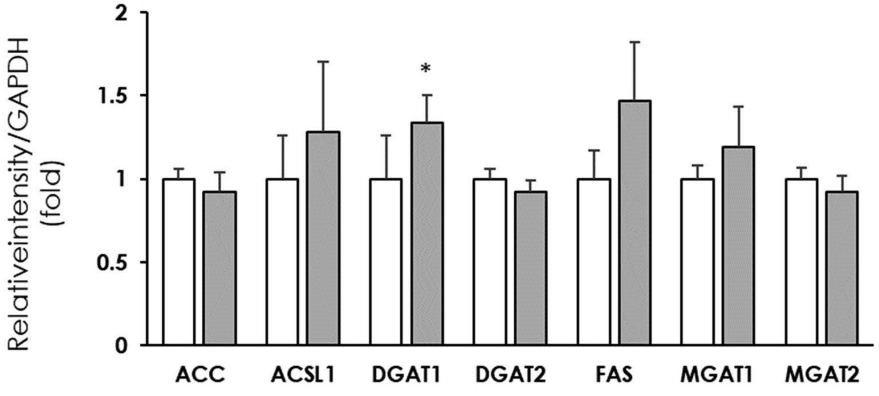


Fig. 9. Expression of Genes Involved in Triglyceride Synthesis

ACC: acetyl-CoA carboxylase, ACSL1: long-chain acyl-CoA synthetases, DGAT: diacylglycerol acyltransferase, FAS: fatty acid synthase, MGAT: monoacylglycerol acyltransferase, White: PXB-cells, Gray: PXB-cells LA, * $P < 0.05$ vs PXB-cells

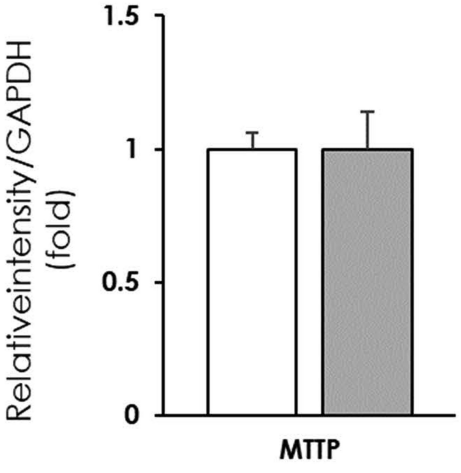


Fig. 10. Expression of Gene Involved in Lipoprotein Synthesis

MTTP: microsomal triglyceride transfer protein, White: PXB-cells, Gray: PXB-cells LA

Evaluation of Lipid Metabolism in PXB-Cells LA Lipid secretion was significantly higher in PXB-cells LA than in PXB-cells, with 1.3-fold higher levels of total cholesterol, 1.6-fold higher levels of total triglycerides, and increased secretion of VLDL ($P < 0.05$) (Table 2). The lipoprotein fraction exhibited a VLDL peak similar to that observed in PXB-cells and frozen human hepatocytes (Fig. 8).[5,9] Therefore, PXB-cells LA were found to secrete higher levels of lipids than the original PXB-cells while still maintaining the synthesis and secretory functions of human lipoproteins. Additionally, the lipoproteins secreted by PXB-cells LA were particularly high in triglycerides, which may mimic the increased triglyceride-rich lipoprotein content seen in the serum of patients with NAFLD.[24]

Evaluation of Triglyceride Synthesis in PXB-Cells LA Although the expression of diacylglycerol acyltransferase (DGAT1) was significantly increased in PXB-cells LA, no significant change was observed in the expression of other triglyceride synthesis-related genes in PXB-cells LA and PXB-cells ($P < 0.05$) (Fig. 9). Traditional models of fatty liver form fatty droplets through the induction of triglyceride synthesis through the uptake of fatty acids, such as oleic acid.[25,26] In contrast, PXB-cells LA exhibited high lipid droplet morphogenesis without increased lipid synthesis (Figs. 4a and 4b), suggesting that the characteristic of NAFLD-like changes in PXB-cells LA is the accumulation of LDs without concurrent lipid synthesis.

Evaluation of Apolipoprotein Levels in PXB-Cells LA VLDL synthesis involves ApoB100, which forms primordial particles (pre-VLDL), a process assisted by microsomal triglyceride transfer proteins (MTTP) in the endoplasmic reticulum. As the translation of ApoB100 continues, pre-VLDL particles expand by incorporating more lipids. The Golgi apparatus and ApoC3 are involved in synthesizing triglyceride-rich mature VLDL particles, which are then secreted into the extracellular space.[27] The gene expression of *MTTP* and *ApoB100* (Figs. 10 and 11a) and the levels of ApoB100 in the cell culture supernatant (Fig. 11b) were comparable between PXB-cells LA and PXB-cells. However, increased expression of *MTTP* [28] and *ApoB100* [29] has been observed in traditional fatty liver cell models using fatty acid-supplemented cultures. Additionally, a positive correlation has been reported between serum ApoB100 levels and body mass index in patients with NAFLD.[30] As the MTTP–ApoB interaction is coordinated by triglyceride synthesis,[31] we hypothesized that the reason for this discrepancy was that the lipid synthesis function of PXB-cells LA was similar to that of PXB-cells.

In contrast, PXB-cells LA secreted higher amounts (1.7-fold) of ApoC3, which is involved in mature VLDL synthesis, than PXB-cells (Fig. 11b). Overexpression of the *ApoC3* gene has been shown to increase serum VLDL levels in mice,[32] and the observed increase in VLDL secretion in PXB-cells LA may be attributed to increased ApoC3 secretion. Furthermore, increased serum ApoC3 levels are associated with NAFLD pathogenesis.[33] Given that ApoC3 is abundant on the surface of triglyceride-rich lipoproteins, which are causally related to the development of cardiovascular diseases,[34] the development of therapeutic agents targeting ApoC3 is underway.[35]

The prevalence of NAFLD has been reported to decrease with increasing levels of ApoA1, a major constituent of high-density lipoproteins.[36] In PXB-cells LA, the secretion of

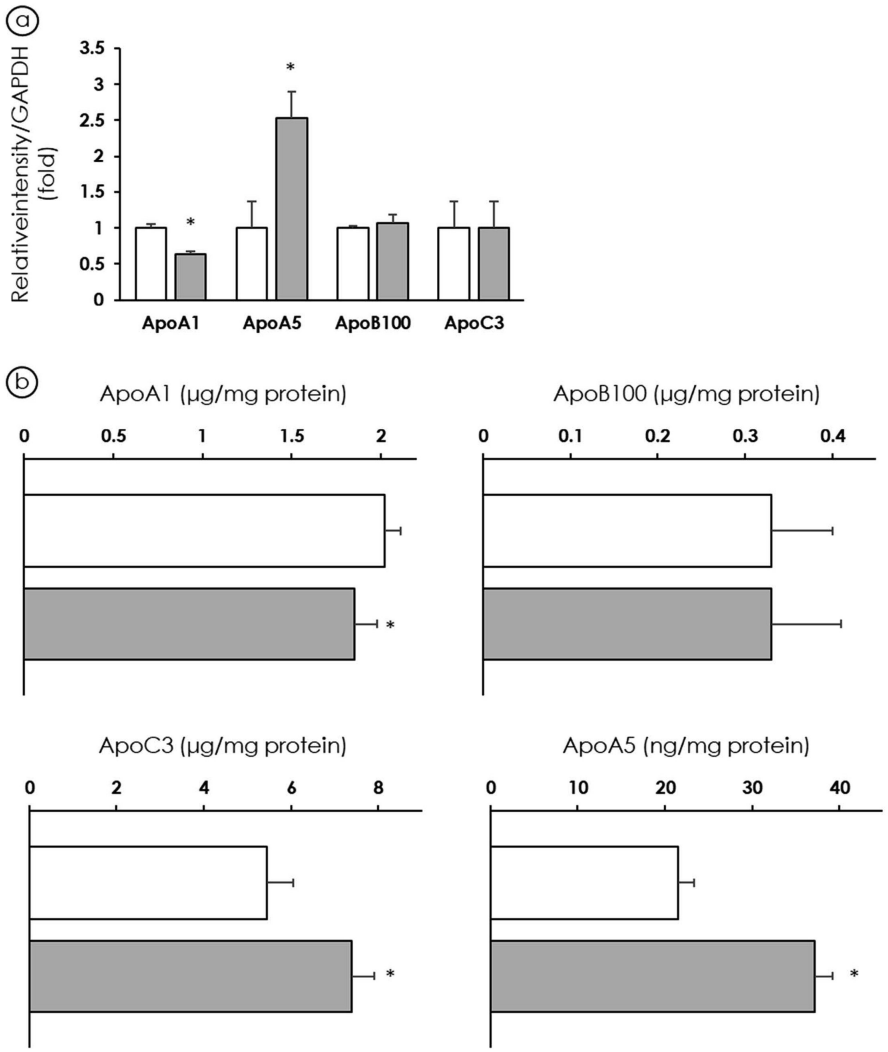


Fig. 11. Genetic and Protein Analysis of Apolipoproteins

a. Gene expression, b: Protein concentration in supernatant, Apo: apolipoprotein, White: PXB-cells, Gray: PXB-cells LA, * $P < 0.05$ vs PXB-cells

ApoA1 was significantly decreased compared to that in PXB-cells ($P < 0.05$) (Fig. 11b). The ApoB/ApoA1 ratio was 1.09-fold higher in PXB-cells LA than in PXB-cells, aligning with the observed decrease in ApoA1 secretion. This increased ratio is associated with a higher risk of coronary heart disease.[37] In summary, PXB-cells LA exhibited altered secretion of apolipoproteins, associated with the risk of atherosclerosis, including cardiovascular diseases, suggesting its utility as a model cell for these conditions.

In this study, we compared the lipid metabolism-related functions of PXB-cells LA, as a fatty liver model, with those of the original cells, PXB-cells, and confirmed that PXB-cells LA formed more LDs, a hallmark of fatty liver, and showed high accumulation of triglycerides and other lipids in the cells. Additionally, PXB-cells LA demonstrated hepatic dysfunction, as indicated by decreased albumin secretion, drug metabolism, bile acid transporters, mitochondria-derived OXPHOS, and elevated levels of inflammatory markers. Collectively, these findings suggest that PXB-cells LA is a cellular model for NAFLD pathology. In many cases, extracellular lipoproteins and apolipoproteins are used as indicators in drug discovery research for dyslipidemia. Herein, PXB-cells LA had a higher secretory capacity than PXB-cells for all measured parameters, suggesting that they function effectively as cellular models of fatty liver. In addition, since PXB-cells LA slowly recovered from steatosis conditions within two weeks of switching to non-lipid-maintained medium (HCGM) in internal experience, these cells are reversible from the lipid-accumulating state.

When using classic frozen *in vitro* human hepatocyte resources, there are some variations in cell function due to the donor's health condition, postmortem time for preparation, and the freeze-thaw process. PXB-cells do not have these disadvantages as they are prepared on demand from healthy PXB-mice. Additionally, PXB-cells provide an advantage in terms of reproducibility as donor-derived PXB-cells can be continuously supplied as long as donor cells are available at PhoenixBio. In addition, commercially available frozen hepatocytes cannot be cultured in a confluent state for long periods of time with high functionality, whereas in many cases, PXB-cells can be cultured for long periods of time (three weeks or more) with high functionality. PXB-cells LA have the same advantages as PXB-cells described above, and also provides a spontaneous steatotic hepatocyte profile without any addition-

al treatments using fatty acids. PXB-cells LA are a valuable *in vitro* material for studying hepatocyte-based human lipid metabolism.

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Conflict of Interest Masaki Takahashi, Mutsumi Inamatsu, and Nami Yoshikawa are employees of PhoenixBio Co., Ltd. Masakazu Kakuni is employer of KMT Hepatech Inc. which is part of PhoenixBio group.

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