様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

令和　５ 年　 ５ 月 ２１ 日現在

機関番号：１２６０１
研究種目：若手研究
研究期間：2021～2022
課題番号：２１Ｋ１６３７７

研究課題名（和文）Generation of mini liver tissue with complex cellular and architectural properties for disease modeling and drug screening

研究課題名（英文）Generation of mini liver tissue with complex cellular and architectural properties for disease modeling and drug screening

研究代表者

聶　運中（NIE, YUNZHONG）

東京大学・医科学研究所・助教

研究者番号：００８３１３３０

交付決定額（研究期間全体）：（直接経費）　　3,600,000円

研究成果の概要（和文）：このプロジェクトではhiPSC由来の肝臓前駆細胞群を用いてミニ肝組織を構築した。培養中に、肝臓前駆細胞は組織様の構造に構成し、肝機能遺伝子の発現およびアルブミンの分泌が増加した。さらに、細胞追跡と遺伝子解析により、ミニ肝組織内に類洞内皮ネットワークに発展し、HSCが静止特性を維持し、単球がクッパー細胞に分化した。また、ミニ肝組織には典型的な肝臓特異的な超微細構造が確認された。遊離脂肪酸で処理すると、ミニ肝組織に脂質蓄積が顕著に検出され、免疫応答が増強し、HSCの活性化および肝障害が観察された。以上より、hiPSCミニ肝組織がヒト非アルコール性脂肪性肝炎の発症と治療の研究に役立つことを示唆した。

研究成果の学術的意義や社会的意義
本研究開発しているヒトミニ肝組織にはヒトの肝臓に非常に近い細胞構成や組織構造を持つことが確認された。In vitroでヒト機能肝臓再構築基礎研究において画期的な進歩である。近年にNASHの発症率は急速に増加し、現代社会において深刻な健康問題となっている。本ヒトミニ肝組織の活用により、NASHの病態生理や病態進行をIn vitroで再現され、NASHの疾患メカニズムや新たな治療法の開発に向けた重要な一歩が踏み出された。これにより、将来的には効果的な予防策や治療法の開発が可能となり、NASH患者の生活の質の向上や医療負担の軽減につながることが期待される。

研究成果の概要（英文）：This project generated mini liver tissue with hiPSCs derived Hepatoblast, fetal hepatic stellate cells (HSC), endothelial progenitors, and monocytes. Within 10 days of culture, these cells organized and matured into tissue-like structures with an increased expression of hepatic function genes and secretion of Albumin. Moreover, lineage tracing and genetic analysis showed that endothelial progenitors gradually developed into sinusoidal endothelial networks, HSC maintained their quiescent characteristics, and monocytes were differentiated into Kupffer cells. The typical liver-specific ultrastructure was also observed in the mini-liver tissues. When treating these liver tissues with free fatty acids, we detected a marked lipid accumulation, resulting in an enhanced immune response, activation of HSC, and hepatic injury. These results suggested that we generated a hiPSC-derived mini liver tissue for studying development and treatment of human nonalcoholic steatohepatitis.

研究分野：幹細胞、再生医学

キーワード：hiPSC　Mini liver tissue　tissue structure　NASH　Kupffer cell　Hepatic stellate cells　LSEC

１．研究開始当初の背景

Liver disease accounts for an actual global burden on health sciences and economies (Asrani et al., *J Hepatol*. 2019; GBD 2017 Cirrhosis Collaborators, *Lancet Gastroenterol Hepatol*. 2020). The developed vaccination and newer drugs help reduce viral related liver diseases, but the global prevalence of viral hepatitis remains high: about 325 million people are still living with hepatitis infection (*www.who.int/health-topics/hepatitis*). Moreover, more than 75 million people are diagnosed with alcohol-use disorders and are at risk of alcohol-associated liver disease (Asrani et al., *J Hepatol*. 2019). Obesity and diabetes are the risk factors for non-alcoholic fatty liver disease and hepatocellular carcinoma, and approximately 2 billion adults are obese or overweight, and over 400 million have diabetes (Younossi, *J Hepatol*. 2019). Notably, viral hepatitis, alcohol-associated liver disease, and non-alcoholic fatty liver disease are the most common causes of liver cirrhosis and liver cancer; both are the most severe liver diseases and cause approximately 2 million people to die per year worldwide. Therefore, to reduce the burden caused by liver cirrhosis and liver cancer, there is an urgent need to develop feasible therapeutic strategies to prevent the progression of viral hepatitis, alcohol-associated liver disease, and non-alcoholic fatty liver disease.

Kinds of human cell-based models have been established for liver disease modeling and drug screening. Hepatoma-derived cell lines are most widely used (Chavez-Tapia et al., *Curr Med Chem*. 2011; Yan et al., *Elife*. 2012). However, these cells are from cancer patients with abnormal genotypes and poorly exhibit hepatic-specific functions. Primary human hepatocytes (PHHs) are the gold standard for *in vitro* modeling liver disease. However, the donor shortage, quality variability, and the loss of xenobiotic biotransformation enzymatic activity in a culture mostly limited their usefulness (Alwahsh et al., *Cell Mol Life Sci*. 2017). Additionally, non-parenchymal cells, such as liver sinusoidal endothelial cells (LSECs), HSCs, and KCs, also play critical roles in the pathological process of liver disease (Bataller et al., *J Clin Invest*. 2005; Connolly et al., *J Immunol*. 2010; Pradere et al., *Hepatology*. 2013). Co-culturing primary non-parenchymal cells with PHHs would be a more natural method to model liver disease progression. However, the usefulness of these non-parenchymal cells is also limited by the donor shortage and quality variability, and co-culturing different donors derived PHHs and non-parenchymal cells may lead to instability of experimental results. So far, there is no human cell-based model that can contain the same background derived hepatocytes, LSECs, HSCs, and KCs.

In the recent decade, significant efforts have been made in the hiPSCs differentiation, and various terminal cell types, including hepatocytes, LSECs, HSCs, and KCs, have successfully been differentiated from hiPSCs (Kajiwara et al., *PNAS*. 2012; Coll et al., *Cell Stem Cell*. 2018; Tasnim et al., *Biomaterials*. 2019; Gage et al., *Cell Stem Cell*. 2020). Studies have found that these hiPSC derived liver-specific cells could also be used for *in vitro* modeling liver diseases (Coll et al., *Cell Stem Cell*. 2018; Tasnim et al., *Biomaterials*. 2019). Nevertheless, how to organize these hiPSCs derived hepatocyte and non-parenchymal cells into a liver model with tissue architectural properties are still unknown. Instead of organizing these hiPSCs derived

liver-specific cells, a multi-cellular human liver organoid composed of hepatocyte-, stellate-, and Kupffer-like cells has been generated to model steatohepatitis under free fatty acid treatment (Ouchi et al., *Cell metabolism.* 2019). However, this multi-cellular liver organoid shows a cyst-like structure that is much different from the liver's regular hepatocyte arrangement. Additionally, the lower population of KCs and the lack of LSECs also make it difficult to simulate disease progression accurately.

２．研究の目的

In this project, we aim to generate a mini liver tissue containing hepatocyte and non-parenchymal cells (LSECs, HSCs, and KCs) by using our advanced organoid technology (Takebe et al. *Nature*. 2013; Takebe et al., *Cell Rep*. 2017; Nie et al., *EBioMedicine*, 2018). Unlike the mixing of multiple cells into a 100 ~ 300μm spheroid, we will generate the mini liver tissue at a centimeter level by step-wisely mimicking the process of liver organogenesis. In this 3D liver tissue, we aim to recapitulate the sinusoid structure among hepatocytes, maintain the quiescent characteristic of HSCs, and create a natural immune environment with KCs. Moreover, with a combination of our developed hiPSCs derived proliferative liver progenitors, we aim to establish a reproducible and productive model that can be used for high-throughput drug screening. The proposed research is highly relevant to public health because it will develop a mini liver tissue with complex cellular and architectural properties observed in the human liver. We will test this mini liver tissue's usefulness in mimicking the progression of injury-induced liver diseases. This work will be critical for developing potential treatments to reduce the health and economic impact of liver disease.

３．研究の方法

In this project, we generated mini liver tissue from hiPSCs derived progenitors, including hepatoblast, fetal hepatic stellate cells, endothelial progenitors, and hematopoietic progenitors, by step-wisely mimicking liver organogenesis. Next, we *in vitro* evaluated the functionality of this mini liver tissue with an extended culture and investigated its usefulness in liver disease modeling and drug screening.

４．研究成果

1) Generation of liver-specific progenitors from hiPSCs

With the optimization of culture conditions, we successfully generated proliferative hepatoblast and hepatic stellate cells from hiPSCs. The hiPSC derived hepatoblast could proliferate more than 10 to the 18th power folds within 15 times of passage. ELISA-, immunostaining-, and flow cytometry-based analyses show that proliferating cells could maintain hepatoblast characteristics during passages. Moreover, the passaged hepatoblast could also be successfully differentiated into hepatic like cells with ALB production, comparable to hiPSC derived hepatocytes. The hiPSC-HSC could proliferate more than 10 to the 8th power folds within 10 times of passage. Q-PCR and RNA sequencing revealed that repeated passages

did not induce the expression of activated HSC related genes.

2) Establishment of an immature mini liver tissue with hiPSCs derived progenitors

To establish an immature mini liver tissue, we co-seeded hiPSC derived hepatoblast, fetal hepatic stellate cells, endothelial progenitors, and monocytes in the 3D culture well, and these cells could organize into liver bud organoids within 24h. To generate an immature mini liver tissue, we transferred these organoids to a cell culture insert, and these organoids could self-merge into a mini tissue.

3) Characterization of the maturation of the mini liver tissue

Next, we developed an air-liquid interface culture method to promote the maturation of the mini liver tissue. Within 10 days of culture, the mini liver tissue increased expression of hepatic function genes and secretion of Albumin. The mini liver tissue also exhibited mature hepatic functions such as Ammonia metabolism and Urea production. Moreover, lineage tracing showed that endothelial progenitors gradually matured into vascular network structures, hepatic stellate cells maintained their quiescent characteristics, and the survival of monocyte was supported within the mini liver tissue microenvironment. Additionally, we found that monocytes could obviously improve the formation of vascular network structures. HE staining revealed that hepatocytes were arranged similarly to liver tissue, and lots of vascular lumens were observed among the hepatocytes.

We also found an enriched expression of genes related to liver sinusoidal endothelial cells and Kupffer cells during maturation. Immunostaining analysis showed that these endothelial cells were positively stained with sinusoidal endothelial markers, LYVE1 and FCGR2B. To confirm the differentiation of monocytes, we performed flow cytometry to sort the monocyte from mini liver tissue and compared their transcriptome with primary human Kupffer cells. The Kupffer cells specific markers, *CD5L*, *VCAM1*, *MACRO*, *KLF4*, *TIMD4*, and *FCGR3A*, were also highly detected in these matured monocytes. These results suggest that the liver microenvironment could significantly promote endothelial progenitors and monocytes to differentiate into liver sinusoidal endothelial cells and Kupffer cells, respectively.

4) Evaluation of the long-term maintenance of tissue structures and functions.

We next evaluated the tissue structures and functions with another 10-day extension culture. Timelapse fluorescence microscopy analysis showed that the endothelial network and characteristics of hepatic stellate cells were well maintained in the mini tissue. Q-PCR revealed the stable expression of hepatic and Kupffer cell related genes. Moreover, we did not observe significant changes in the functions of mini liver tissue, including ALB production, and Ammonia metabolism.

5) Modeling Non-Alcoholic Steatohepatitis (NASH) development with the mini liver tissue

We then investigated whether the mini liver tissue could be used for studying the development of NASH. When treating these liver tissues with free fatty acids, we detected a marked lipid accumulation, resulting in an enhanced immune response, activation of hepatic stellate cells, and

hepatic injury. We also performed drug testing on the NASH mini tissue and found the inhibition of immune response could help to attenuate the free fatty acids induced liver injury and activation of hepatic stellate cells. These results suggested that we successfully established a hiPSC derived mini liver tissue for studying NASH development and screening candidate drugs.

５．主な発表論文等

〔雑誌論文〕　計3件（うち査読付論文　3件／うち国際共著　2件／うちオープンアクセス　2件）

１．著者名 Nie YZ, Zheng YW, Taniguchi H	４．巻 0
２．論文標題 Improving the repopulation capacity of elderly human hepatocytes by decoding aging-associated hepatocyte plasticity	５．発行年 2022年
３．雑誌名 Hepatology	６．最初と最後の頁 1-16
掲載論文のDOI（デジタルオブジェクト識別子） 10.1002/hep.32443.	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

１．著者名 Li Y, Yang X, Plummer R, Hayashi Y, Deng XS, Nie YZ, Taniguchi H.	４．巻 22
２．論文標題 Human Pluripotent Stem Cell-Derived Hepatocyte-Like Cells and Organoids for Liver Disease and Therapy	５．発行年 2021年
３．雑誌名 Int J Mol Sci	６．最初と最後の頁 1-15
掲載論文のDOI（デジタルオブジェクト識別子） 10.3390/ijms221910471.	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

１．著者名 Qiu R, Murata S, Cheng C, Mori A, Nie YZ, Mikami S, Hasegawa S, Tadokoro T, Okamoto S, Taniguchi H.	４．巻 13
２．論文標題 A Novel Orthotopic Liver Cancer Model for Creating a Human-like Tumor Microenvironment	５．発行年 2021年
３．雑誌名 Cancers (Basel)	６．最初と最後の頁 1-23
掲載論文のDOI（デジタルオブジェクト識別子） 10.3390/cancers13163997.	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

〔学会発表〕　計5件（うち招待講演　0件／うち国際学会　3件）

１．発表者名 Li Y, Nie YZ, et al.,
２．発表標題 Recapitulation of hepatic hematopoiesis with human liver organoids
３．学会等名 ISSCR 2021 Virtual Annual Meeting（国際学会）
４．発表年 2021年

１．発表者名
Yang X, Nie YZ, et al.,

２．発表標題
Generation of quiescent hepatic stellate cells from human induced pluripotent stem cells.

３．学会等名
ISSCR 2021 Virtual Annual Meeting（国際学会）

４．発表年
2021年

１．発表者名
Plummer RT, Nie YZ, et al.,

２．発表標題
In-vitro generation of a functional and vascularized liver organoid from human iPS cells.

３．学会等名
ISSCR Tokyo 2021 International Symposium（国際学会）

４．発表年
2021年

１．発表者名
Nie YZ, Hideki T

２．発表標題
Decoding hepatocyte plasticity in aging with human induced pluripotent stem cells

３．学会等名
東京大学生命科学シンポジウム

４．発表年
2022年

１．発表者名
聶　運中、谷口　英樹

２．発表標題
ヒトiPS細胞を用いて老化に伴う肝細胞可塑性変化メカニズムの解明

３．学会等名
第22回日本再生医療学会総会

４．発表年
2023年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

―

６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関