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研究課題名（和文）The development of human iPSC derived proliferative progenitors for the effectively massive production of liver organoid

研究課題名（英文）The development of human iPSC derived proliferative progenitors for the effectively massive production of liver organoid

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

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研究成果の概要（和文）：肝オルガノイド（LO）は肝移植の潜在的な代替手段である。、ここでは、hiPSC由来の増殖性前駆細胞を分化誘導し、効率的なLO産生システムの構築を目指す。hiPSCから増殖性ある肝芽細胞と肝星細胞への誘導を成功した。免疫不全マウスに増殖性肝芽細胞および肝星細胞の安全性を移植し、移植部位での腫瘍形成を観察しなかった。さらに、肝芽細胞は慢性肝障害モデルの肝臓に再増殖し、機能的な肝細胞への成熟を確認した。さらに、生産効率を向上させるために、増殖性前駆細胞を用いて新規LOs作製法（マトリックスおよび3Dマイクロウェルフリー）を開発した。従来LOsと比較して、新規法作製したLOsは高く肝機能を確認した。

研究成果の学術的意義や社会的意義
末期肝疾患を治療するための移植可能なドナーの持続的な深刻な不足のために、新しい移植可能なヒト肝臓の開発が緊急に必要とされている。ヒトiPS細胞由来の肝臓オルガノイド（LO）は肝移植の潜在的な代替手段である。効率的な低安全で低コストのLO作製法の開発は臨床への応用における最重要なステップである。この研究では、増殖性前駆細胞に基づいてLO作製基盤を構築した。増殖性前駆細胞の技術は、未分化のiPSの排除と細胞分化誘導コストの削減に有益と考えられ、より安全、低コスト、高機能のLOを製造が促進されることが期待される。

研究成果の概要（英文）：We previously found that liver organoids (LOs) derived from hiPSC might be a potential alternative for liver transplantation. This project aimed to generate hiPSC derived proliferative liver progenitors and establish an efficient LO production system. With the optimization of culture conditions, we have generated proliferative Hepatoblast and fetal hepatic stellate cells, which could be proliferated 10 times and 10 times, respectively. We also transplanted these proliferative progenitors into immunodeficiency mice and did not observe tumor formation at the transplant sites. Moreover, we found the transplanted Hepatoblast could repopulate in the liver of chronic liver injury models and mature into functional hepatocytes. To further improve the production efficiency, we developed a matrix- and 3D microwell-free method to generate LOs with these progenitors, which exhibited an improved hepatic function compared with the conventional method derived LOs.

研究分野：再生医学

キーワード：Stem cell　Liver regenration　Liver organoid　Liver reconsturction

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９（共通）

１．研究開始当初の背景

Liver disease causes a global economic burden and accounts for approximately 2 million deaths per year worldwide (*Asrani et al., J Hepatol. 2019*). For end-stage liver diseases, the only proven treatment is liver transplantation, while there is still a persistent profound shortage of transplantable donors in the recent 20 years (www.organdonor.gov). Therefore, the development of new transplantable human livers is urgently needed. By recapitulation of the early liver organogenesis, Our lab has established a three-dimensional vascularized LO from hiPSC that can grow and help to rescue the drug-induced lethal liver failure model in vivo. (*Takebe et al., Nature. 2013*; *Takebe et al., Nat Protoc. 2014*; *Takebe et al., Cell Rep. 2017*). Current, we are investigating this clinical application of this LO for the treatment of liver diseases, while this advanced method for LOs could not meet the needs of human-scale production of LOs because of the following problems: 1)It lacks a stable hiPSC culture and differentiation system that can culture and differentiate 10^9 cells at the same time; 2) The undifferentiated hiPSC could not be excluded during the process; 3) It is too expensive and time-costing for LOs production; 4) There is no hepatic stellate cells, a critical mesenchymal population during liver development, in the advanced LOs.

２．研究の目的

In this project, we aim to develop original and robust methods for the generation of hiPSC derived proliferative progenitors that exist in the fetal liver, including hepatoblast, fetal hepatic stellate cells, and endothelial progenitors, and then to establish LOs with these proliferative progenitors. These progenitors derived LOs will be much close to the characteristic of the fetal liver that will be beneficial to improving the effectiveness of treatment. Since these progenitors acquire the proliferative capacity, the massive culture and differentiation of hiPSC are unnecessary, and the human-scale production of LOs will be much more costless and accessible. Moreover, it is easy to purify the progenitor cells without the contamination of undifferentiated hiPSCs, which will significantly improve the transplantation safety of LOs. Therefore, the development of proliferative progenitors derived LOs will provide a promising, safe, and effective therapeutic strategy for liver diseases.

３．研究の方法

First of all, we differentiated proliferative progenitors, hepatoblast, septum transversum (a progenitor cell of the fetal hepatic stellate cell), and endothelial progenitors with our published protocol (*Takebe et al., Cell Rep. 2017; Nie et al., Hepatology, 2022*). To maintain the proliferative capacity of these progenitors, we developed and optimized a combination of cytokines and small-molecule compounds in chemically defined media. The characteristics of these progenitors were detected with gene analysis (Q-PCR and RNA sequence), flow cytometry, and immunostaining. Moreover, we evaluated the proliferative capacity of these progenitors with a repeated passage and investigated their tumorigenicity with NOG mice. In addition, we explored the repopulation capacity of the proliferative hepatoblast by transplanting them into TK-NOG mice with chronic liver injury. Finally, we developed a matrix-free method to generate LOs with these

progenitors. Q-PCR, ELISA, and transplantation experiments were used to confirm the newly generated LOs *in vitro* and *in vivo*.

４．研究成果

(1) Generation of proliferative hepatoblast from hiPSCs

To differentiate hepatoblast, we first cultured hiPSC in an endoderm differentiation medium, and then changed it into a hepatic specification medium (*Nie et al., Hepatology, 2022*). When tracing the expression of lineage specific genes during the differentiation process, we found the hiPSC derived hepatoblast showed a low expression of Pluripotent and endoderm related genes, and a high expression of *AFP*, *ALB* as well as hepatoblast specific genes (*TBX3*, *DLK* and *C-MET*). We also confirmed the hepatobalst cell fate with Immunostaining and flow cytometry. Immunostaining revealed these cells were positively stained HNF4A, SOX9, TBX3 and A1AT, ALB, AFP, CK19, and KI67. Flow cytometry showed that more than 90% of cells express EpCAM, CD13, CD54, CD324, and CD133. Additionally, these cells were negatively stained by Pluripotent, mesoderm, endoderm related makers.

When sub-cultured hepatoblast in a proliferation medium (*Nie et al., Hepatology, 2022*), we observed rapid cellular growth within 7 days. Q-PCR revealed that the proliferative cells could maintain the expression of hepatoblast specific genes but lost the expression of hepatic lineage genes (*AFP* and *ALB*). To improve the hepatic characteristics of the proliferative cells, we optimized the combination of cytokines and small-molecular compounds and found a suitable combination to maintain the expression of hepatic lineage genes as well as hepatoblast specific genes. By optimizing the culture medium, we found that 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 proliferate 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 hepatocyte.

Next, we investigated the tumorigenicity and repopulation capacity of hepatobalst *in vivo*. Proliferative hepatoblasts were intrasplenically transplanted into TK-NOG mice after the gancyclovir-induced liver failure. After 8 weeks of transplantation, we detected a noticeable increase of human ALB production in mice serum. The production of human ALB could be maintained for more than 40 weeks. Histology analysis showed that Hepatobalst were matured into functional hepatocytes, with positive staining of CYP3A4 and CYP2E1, but negative staining of AFP and CK19. Additionally, we did not detect an abnormal proliferation of Hepatobalst in transplanted grafts.

(2) Generation of proliferative hepatic stellate cells from hiPSCs

We previously successfully differentiated hiPSC into septum transversum mesenchyme, a progenitor of hepatic stellate cells. However, the differentiation of septum transversum mesenchyme (STM) into hepatic stellate cells (HSCs) has not been achieved (*Takebe et al., Cell Rep. 2017*). By screening kinds of cytokines and small-molecule compounds, we successfully promote STM to mature into HSCs, with the capacity of retinol uptake. RNA sequencing showed that the characteristics of hiPSC-HSCs were much close to primary

human HSCs, but with lower expression of activated HSC related genes. Moreover, we found that HSC could proliferate more than 10 to the 8th power folds within 10 times of passage. Q-PCR revealed that repeated passages did not induce the expression of activated HSC related genes. To evaluate the tumorigenicity of HSCs, we transplanted GFP labeled HSCs into the sub-renal capsule of mice. After 4 weeks of transplantation, the transplanted grafts were detected with the confocal microscope, and no tumor formation was observed in these grafts.

(3) Generation of liver organoid with proliferative progenitors

Here, we also developed a matrix-free method to generate liver organoids. When seeding hepatoblast, hepatic stellate cells, and endothelial progenitors on a pretreated culture plate, these three types of cells could self-organize into 3D structures within 24 hours. Live imaging showed endothelial network formation in the organoid, and hepatic stellate cells were next to the endothelial network. Compared to our previous LOs generated with hepatic endoderm, STM, and endothelial progenitors, the proliferative progenitors derived LOs had a much higher expression of hepatic lineage genes as well as ALB production. To confirm the in vivo functions of proliferative progenitors derived LOs, we transplanted the progenitors derived LOs into mouse cranium, and found the human ALB production in proliferative progenitors derived LOs was much higher than our previous LOs. 28 days later, we found the endothelial progenitors could mature into functional vascular, and hepatic lineages were mature into functional hepatocytes by positive staining of CYP3A4.

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4件（うち招待講演　0件／うち国際学会　4件）

１．発表者名 Yun-Zhong Nie, Yun-Wen Zheng, Hideki Taniguchi
２．発表標題 Ex vivo evaluation of human hepatocyte plasticity
３．学会等名 The International Society for Stem Cell Research（国際学会）
４．発表年 2020年

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

〔図書〕　計0件

〔出願〕　計1件

産業財産権の名称	発明者	権利者
肝細胞の可塑性誘導法	谷口英樹,聶運中,鄭允文	同左
産業財産権の種類、番号	出願年	国内・外国の別
特許、PCT/JP2020/36043	2020年	国内

〔取得〕　計0件

〔その他〕

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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