Center for Stem Cell Biology and Regenerative Medicine

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Currently, organ transplantation is the only effective treatment for patients with end-stage organ failure. Unfortunately, the limited number of transplantable organs hinders the extensive application of this treatment. On the other hand, recent development of regenerative medicine that aims to generate transplantable organs on a dish has attracted much attention. Regenerative medicine is a challenging scientific field that attempts to convert knowledge from developmental biology and stem cell biology into clinical application. Our established novel organoid culture technologies reconstruct functional human organs derived from human induced pluripotent stem cells (hiPSCs), and finally aim to develop ex vivo human liver disease models and a substitute for organ transplantation therapy. Currently, we are trying to conduct the transplantation of human liver organoids (LOs) generated from hiPSCs to treat liver diseases, such as metabolic disorders and liver fibrosis. Moreover, we expand the application of our technologies to reconstruct artificial cancer tissue (cancer organoid) with a refractory tumor microenvironment for developing a new drug-screening platform to discover candidate compounds that could prevent cancer relapse and metastasis.

1. Development of treatment for metabolic liver disease by transplantation of human iPS cell derived 3D-organoids.

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The liver plays a crucial role in maintaining homeostasis in the living organism by performing various metabolic functions such as glucose metabolism, lipid metabolism, and ammonia metabolism. On the other hand, abnormalities in these metabolic functions can lead to a variety of diseases of the liver. Liver transplantation is the only curative therapy for end-stage liver disease, but the absolute shortage of donor organs is a serious challenge, and alternative treatments are clinically highly demanded. We established a technique to produce and evaluate human pluripotent stem cell (hiPSC) derived liver organoids (hiPSC-LO) by inducing differentiation of hepatic endodermal cells, vascular endothelial cells, and mesenchymal cells from hiPSCs and co-culturing them in a 3D manner (***Nature*** 2013, ***Nature*** 2017, ***Cell Reports*** 2017, ***Sci Rep*** 2020, ***Stem Cell Rev Rep.*** 2022).

Currently, we are developing a novel therapeutic method using hiPSC-LO transplantation for urea cycle disorders, a serious liver disease, and metabolic dysfunction-associated steatohepatitis (MASH), (***Sci Transl Med.*** 2024 Jul 24;16(757):eadg0338.).

Liver cirrhosis is the end stage pathological condition of chronic liver diseases such as MASH. MASH is characterized by reduced liver function and regenerative capacity and is expected to explode in the number of patients worldwide. With the support of AMED, we are currently developing a novel treatment for MASH cirrhosis by transplantation of a newly developed fused-type hiPSC-LO based on hiPSC-LO production technology. To this end, we have established a stable method for creating fused-type hiPSC-LOs and now we are examining its efficacy as MASH treatment by transplanting them into MASH liver cirrhosis model animals. Given that no effective treatment has been developed for MASH cirrhosis, there is great hope for fused-type hiPSC-LOs transplantation.

2. Liver repopulation with hiPSC derived proliferative progenitors

Yun-Zhong Nie[1], Yoshihito Hayashi[1], Qing-Lin LI[1], Xiao-Shan Deng[1], Luo Na[1], Yang Li[1], Xia Yang[1], Riana Plummer[1], Naoki Tanimizu[1], Yasuharu Ueno[1], Hideki Taniguchi[1,2]

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hiPSCs have shown immense potential for cell replacement therapy for disease treatment. However, hiPSC-derived cells that can effectively repopulate in the damaged tissues such as liver have not been reported. Here, we present the generation of expandable hiPSC-derived hepatoblast (hiPSC-HB) with robust repopulation capacity after transplantation. These hiPSC-HB exhibited an impressive expansion capability and displayed bipotential differentiation abilities both *in vitro* and *in vivo*. Notably, we found that hiPSC-HB transplantation could rescue mice from liver failure, demonstrating a repopulation capacity comparable to that of primary human hepatocytes (PHHs). Further, the engrafted hiPSC-HB matured into functional human hepatocytes with tissue-specific structural features in kinds of disease models. This study marks a breakthrough as the first successful generation of lineages from pluripotent stem cells capable of *in vivo* repopulating and restoring tissue function. Moving forward, we aim to explore the potential clinical applications of hiPSC-HB transplantation in the treatment of liver diseases.

3. Modeling liver diseases with hiPSC-derived organoid

Yun-Zhong Nie[1], Yang Li[1], Xia Yang[1], Riana Plummer[1], Xiao-Shan Deng[1], Yoshihito Hayashi[1,] Qing-Lin LI[1], Luo Na[1], Toshiharu Kasai[1], Takashi Okumura[1], Naoki Tanimizu[1], Yasuharu Ueno[1], Hideki Taniguchi[1,2]

Maximizing the potential of human liver organoids (LOs) for modeling human septic liver requires the integration of innate immune cells, particularly resident macrophage Kupffer cells. In this study, we present a strategy to generate LOs containing Kupffer cells (KuLOs) by recapitulating fetal liver hematopoiesis using hiPSC-derived erythro-myeloid progenitors (EMPs), the origin of tissue-resident macrophages. Remarkably, LOs actively promote EMP hematopoiesis toward myeloid and erythroid lineages. Moreover, supplementing M-CSF proves crucial in sustaining the hematopoietic population during the establishment of KuLOs. Exposing KuLOs to sepsis-like endotoxins leads to significant organoid dysfunction that closely resembles the pathological characteristics of the human septic liver. Furthermore, we observe a notable functional recovery in KuLOs upon endotoxin elimination, which is accelerated by using Toll-like receptor 4-directed endotoxin antagonist. Our study represents a comprehensive framework for integrating hematopoietic cells into organoids, facilitating in-depth investigations into inflammation-mediated liver pathologies. Moving forward, we aim to enhance the complexity of liver organoids by incorporating hepatic stellate cells and sinusoidal endothelial cells, thereby establishing organoids with tissue-specific features for studying disease progression and identifying potential treatments.

4. hiPSC-liver bud *in vitro* growth enhanced by perfusion culture

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To overcome the critical shortage of organ donors, the generation of hiPSC-derived organs with structures and functions is urgently needed. Although hiPSC-organoid is an innovative technology to reconsti-

tute tissue structure and function, an alternative for organ transplantation. Blood perfusion is a critical event for organ growth by supplying nutrients and oxygen. However, blood perfusion is still lacking in the present organoid culture system. We are developing perfusion culture systems using two approaches; hiPSC-liver buds (LBs) connected with pefusable hiPSC-blood vessel, and the decellularized liver tissue infused with hiPSC-LBs.

From our first approach, we generated the perfusable hiPSC-derived blood vessel using collagen gels, hiPSC-derived vascular smooth muscle cells (SMC), and vascular endothelial cells (EC). Although we clarified that hiPSC-derived blood vessel is histologically similar to the vascular structure of *in vivo* blood vessels, EC-seeded blood vessels did not show angiogenesis, resulting no contact/connection with capillaries within hiPSC-LBs under co-culture. Therefore, we established a new culture protocol to differentiate hiPSC into specific EC lineages that exist around the fetal liver. We demonstrated that hiPSC-derived blood vessel containing those specific ECs had higher angiogenic potentials. Under an optimized culture condition, we successfully induced the connection between the hiPSC-derived blood vessels with the capillaries in hiPSC-LBs. Therefore, we recently tried to establish the organoid perfusion system. Our perfusion system enabled us to culture the hiPSC-LBs by 14 days from co-culture and increase the area of hepatic progenitors/hepatocyte within hiPSC-LBs. Optimizing the perfusion culture system to mimic *in vivo* blood perfusion during organogenesis, we are trying to enhance hiPSC-LB growth more efficiently.

As the second approach, we utilize decellularized liver tissue which retains *in vivo* vascular structures. The decellularization technique has been established to prepare the scaffold for organ reconstitution. Decellularized organs potentially retain the architecture of the original tissue, including the extracellular matrix. A recent report shows how the recellularized liver using hepatocytes could exert liver-specific functions after transplantation. However, the vascular structures within this recellularized liver remain unreconstructed, which might explain limited hepatocyte functions in the recellularized liver. Our current study attempts to generate a more functional recellularized liver by adding oxygen supply into our perfusion culture system of the recellularized liver containing hiPSC-LBs.

5. Generation of 3D cancer tissue using patient-derived pancreatic cancer cells

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Pancreatic ductal adenocarcinoma (PDAC) has a poor prognosis, with a 5-year survival rate of about 10% due to delayed diagnosis, drug resistance, and recurrence. Organoid technologies have been applied to investigate the properties of PDACs. To recapitulate tumor microenvironment (TME), which is highly correlated with the poor prognosis of PDAC, we generated multicellular spheroids consisting of primary PDAC cells isolated from Japanese pancreatic cancer patients with hiPSC-mesenchymal cells (MCs) and -endothelial cells (ECs), and then fused them to construct fused pancreatic cancer organoid (FPCO). Our FPCO resembles the tissue structure of clinical tissue including PDAC ductal structures, dense deposition of extracellular matrix components compared to conventional organoids, and heterogeneous cancer-associated fibroblasts (CAFs) namely immunological CAF (iCAF), myofibroblastic (myCAF), and antigen presenting ones (apCAFs). In addition to CAFs, we recently developed FPCO containing tumor associated macrophages (TAMs) to recapitulate immunosuppressive TME. Since the PDAC organoid showed strong resistance to anti-cancer drugs, we apply this new cancer organoid in drug screening and biological analysis to develop effective therapies against PDAC.

6. Space Organogenesis (Development of advanced 3D organ culture system utilizing microgravity environment)

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Microgravity in orbit does not cause subsidence or convection and is considered advantageous in expanding cells in three dimensions. Utilizing this microgravity environment, we aim to develop a novel method for generating human iPSCs-derived liver tissue in collaboration with Japan Aerospace Exploration Agency (JAXA). In particular, we attempt to establish a new technique for generating three-dimensional organs containing large blood vessels. The second space experiment was conducted in March 2024 to uncover the effects of microgravity on cell growth and differentiation of hiPSC-derived liver tissue. After we prepared hiPSC-liver buds (LBs) and

hiPSC-derived blood vessels (BVs) on the earth, we placed those organoids into the culture container and launched them to the International Space Station "KIBO". We confirmed that hiPSC-LBs were successfully assembled around the hiPSC-BVs under microgravity, as how *in silico* simulation suggested. After the perfusion culture of BV-equipped hiPSC-liver organoids (LOs) for a predetermined period in the incubator installed in "KIBO", the samples were transported back to the earth. Adherence of hiPSC-LBs to the BVs and the formation of hiPSC-LOs by fusion of hiPSC-LBs were observed in the post-flight samples. Moreover, endothelial cells formed more vascular networks within the hiPSC-LOs in the post-flight samples than the ground controls. Gene ontology analysis of RNA-seq data revealed that genes related to hepatic functions such as complement and coagulation cascades, fat digestion and absorption, bile secretion, steroid hormone biosynthesis, and retinol metabolism were enriched in the post-flight samples, indicating how the space environment could provide an optimal condition for tissue reconstruction. We hope these results from space experiments will contribute to the subsequent development and understanding of (1) The development of a new technique in human three-dimensional tissue preservation and transportation, which is crucial to the practical use of regenerative medicine products. (2) The establishment of a novel technique for generating human organs equipped with large blood vessels. (3) The development of a new three-dimensional culture device simulating the microgravity environment on earth.

7. Generation of bile duct tubules in hiPSC-liver buds

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The biliary system consisting of intrahepatic bile duct (IHBD), extrahepatic bile ducts (EHBDs), and gallbladder, is a crucial tissue structure for maintaining liver homeostasis by providing the excretion route for the bile secreted from hepatocytes. Although various types of liver organoids have been established, the generation of hiPSC-liver organoids associated with the bile drainage system consisting of IHBD and EHBD has not been reported so far.

Previous works demonstrated that IHBD formation depends on JAGGED1(JAG1)-NOTCH signal activated by vascular smooth muscle cells of the portal vein. Therefore, to generate liver organoid containing IHBD-like structures, we developed a new co-culture system in which the hiPSC-liver progenitors are located next to the hiPSC-blood vessel (BV) to recapitulate the fetal portal vein-IHBD tissue interaction. In this condition, hiPSC-liver progenitors differentiated into cholangiocytes and formed duct structures. We named this organoid as blood vessel incorporated liver organoid (BVLO). hiPSC-cholangiocytes in BVLO showed secretory functions in vitro and formed duct structures within the recipient liver after organoid transplantation to immunodeficient mice. Furthermore, when BVLO was transplanted to bile duct ligated mice, it temporally attenuated cholestatic symptoms and extended the survival period of injured mice. Finally, we introduced the artificial BV containing mesenchymal cells derived from JAG1 KO hiPSCs and found that bile duct formation was attenuated. This could be an in vitro model for human Alagille syndrome, a human congenital biliary disease caused by JAG1 mutation for understating underlying mechanisms.

Now, we focus on the establishment of EHBD organoids. We induced EHBD progenitor cells from hiPSC-definitive endoderm cells and generated 3D cystic structures. Currently, we are further optimizing culture condition to confer EHBD characteristics on those cystic structures. Our final goal is to eventually connect these two tubular structures with hiPSC-derived hepatocytes on a dish to generate Hepatobiliary Tubular Organoids (HBTO) that possess a long-term hepatic function *in vitro* as well as *in vivo*.

8. Generation of a novel treatment for pediatric craniofacial deformity using human auricular perichondrium-derived elastic cartilage devices

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Auto-transplantation of rib cartilage segments is the current most popular treatment for patients with craniofacial deformity. However, major disadvantages such as limited harvestable amounts and post-operative pain of the donor site remain to be solved. To this end, a none-invasive, morphologically stable scaffold-free elastic cartilage implantation treatment for patients with craniofacial deformity is essential.

Our previous study showed the world's first technology of separating and identifying chondroprogenitor cells from the human auricular perichondrium (Kobayashi S et al. PNAS 2011, Patent registration no. 4748222; PCT/JP2008/051327). We succeeded in developing non-scaffold elastic cartilage, which is obtainable in vitro, by using three-dimensional rotation culture and U–bottomed micropatterned plate culture (Enomura M et al. Int J Mol Sci 2020, Oba T et al. J Tissue Eng 2022, Patent application no. 2021-141210; PCT/JP2022/25582). Furthermore, the size and elasticity of the tissue were maintained after craniofacial transplantation in immunodeficient mice, indicating the tissue to be morphologically stable.

Our major goal is to establish a non-invasive novel promising treatment for pediatric patients with nasal deformity by transplanting morphologically stable non-scaffold elastic cartilage. To obtain the clinical POC of the novel treatment, we established the manufacturing system, quality control methods, product specification, evaluation of nonclinical safeness and determination of clinical protocol with Japan Tissue Engineering and JTEC. Currently we are discussing with the Pharmaceuticals and Medical Devices Agency (PMDA) to obtain an approval of the clinical trial which is planned to be carried out next summer.

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