Center for Stem Cell Biology and Regenerative Medicine

Division of Regenerative Medicine
再生医学分野

Professor	Hideki Taniguchi, M.D., Ph.D.	教　授	博士(医学)	谷　口　英　樹
Associate Professor	Naoki Tanimizu, Ph.D.	准教授	博士(農学)	谷　水　直　樹
Assistant Professor	Yun-Zhong Nie, Ph.D.	助　教	博士(医学)	聶　　運　中
Project Assistant Professor	Yasuharu Ueno, Ph.D.	特任助教	博士(医学)	上　野　康　晴
Project Assistant Professor	Takayoshi Oba, M.D., Ph.D.	特任助教	博士(医学)	大　場　敬　義

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 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.

Yasuharu Ueno[1], Naoki Tanimizu[1], Yunzhong Nie[1], Satoshi Okamoto[1], Yoshiki Kuse[1], Takashi Okumura[1], Toshiharu Kasai[1], Marina Zushi[1], Emi Yajima[1], Erika Jinbo[1], Kerrigan Kilpatrick[1], Tomomi Tadokoro[2] and Hideki Taniguchi[1,2]:

[1] Division of Regenerative Medicine, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo

[2] Department of Regenerative Medicine, Graduate School of Medical Science, Yokohama City University

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 and rapid availability of donor organs is a serious challenge and alternative treatments are clinically highly demanded. We established a technique to produce 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). Currently, we are developing a novel therapeutic method using hiPSC-LO transplantation for urea cycle disorders, a serious liver disease,

and metabolic insufficiency steatohepatitis (MASH), for which there is a huge number of patients.

Urea cycle disorders is a severe metabolic liver disease resulting from the dysfunction of ornithine carbamoylase (OTC) and other enzymes, which is associated with hyperammonemia. We have optimized the ECM coating on the culture dishes and further optimized the organoid culture method to establish a robust production method for hiPSC-LO with appropriate ammonia metabolic capacity (***Biol Methods Protoc*** 2022). In addition, to evaluate the safety of the hiPSC-LOs, we established a highly sensitive method detecting undifferentiated cells after induction of LO component cells from hiPSCs (***Stem Cell Rev Rep*** 2022). Next, we evaluated the efficacy of hiPSC-LO transplantation in a severe OTCD animal model with a background of immunodeficiency. Subadrenocortical transplantation of hiPSC-LOs into immunocompromised OTCD mice significantly improved their hyperammonemic state, suggesting that iPSC-LO transplantation is effective as the treatment of OTCD.

Liver cirrhosis is the end stage pathological condition of chronic liver diseases such 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]

[1] Division of Regenerative Medicine, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo

[2] Department of Regenerative Medicine, Graduate School of Medical Science, Yokohama City University

Liver transplantation is a proven therapy for genetic liver disorders. Yet, its clinical application faces the persistent shortage of transplantable livers. 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*. Moreover, we noticed that the repopulation capacity of hiPSC-HB could be enhanced with specific conditions, leading to engraftment levels comparable to primary human hepatocytes (PHHs) in liver failure mice. Further, the engrafted hiPSC-HB matured into functional human hepatocytes with tissue-specific structural features. 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, offering promising prospects for innovative disease treatment through regenerative medicine.

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]

The developed human cell-based liver *ex vivo* models still could not accurately recapitulate liver physiology and disease progression due to lack of non-parenchymal cell, including Kupffer cell, hepatic stellate cells and sinusoidal endothelial cells. Currently, we generated LOs containing Kupffer cells (KuLOs) by recapitulating fetal liver hematopoiesis using hiPSC-derived erythro-myeloid progenitors (EMPs), origin of tissue-resident macrophages, and hiPSC-derived LOs. Exposing KuLOs to sepsis-like endotoxins led to significant organoid dysfunction that closely resembled the pathological characteristics of the human septic liver. Furthermore, we observed a notable functional recovery in KuLOs upon endotoxin elimination, which was accelerated by using Toll-like receptor 4-directed endotoxin antagonist. Moreover, we are trying to establish a mini liver with hepatocytes, Kupffer cells, hepatic stellate cells, and sinusoidal endothelial cells, which will allow a more precise understanding of the cell-to-cell interactions during liver disease progression, and provide a novel platform to explore potential targets for alleviating liver disease progression.

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

Yoshiki Kuse[1], Naoki Tanimizu[1], Yasuharu Ueno[1], Nie Yunzhong[1], Shinya Matsumoto[1], Takashi Okumura[1], Erica Carolina[1], Soichiro Yamabe[1], Toshiharu Kasai[1], Tomomi Tadokoro[2], Satoshi Okamoto[2],

and Hideki Taniguchi[1,2]:
[1] Division of Regenerative Medicine, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo
[2] Department of Regenerative Medicine, Graduate School of Medical Science, Yokohama City University

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 reconstitute 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 reconstructed hiPSC-blood vessel, and the decellularized liver tissue infused with hiPSC-LBs.

From our first approach, we generated the hiPSC-derived macrovessel using collagen gels, hiPSC-derived vascular smooth muscle cells (SMC), and vascular endothelial cells (EC). Although we clarified that hiPSC-derived macrovessel is histologically similar to the vascular structure of *in vivo* blood vessels, EC-seeded macrovessels did not show angiogenesis after coculturing with hiPSC-LBs. Therefore, we established a new induction method to differentiate hiPSC into specific EC lineages that exist around the fetal liver. We demonstrated that hiPSC-derived macrovessel containing those specific ECs had higher angiogenic potentials. Under an optimized culture condition, we successfully induced the connection between the hiPSC-derived macrovessel with the microvessels within hiPSC-LBs. Therefore, we recently tried to establish the organoid perfusion system. The established perfusion system enabled us to culture the hiPSC-LBs by 14 days from co-culture and increase $CD31^+$ blood vessels in LBs. Optimizing the perfusion culture system, we are trying to enhance hiPSC-LB growth.

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

Kenta Takeuchi[1],, Shunsuke Tabe[1], Yuya Yamomoto[1], Yasuharu Ueno[1], Hideki Taniguchi[1,2], and Naoki Tanimizu[1]
[1] Division of Regenerative Medicine, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo
[2] Department of Regenerative Medicine, Graduate School of Medical Science, Yokohama City University

Pancreatic 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 thought to be crucial for 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 and dense deposition of extracellular matrix components compared to conventional organoids. Single cell RNA sequence analysis demonstrated that FPCO contains various types of cancer-related fibroblasts (CAFs) namely immunological CAF (iCAF), myofibroblastic (myCAF), and antigen presenting ones (apCAFs). Moreover, the PDAC organoid showed strong resistance to anti-cancer drugs and re-proliferative capacity after drug treatment indicating its close resemblance to frequent relapse in PDAC patients. We are currently making FPCO containing tumor associated macrophages (TAMs) to recapitulate immunosuppressive TME. We will 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)

Tomomi Tadokoro[2], Tatsuya Kobayashi[1], Yoshiki Kuse[1], Yasuharu Ueno[1], Takashi Okumura[1], and Hideki Taniguchi[1,2]:
[1] Division of Regenerative Medicine, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo
[2] Department of Regenerative Medicine, Graduate School of Medical Science, Yokohama City Universi-

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Microgravity in orbit does not cause subsidence or convection and is considered advantageous in expanding cells in three-dimension. By 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. After we prepared hiPSC-LBs and artificial vessels on the earth, we placed those organoids into the culture container and launched to the International Space Station "KIBO". We confirmed that hiPSC-LBs were successfully assembled around the artificial vessel under microgravity, as how *in silico* simulation suggested. After culturing hiPSC-LBs for a predetermined period in the incubator installed in "KIBO", the samples were then transported back to the earth. Adherence and fusion of hiPSC-LBs to the artificial vessels were observed in the post-flight samples cultured in orbit. Moreover, endothelial cells started to extend their filopodia-like structure. Using qRT-PCR analysis of ground controls and post-flight samples, comparable expressions of hepatic, endothelial cell-related, and mesenchymal cell-related genes were observed in both samples. In addition, gene ontology analysis of RNA-seq data revealed that genes related to triglyceride homeostasis, cholesterol biosynthetic process, MAPK pathway, and angiogenesis were enriched in the post-flight samples, indicating how the space environment could provide an optimal condition for tissue reconstruction. Next, we upgraded our system by using a large blood vessel composed of hiPSC-derived smooth muscle cells and endothelial cells which resulted in the improvement of hiPSC-LB functions. The second space experiment is planned to be conducted in March 2024. These findings will uncover the effects of gravity on cell growth and differentiation. We hope these space experiment results 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 joined 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

Ayumu Okumura[1], Erica Carolina[1], Kenji Aoshima[1], Taichi Tsuyuki[1], Minjia Zhong[1], Kazuki Yanagisawa[1], Yoshiki Kuse[1], Takashi Okumura[1], Toshiharu Kasai[1], Tomomi Tadokoro[2], and Hideki Taniguchi[1,2], and Naoki Tanimizu[1]

[1] Division of Regenerative Medicine, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo

[2] Department of Regenerative Medicine, Graduate School of Medical Science, Yokohama City University

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.

First, to generate liver organoid containing IHBD-like structures, we developed a new co-culture system in which the hiPSC-liver progenitors are located in 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. We applied BLVO technology to modelling a human congenital biliary disease for understating underlying mechanisms.

Second, to generate EHBDs, we induced EHBD progenitor cells from hiPSC- definitive endoderm cells and generated 3D cystic structures. We are currently analyzing transcriptome data of mouse fetal EHBD epithelial cells to acquire information about proliferation signal for EHBD progenitors and their functional maturation process. 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

Takayoshi Oba[1], Satoshi Okamoto[1], Yasuharu Ueno[1], Chie Ikezuki[1], Takuya Ohkuma[1], Tenta Kubo[2], and Hideki Taniguchi[1,2]

[1] Division of Regenerative Medicine, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo

[2] Department of Regenerative Medicine, Graduate

School of Medical Science, Yokohama City University

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 are currently establishing the manufacturing system, quality control methods, product specification, evaluation of non-clinical safeness and determination of clinical protocol.

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