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| No.   | K22-1040                                                                                       |                                                                                                                                                                                       |
| 研究課題名 | Reconstruction of human liver in Fah-/-Rag2-/-IL2rg-/- rats with hiPSC derived liver organoids |                                                                                                                                                                                       |
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IMSUT International Joint Usage/Research Center Project <International>

Joint Research Report (Project Completion)

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| Project Completion Report                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
| Report                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
| <p>To address the critical shortage of donor organs for treating end-stage liver failure, Prof. Taniguchi's team has successfully developed a vascularized and functional human liver through ectopic transplantation of human iPSC-derived liver organoids (hiPSC-LOs) <i>in vivo</i>. Moreover, transplanted hiPSC-LO could improve the survival rate and alleviate fibrosis in the liver fibrosis model. However, the gradual decline in albumin (ALB) production following transplantation indicated that the regenerative capacity of hiPSC-LOs remains limited. In this study, we combined the newly developed hiPSC-derived hepatoblast (hiPSC-HBs) from Prof. Taniguchi's team with our established <i>Fah<sup>-/-</sup>Rag2<sup>-/-</sup>IL2rg<sup>-/-</sup></i> (FRG) rats model to explore strategies for enhancing the regenerative capacity of hiPSC-LOs.</p> <p><b>Characterization of hiPSC-HB</b></p> <p>We first characterized the differentiation of hiPSCs into hepatoblast. After 13 days of differentiation, we observed significant induction of hepatoblast-related gene expression. Bulk RNA sequencing confirmed strong enrichment of pan-hepatoblast transcriptional signatures in hiPSC-HB. Immunostaining revealed expression of bipotential markers, and flow cytometry analysis demonstrated a cell purity exceeding 99%, with minimal contamination from mesenchymal and definitive endoderm lineages. Single-cell RNA sequencing further revealed that the transcriptional profile of hiPSC-HBs closely resembled that of hepatoblasts found in the human fetal liver. Moreover, these hiPSC-HB exhibited robust expansion potential, with over a 10<sup>16</sup>-fold increase in cell number <i>in vitro</i>, while maintaining bipotential marker expression and high purity through multiple passages. Transcriptomic profiles remained consistent across differentiated passages, and cryopreservation did not impair their expansion capacity.</p> <p><b>Generation and transplantation of hiPSC-HB-Derived Liver Organoids (hiPSC-HB-LO)</b></p> <p>Next, we generated liver organoids using hiPSC-HB. Compared to conventional organoids derived from hiPSC-derived hepatic endoderm (hiPSC-HE), the hiPSC-HB-derived liver organoids (hiPSC-HB-LOs) exhibited more pronounced vascular structures. Histological analysis revealed an abundance of parenchymal cells within the hiPSC-HB-LOs. Additionally, these organoids showed enhanced expression of hepatic functional genes and improved liver-specific functions, including albumin and urea production, CYP3A4 enzymatic activity, and ammonia metabolism, relative to hiPSC-HE-LOs. Due to breeding limitations with the FRG rat model, we conducted transplantation studies in mouse models. By monitoring human ALB production, we noticed that transplantation of hiPSC-HB-LOs led to high levels of human ALB production in recipient mice. Histological analysis at four weeks post-transplantation confirmed successful engraftment, with evidence of <i>in vivo</i> tissue repopulation and reconstruction of human liver tissue. Immunostaining analyses revealed that the reconstructed tissues were negative for KRT19, an immature hepatic marker, and strongly positive for CYP3A4, indicating functional maturation. Moreover, we observed the integration of mouse CD31-positive endothelial cells within the engrafted tissue,</p> |

suggesting vascular remodeling and host-derived endothelial incorporation. These results support the *in vivo* maturation of hiPSC-HB-LOs into functional hepatic tissue.

### **Analysis of Regenerative Capacity**

We then compared the repopulation potential of hiPSC-HB-LOs versus single-cell hiPSC-HB in mouse models. Mice transplanted with hiPSC-HB exhibited a more rapid and significantly higher increase in human ALB levels than those transplanted with organoids. Immunohistology analyses confirmed more efficient liver tissue reconstruction by hiPSC-HB than by hiPSC-HB-LOs.

Moreover, we assessed hiPSC-HB transplantation in the FRG model under optimized conditions. The transplanted hiPSC-HB successfully engrafted and differentiated into functional hepatocytes, as evidenced by widespread expression of human fumarylacetoacetate hydrolase (FAH). Immunostaining confirmed a broad distribution of FAH-positive human hepatocytes in the host liver.

### **Conclusion**

In summary, our study confirms the robust and stable expansion capacity of hiPSC-HBs and their applicability for generating liver organoids. While hiPSC-HB-derived organoids are capable of engrafting, maturing, and contributing to functional liver tissue, their repopulation efficiency is lower than that of direct hiPSC-HB transplantation. Importantly, hiPSC-HB demonstrated strong regenerative potential and functional maturation in different models. These findings highlight both hiPSC-HB and hiPSC-HB-derived liver organoids as promising cell sources for liver regeneration and treating liver diseases.