

Auxiliary xenotransplantation as an in vivo bioreactor— Development of a transplantable liver graft from a tiny partial liver

Yuki Masano¹  | Shintaro Yagi¹  | Yosuke Miyachi¹ | Shinya Okumura¹ |
Toshimi Kaido¹ | Hironori Haga² | Eiji Kobayashi³  | Shinji Uemoto¹

¹Division of Hepato-Biliary-Pancreatic and Transplant Surgery, Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan

²Department of Diagnostic Pathology, Kyoto University Hospital, Kyoto, Japan

³Department of Organ Fabrication, Keio University School of Medicine, Tokyo, Japan

Correspondence

Shintaro Yagi, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto, 606-8507, Japan.

Email: shintaro@kuhp.kyoto-u.ac.jp

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Abstract

Background: We established a completely novel method of auxiliary xenogeneic partial liver transplantation and examined whether liver grafts procured from Syrian hamsters regenerated in nude rats, which were used as in vivo bioreactors.

Methods: The hamsters and the rats were all males (n = 10). Partial liver grafts from hamsters were transplanted into nude rats in an auxiliary manner. We evaluated liver graft injury, rejection, and regeneration during 7 days after auxiliary xenogeneic partial liver transplantation.

Results: All rats survived until sacrifice on post-operative day (POD) 1, 3, and 7. HE-staining showed normal at POD1, mild periportal edema, and slight bile duct and venous endothelial inflammation at POD3, and moderate acute cellular rejection at POD7 without parenchymal necrosis. The liver regeneration rates at POD3 and 7 were 1.54 ± 0.23 and 2.54 ± 0.43 , respectively. The Ki-67 labeling index was also elevated at POD3 ($27.5 \pm 4.1\%$). Serum HGF and VEGF were elevated at POD1 and 3. ATP levels of liver grafts recovered at POD7.

Conclusions: These results revealed that with appropriate immunosuppressive therapy, partial liver graft regeneration occurred in a xenogeneic animal, which suggests liver grafts regenerated in xenogeneic environments, such as an in vivo bioreactor, have potential to be transplantable liver grafts for humans.

KEYWORDS

in vivo bioreactor, liver regeneration, xenotransplantation

Abbreviations: ALT, alanine aminotransferase; APLT, auxiliary partial liver transplantation; AST, aspartate aminotransferase; ATP, adenosine triphosphate; AxPLT, auxiliary xenogeneic partial liver transplantation; GTKO, α -1,3-galactosyltransferase knock out; HAR, hyper-acute rejection; HE, hematoxylin and eosin; HGF, hepatocyte growth factor; HTK, histidine-tryptophan-ketoglutarate; IHIVC, infrahepatic inferior vena cava; IL-6, interleukin-6; LDH, lactate dehydrogenase; LDLT, living donor liver transplantation; LT, liver transplantation; MMF, mycophenolate mofetil; POD, post-operative day; PSCs, pluripotent stem cells; PV, portal vein; SCID, severe-combined immunodeficiency; SEM, standard error of the mean; SFSG, small-for-size graft; SFSS, small-for-size syndrome; SHIVC, suprahepatic inferior vena cava; TAC, tacrolimus; T-Bil, total bilirubin; TNF α , tumor necrosis factor α ; uPA, urokinase-type plasminogen activator; VEGF, vascular endothelial growth factor; α -Gal, galactose- α 1,3-galactose.

1 | INTRODUCTION

Liver transplantation (LT) is the only ultimate treatment for end-stage liver failure. Because of the shortage of organ donors worldwide, the demand for usage of marginal grafts, including partial small grafts, is increasing. However, insufficient liver graft volume compared with recipient body weight, that is, "small-for-size graft (SFSG)", is an inevitable consequence in some cases of living donor liver transplantation (LDLT). Strategies to satisfy the insufficient numbers of donor organs for LT have been continuously investigated.

One approach is to promote small partial liver graft regeneration after LT. The liver is alone among solid organs in its ability to regenerate full mass and function following extensive acute injury (as in acute liver failure), partial resection, or partial liver transplantation.¹⁻⁴ Furthermore, if we can use an extremely smaller liver graft successfully from a living or deceased donor, it will contribute not only donor safety but also expansion of donor pool. Numerous research projects using various agents to promote partial small liver grafts after LT have been implemented, all of which have only a limited effect for liver graft regeneration.⁵⁻⁷ Another approach is a challenging project to make transplantable livers using scaffolding and human embryonic stem cells (PSCs) through a tissue-engineering technique.^{8,9} Because the structure is 3-dimensional and several kinds of cells are involved intricately in the liver, projects attempting this approach may require more time.

Yet another approach is the use of xenografts instead of human organs. This approach has been attempted for several decades in clinical and experimental studies. To conquer the hyper-acute rejection (HAR) due to galactose- α 1,3-galactose (α -Gal) after pig-to-human or non-human primates transplantation, α -1,3-galactosyltransferase knock out (GTKO) pigs have been used as a source of liver grafts. However, xenogeneic rejection remains uncontrollable because of non- α -Gal antigens, even when using GTKO pig liver

grafts.¹⁰ Whether by xenotransplantation or tissue-engineering, development of transplantable liver grafts remains difficult.

Another approach is to use the xenogeneic animal as an *in vivo* bioreactor. This *in vivo* bioreactor concept has attracted attention from the field of regenerative medicine. Hata et al reported that they generated mice with chimeric livers with a rat-origin hepatocyte.¹¹ An additional emerging perspective is Yamaguchi et al reported the possibility of humanized organs in pigs using blastocyst complementation.¹² Additionally, Wu et al reported that human pluripotent stem cells (PSCs) robustly engrafted into both cattle and pig pre-implantation blastocysts but showed limited chimeric contribution to post-implantation pig embryos.¹³ However, there has been a lot of ethical argument against the development of chimeric embryos.

Accordingly, we intended to promote regeneration of a tiny liver graft in a xenogeneic animal as an *in vivo* bioreactor, which is completely novel idea for regeneration before LT. In this study, we aimed to investigate the possibility of liver graft regeneration using a xenogeneic animal as an *in vivo* bioreactor. For this, we used a hamster as the donor and a nude rat as the recipient in a newly developed auxiliary xenogeneic partial liver transplantation (AxPLT) technique.

2 | MATERIALS AND METHODS

2.1 | Animals

We used 10 male Syrian hamsters (8-10 weeks old; weighing 100-140 g, Japan SLC, Inc) as donors and 10 inbred male F344/NJcl-rnu/rnu nude rats (9-11 weeks old; weighing 180-230 g, CLEA Japan Inc) as recipients (Figure 1A). These animals were housed under standard animal laboratory conditions (constant environment, 12-hour/12-hour light-dark cycle) with activity freedom, and unlimited access to water and chow. All animal experiments were approved by the animal research committee of Kyoto University (Medkyo-16125)

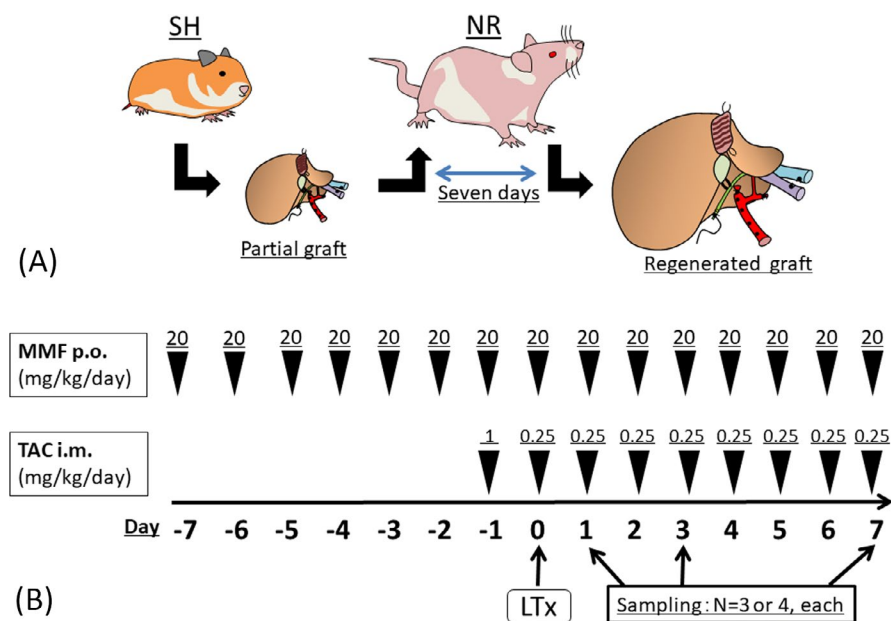


FIGURE 1 A, Experimental protocol used for liver transplantation. Male Syrian hamsters were used as donors and male nude rats as recipients ($n = 10$ and 10 , respectively). B, Immunosuppressive protocol for nude rats.

Immunosuppressive protocols with TAC and MMF were used. Abbreviations: i.m., intramuscularly; MMF, mycophenolate mofetil; NR, nude rat; p.o., per os; SH, Syrian hamster; TAC, tacrolimus

and all animals were cared for in accordance with Guide for the Care and Use of Laboratory Animals from the National Institutes of Health.

2.2 | Experimental design

Auxiliary xenogeneic partial liver transplantation (AxPLT) was performed with partial liver grafts procured from Syrian hamsters

(Figure 1A; Movie S1). Rats were sacrificed on post-operative day (POD) 1 ($n = 4$), 3 ($n = 3$), and 7 ($n = 3$) to evaluate graft liver injury, rejection, and regeneration.

2.3 | Immunosuppressive protocols

Immunosuppressive protocols with tacrolimus (TAC, Prograf; Astellas) and mycophenolate mofetil (MMF, CELLCEPT; Chugai) were used. The

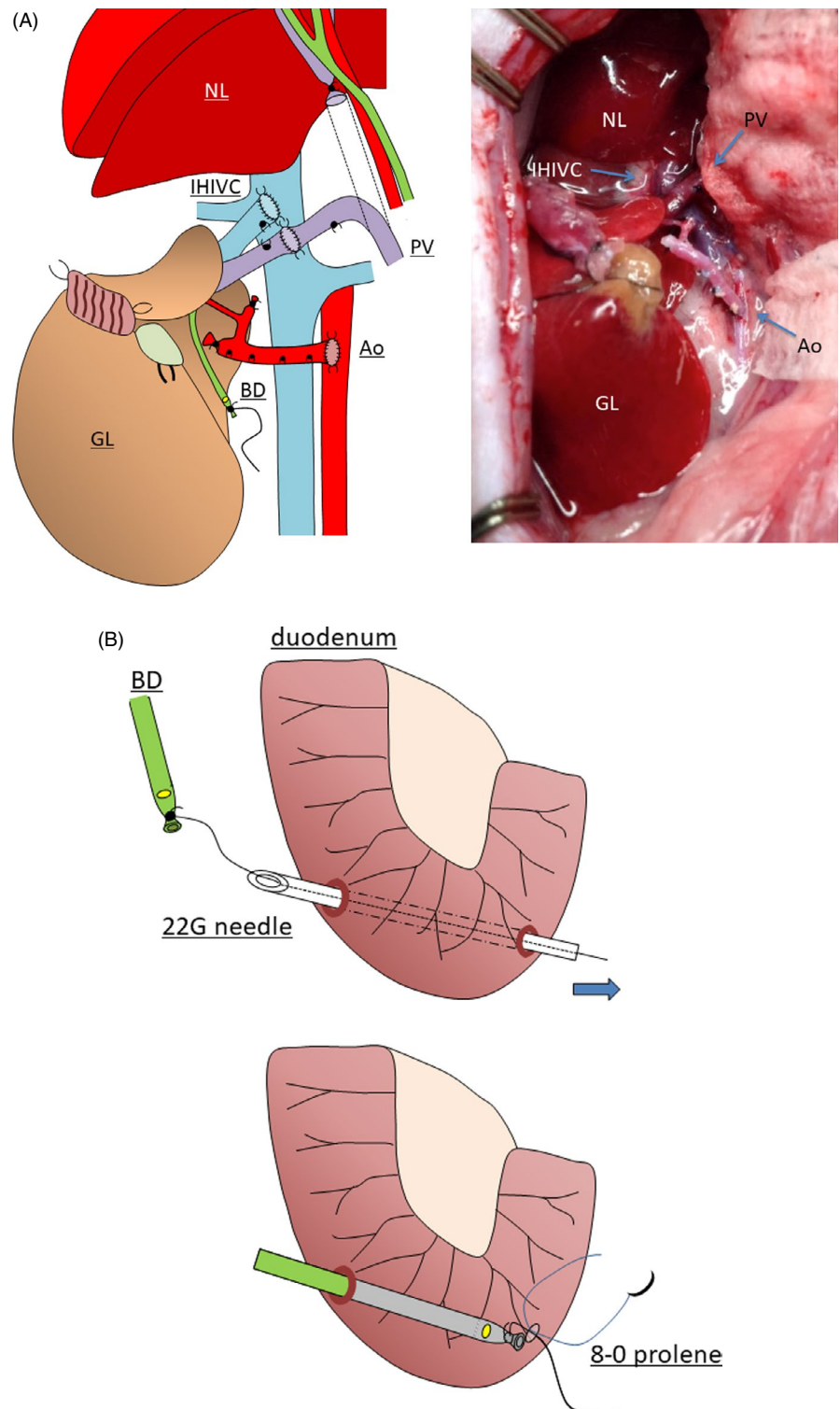


FIGURE 2 A, Images and scheme of auxiliary xenogeneic partial liver transplantation (AxPLT) just after vessel reconstructions. B, Bile duct reconstruction. Abbreviations: Ao, aorta; BD, bile duct; GL, graft liver from hamster; IHIVC, infrahepatic inferior vena cava; NL, native liver of nude rat; PV, portal vein

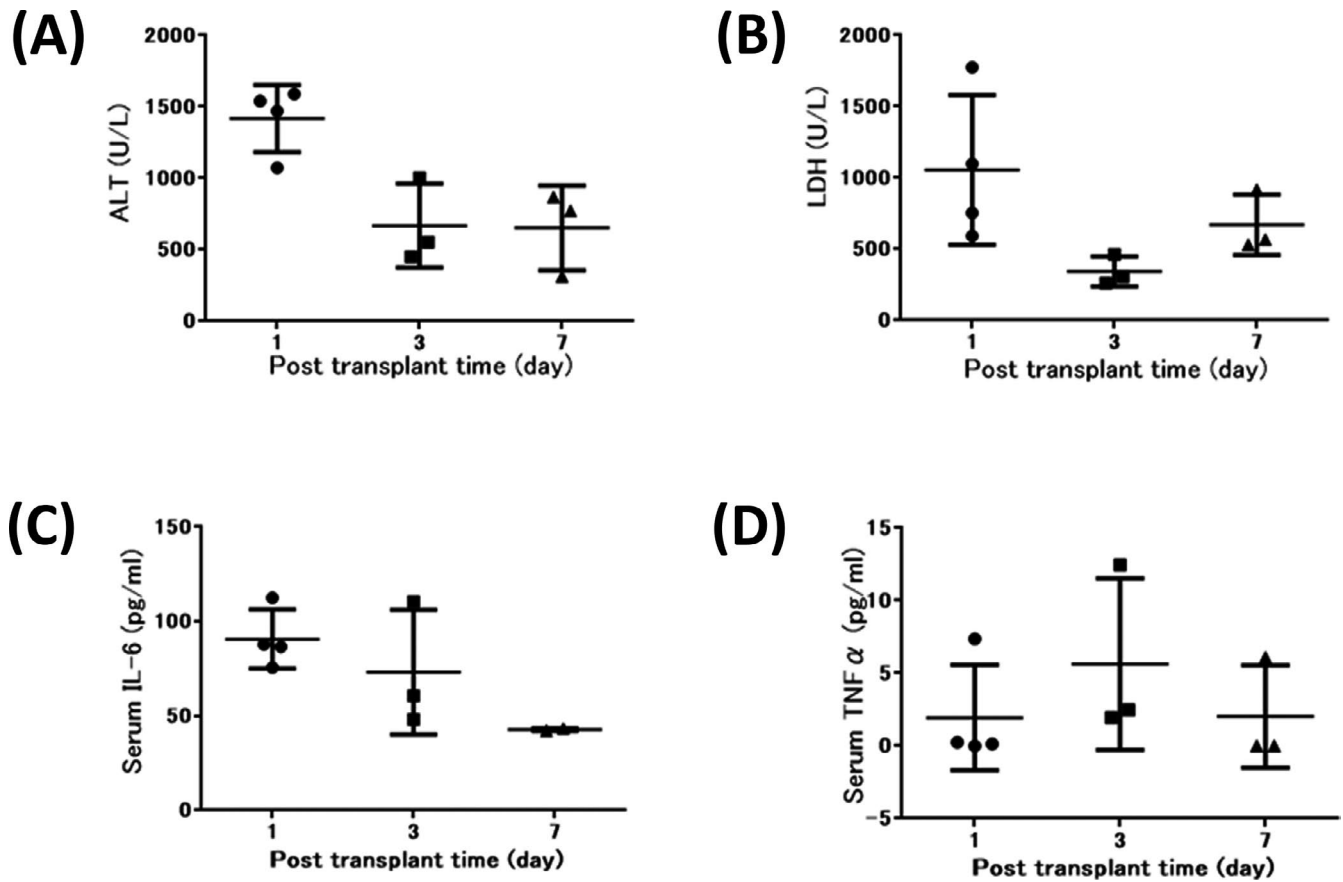


FIGURE 3 Change of serum ALT, LDH, IL-6, and TNF-α. A, ALT. Kruskal-Wallis test: $P = .0171$. B, LDH, C, IL-6, and D, TNF-α

nude rats were treated with the following protocol: TAC was injected intramuscularly into the hind legs at a dose of 1 mg/kg/d 1 day before LT and at a dose of 0.25 mg/kg/d from just before LT until sacrifice. MMF was also administered by gavage at a dose of 20 mg/kg/d from 7 days before LT until sacrifice once a day (Figure 1B).

2.4 | Anesthesia

We anesthetized each hamster and rat with inhalation of 5% isoflurane (Escain; Mylan) in air, and then intubation was performed by inserting a 16-gauge catheter (16G SURFLO intravenous catheter; Terumo). The

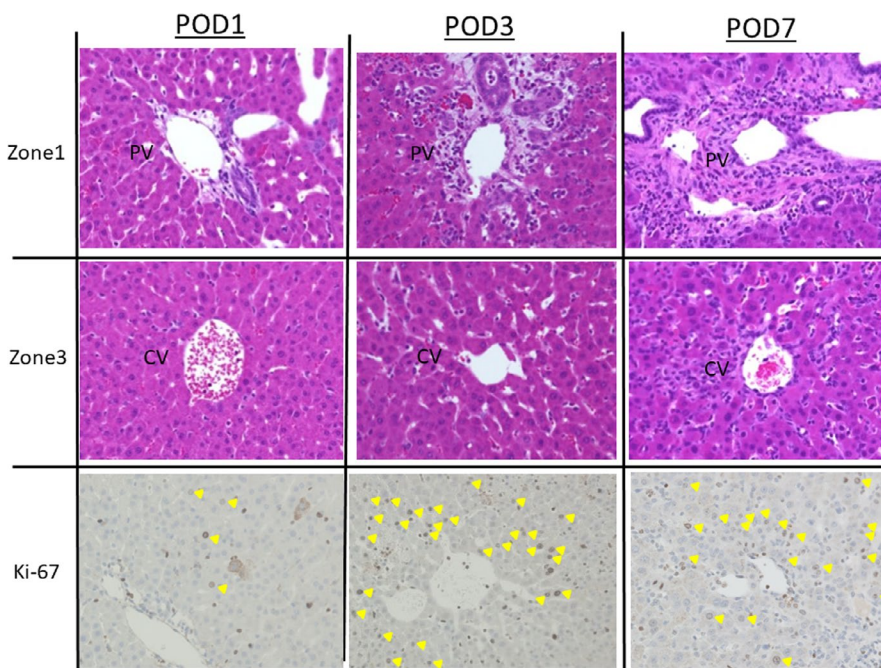


FIGURE 4 Histopathological changes of hamster's liver graft (HE and Ki-67 staining). Yellow arrowheads show the Ki-67 positive cells

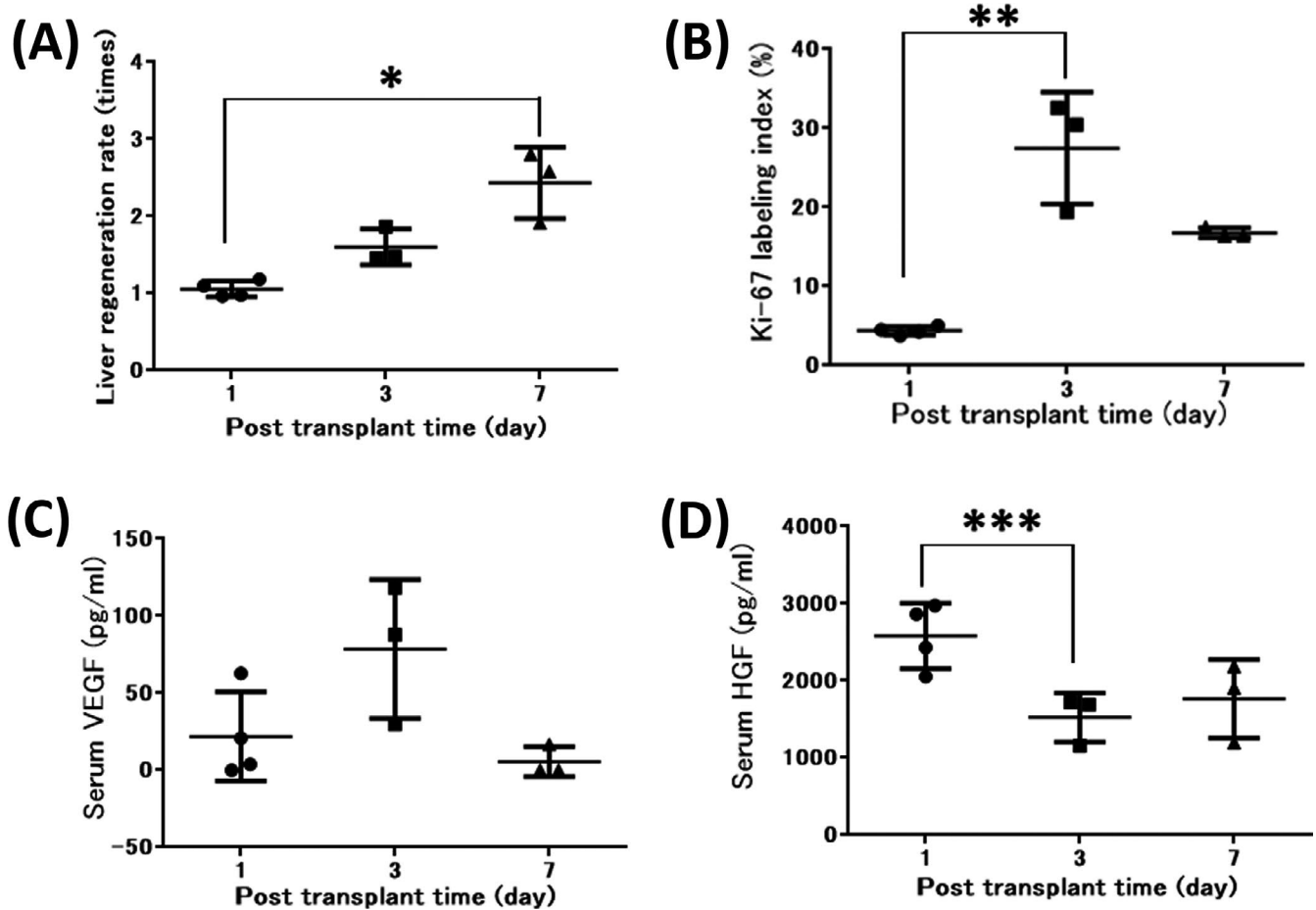


FIGURE 5 Liver graft regeneration. A, Liver graft regeneration rate; graft liver weight at sacrifice/graft liver weight pre-transplant. Kruskal-Wallis test; $P = .0014$; *Post-test $P < .05$, POD1 vs POD7. B, Ki-67 labeling index. Kruskal-Wallis test; ** $P = .0014$; post-test $P < .05$, POD1 vs POD3. C, Serum VEGF. D, Serum HGF. Kruskal-Wallis test; $P = .0248$; ***Post-test $P < .05$; POD1 vs POD3. Abbreviations: AxPLT: auxiliary xenogeneic partial liver transplantation

animals were kept on a ventilator (SN-480-7; Shinano) throughout the operation with inhalation of 1.5%-3% isoflurane in air.

2.5 | Hamster liver procurement

After midline laparotomy followed by bilateral subcostal incisions, the liver was carefully mobilized from all ligamentous attachments. The cystic duct was ligated with 8-0 polypropylene, and then cholecystectomy was performed. The common bile duct was ligated with 6-0 silk thread and cut to create an opening for bile drainage. The subphrenic vein, right renal artery and vein, splenic vein, pyloric vein, and gastroduodenal artery were ligated with 8-0 silk thread and divided. The hamster was heparinized with 200 IU of heparin (Mochida Pharmaceutical Co., Ltd.). The infra-renal abdominal aorta was ligated and a 20-gauge catheter (20G SURFLO intravenous catheter; Terumo) was inserted, followed immediately by opening of the diaphragm, division of the thoracic suprahepatic inferior vena cava (SHIVC), cross-clamp of the aorta, and washout with 20 mL histidine-tryptophan-ketoglutarate (HTK) solution. After perfusion, the abdominal aorta and its tributaries were ligated and divided.

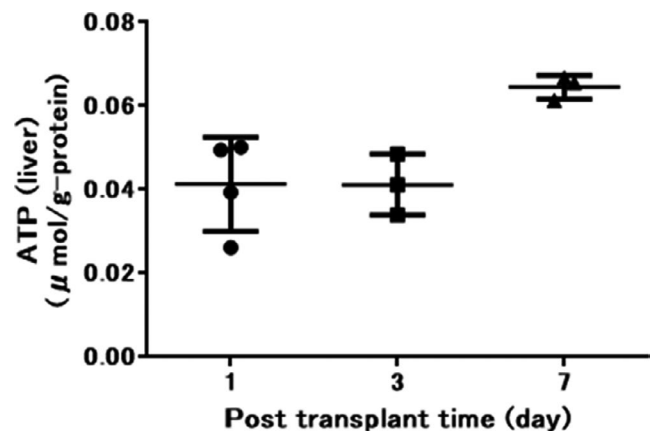


FIGURE 6 Change of liver graft ATP level after auxiliary xenogeneic partial liver transplantation. ATP levels improved during 7 d after AxPLT, (Kruskal-Wallis test; * $P = .0457$)

The thoracic aorta was cut sharply just below the cross-clamp. The liver was then removed and transferred into a back-table on ice. The SHIVC was ligated and the diaphragm was removed. Thereafter, the left middle lobe and left lateral lobe were ligated with 4-0 silk

threads and removed. Accordingly, the 50%-reduced partial liver graft was prepared.

2.6 | Hamster to rat auxiliary xenogeneic partial liver transplantation

In this study, partial liver grafts of hamsters were transplanted into rats in an auxiliary manner with arterial reconstruction. After laparotomy, the pyloric vein was ligated and divided and the portal vein (PV) was isolated. All intestinal tracts were moved out of abdominal cavity. Just after clamping the infrahepatic inferior vena cava (IHIVC) at the level of the right renal vein, the graft was placed above the right kidney of the recipient. The donor IHIVC was connected by an 8-0 polypropylene continuous suture to the recipient IHIVC with end-to-side anastomosis. Then, the PV of the recipient was ligated completely and divided, and was connected by a 10-0 polypropylene continuous suture to the donor PV using an end-to-end fashion; total portal venous flow of the recipient entered the hamster liver graft. The graft aorta was connected with a 10-0 polypropylene continuous suture to the recipient aorta by end-to-side anastomosis (Figure 2A). As shown in Figure 2B, the bile duct was reconstructed using choledochoduodenostomy. A 22-gauge needle was inserted into the duodenum and exited from the duodenum lumen 1 cm proximal to the previous puncture. Silk thread, which was used for common bile duct ligation, was introduced into the needle from the proximal side to the distal side. When the thread was pulled, the bile duct was pulled into the duodenum lumen. The needle was removed and the distal small hole was closed with 8-0 polypropylene sutures. The silk thread and this 8-0 polypropylene suture were tied to prevent bile leakage.

2.7 | Evaluation of liver injury

We collected blood samples from rats at POD1, 3, and 7. Serum samples were stored at -80°C , and then serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (T-Bil), and lactate dehydrogenase (LDH) levels were analyzed using the standard spectrophotometric method with an automated clinical analyzer (JCA-BM9030; JEOL Ltd.).

2.8 | Histopathology of the native rat's liver and hamster's liver graft

For histological examinations, liver tissue specimens from each group were fixed in buffered formalin and embedded in paraffin, then cut and stained with hematoxylin and eosin (HE) and used for microscopic observations. Tissue sections were evaluated by three independent investigators including an expert pathologist of liver transplantation (HH).

2.9 | Liver graft regeneration rate

The liver graft regeneration rate was calculated as graft liver weight at sacrifice/graft liver weight at pre-transplant.

2.10 | Immunohistochemistry for the Ki-67 Labeling Index of Hamster's Graft Liver

To elucidate cell proliferation, we investigate the Ki-67 labeling index, which indicates DNA synthesis in the cell cycle. Deparaffinized hepatic sections (4- μm thickness) were stained for Ki-67 using the Ki-67 antibody (ab15580; Abcam) and the EnVision + System Horseradish Peroxidase Kit (Dako). Immunoreactivity was detected with a diaminobenzidine substrate kit (liquid 3,3'-diaminobenzidine Substrate-Chromogen System; Dako), and the sections were counterstained with hematoxylin. The high-power fields ($\times 200$) were randomly selected, and the Ki-67 labeling index (%) was calculated in accordance with the percentage of positive cells.

2.11 | Evaluation of cytokine and growth factor production

Serum concentration of interleukin-6 (IL-6), tumor necrosis factor α (TNF- α), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) were determined with R&D Systems Quantikine ELISA Kits (catalogue numbers R6000B, RTA00, RRV00, MHG00, respectively; R&D Systems) according to the manufacturer's protocol.

2.12 | Tissue ATP concentrations

Just after sacrifice of the recipient rats, liver tissue samples were snap-frozen in liquid nitrogen and preserved below -80°C until analysis. Tissue adenosine triphosphate (ATP) concentrations were determined by the luciferase method with an ATP bioluminescence assay kit (Tokyo B-Net Co., Ltd.). The results were normalized to protein concentrations, measured by a BCA protein assay kit (Thermo Fisher Scientific KK).

2.13 | Statistical analysis

All data are expressed as the mean $\pm$ standard error of the mean (SEM). The statistical analyses between time points were tested using Kruskal-Wallis test and Bonferroni's post-hoc correction. When applicable, Mann-Whitney's *U* test was performed. A *P*-value of $<.05$ was considered statistically significant. All statistical analyses were performed using Prism 6 (GraphPad Software, Inc).

3 | RESULTS

All rats survived until sacrifice on POD1, 3, and 7.

3.1 | Serum AST, ALT, T-Bil, LDH, IL-6, and TNF- α

As shown in Figure 3, the serum ALT levels at POD1, 3, and 7 were 1471 ± 118 U/L, 669 ± 169 U/L, and 651 ± 171 U/L, respectively. Serum ALT levels significantly decreased during 7 days after

transplantation ($P = .0171$). Serum LDH and AST levels decreased at POD3 and 7; however, there were no significant difference. Serum T-Bil level was 0.2 mg/dL or less during the 7 days after AxPLT (data not shown). Serum IL-6 concentration gradually decreased until POD7. Serum TNF- α concentration was slightly elevated at POD3 and gradually decreased at POD7.

3.2 | Histopathological analysis and graft rejection

Histopathological changes during the 7 days after AxPLT are shown in Figure 4. No portal, bile duct, or venous endothelial inflammation was found at POD1. Mild periportal edema, slight bile duct inflammation, and venous endothelial inflammation emerged on POD3. There was no venous endothelial inflammation but there was some perivenular hepatocyte necrosis on POD7, which indicated moderate acute cellular rejection. However, there was no focal necrosis of hepatocytes in any specimen.

3.3 | Hamster's liver graft regeneration

The liver regeneration rates of the hamster's liver graft in the rat on POD1, 3, and 7 are shown in Figure 5A. The liver regeneration rates on POD1, 3, and 7 were 1.06 ± 0.10 , 1.54 ± 0.23 , 2.54 ± 0.43 , respectively. The liver regenerating rate at POD7 exhibited significantly higher than POD1 (Kruskal-Wallis test; $P = .0014$; post-test $P < .05$, POD1 vs POD7). A representative liver section stained by Ki-67 was shown in Figure 4. The Ki-67 labeling index on POD1, 3, and 7 are shown in Figure 5B. The Ki-67 labeling index on POD1, 3, and 7 were $4.40 \pm 0.26\%$, $27.47 \pm 4.08\%$, $16.78 \pm 0.36\%$, respectively. The Ki-67 labeling index on POD3 was significantly higher on POD3 than at POD1 (Kruskal-Wallis test; $P = .0014$; post-test $P < .05$; POD1 vs POD3).

Serum VEGF and HGF levels were elevated on POD1 and 3 (Figure 5). Serum VEGF at POD1, 3, and 7 was 21.9 ± 14.4 pg/mL, 78.6 ± 26.0 pg/mL, and 5.6 ± 5.6 pg/mL (Figure 5C). Serum HGF at POD1, 3, and 7 was 2581 ± 212 pg/mL, 1525 ± 183 pg/mL, and 1764 ± 294 pg/mL (Figure 5D). Serum value of HGF was significantly higher on POD1 (Kruskal-Wallis test; $P = .0248$; post-test $P < .05$; POD1 vs POD3).

3.4 | Liver graft viability

As a parameter for functional recovery of the hamster's liver graft viability, we measured tissue ATP concentrations at POD1, 3 and 7. ATP levels improved during the 7 days after AxPLT; ATP level on POD7 (0.066 ± 0.002 $\mu\text{mol/g-albumin}$) was higher than on POD1 (0.044 ± 0.006 $\mu\text{mol/g-albumin}$) or POD3 (0.041 ± 0.004 $\mu\text{mol/g-albumin}$) (Kruskal-Wallis test; $P = .0457$; Figure 6).

4 | DISCUSSION

The results of this study revealed that under the appropriate immunosuppressive therapy, small liver grafts regenerated sufficiently in

a xenogeneic in vivo bioreactor without hepatocyte necrosis related to hyper-acute rejection. As far as we know, this is the first report to investigate the possibility of small liver grafts to regenerate in a xenogeneic animal, which has fewer ethical concerns compared with developing of chimeric embryos.

We established AxPLT based on auxiliary whole liver transplantation model published by Lee et al and Ishikawa et al.^{14,15} We transposed recipient's portal vein to the graft, which enabled the portal blood to completely flow into the graft. Yabe et al and Rela et al reported one of the problems of auxiliary partial orthotopic liver transplantation is that portal blood is difficult to flow into the graft and that it requires a long period for liver regeneration.^{16,17} Based on this problem, "portal blood steal", we therefore changed the whole portal blood flow into the graft.

Liver regeneration after the loss of hepatic tissue is a fundamental parameter of liver response to injury. Many growth factors and cytokines, such as IL-6, TNF- α , HGF, or VEGF, play important roles in liver regeneration.² Several authors reported that liver regeneration was completed by 5-7 days after partial liver graft transplantation with a peak level of the bromodeoxyuridine, proliferating cell nuclear antigen, or Ki-67 labeling index at 1-3 days after operation in rodents.^{7,18-20} However, our experiment was the first trial to evaluate the possibility of liver regeneration in xenogeneic animals. The results of Ki-67 labeling index suggested that liver regeneration of xenogeneic liver peaked on POD3 and continued even after POD7. The Ki-67 labeling index on POD7 was higher when compared with the result of syngeneic rat to rat liver transplantation.^{7,19} This was in part related to our result of the serum level of HGF on POD7, which was still slightly higher as a result of the persistence of possible mild liver injury which could be due to the rejection. Generally, the serum levels of HGF increase immediately after partial liver resection or partial liver transplantation and after gradually decrease.²¹ HGF elevation does not only indicate the liver regeneration but also liver injury.²¹ VEGF plays a major role in angiogenesis which is essential for healing of injured tissue and VEGF expression reaches a maximal level 48-72 hours after liver injury.²²

In our study, regarding the liver graft regeneration, not only did the graft weight increase by about 2.4 times after 7 days after liver transplantation, but also the Ki-67 labeling index markedly increased at POD3 with restoration of ATP at POD7 compared with POD1 or 3 with histological graft integrity. These findings indicated that the regenerated liver graft would be transplantable to an allogeneic hamster.

In our preliminary study I, we chose to perform Lewis to Lewis rat auxiliary 20% partial liver transplantation (APLT; Figure S1A). We showed that the graft weight increased from 3.1 to 6.8 g, during 7 days after transplantation (Figure S1B). Therefore, we chose this APLT model for our thesis. Previous papers have shown that the age of the donors and the recipients,²³ and the regenerative effects of TAC^{24,25} have some influence on the regenerative ability in allogeneic partial liver transplantation. Further studies are needed to compare the regenerative ability of xenogeneic liver graft and that of the syngeneic graft.

Although the combination of hamster to rat is concordant,²⁶ appropriate immunosuppressive therapy remains crucial for the success of liver transplantation. The athymic homozygous nude rat is T-cell-deficient and shows depleted cell populations in the thymus-dependent areas of peripheral lymphoid organs. Although it lacks T cells, the nude rat has a normal complement of bone-marrow dependent B cells.²⁷ Untreated nude rats reject hamster heart grafts at the same 3 days as in normal rats, in accordance with a T-cell independent mechanism of humoral xenograft rejection.²⁸⁻³⁰ Furthermore, we evaluated optimal immunosuppression in hamster to rat AxPLT in our preliminary study II. Firstly, we performed the hamster to wild Lewis rat AxPLT using TAC and Endoxan. Although the wild rats treated with TAC and Endoxan survived until POD7, severe rejection observed in the liver graft (Figure S2A,B). Accordingly, we changed from wild Lewis rats to nude rats. We evaluated two doses of MMF in the hamster to nude rat AxPLT, 20 mg/kg/d: day-2 to 7, and 20 mg/kg/d: day-7 to 7. The histology of the liver graft at POD7 showed also rejection but it improved in the latter group (MMF day-7 to 7) rather than in the former group (MMF day-2 to 7) (Figure S2C-F). However, since rejection still remained, additional administration of TAC was considered. Mollevi et al³¹ reported the immunosuppressive treatment with MMF plus TAC. They used male Lewis rats and Syrian hamsters as recipients and donors, respectively, for heart transplantation and orthotopic liver transplantation. Their immunosuppressive protocol was the combination of TAC and MMF. TAC was injected at a dose of 0.2 mg/kg/d intramuscularly for 31 days and MMF was administered at a dose of 25 mg/kg/d for 8 days. In their research, all the recipients survived over 50 days.³¹ Therefore, we modified this immunosuppressive regimen in our study. The mechanism that TAC suppressed the xenograft rejection in nude rats of our experiment is enigmatic. One possibility for this mechanism could be that TAC impairs Toll-like receptor function after xenogeneic liver transplantation.³² Although mild rejection was detected in liver grafts at POD7, it was well-controlled, and the regenerated liver grafts were thought to be transplantable to the next recipient.

There are some limitations to our study. Firstly, we have not shown the data of antibodies related to this rejection. However, it is the first step and the most important information in this study to show the increased graft volume with increased Ki-67 index, histological integrity and tissue viability in xenogeneic environment. Secondary, hamster and rat are phylogenetically closer species than human and pig. Thirdly, to apply this concept to humans, we have many problems to solve, such as ethical issues, infection, and immunosuppression. Fourthly, we did not re-transplant the explanted grafts after removal from the bioreactor because of severe adhesion in the small abdominal cavity.

Currently, based on the results of this rodent model experiment, we have begun planning a large animal experiment using an operational SCID pig. In large animals, we may achieve regenerated graft procurement even with severe adhesion around the transplanted liver. Hsu et al³³ reported enhancing the survival of human hepatocytes in an operational SCID pig. The final goal of our research is to regenerate small human liver grafts in operational SCID pigs and to

transplant these regenerated liver grafts into humans again. This is a challenging and completely novel idea.

In conclusion, we demonstrated that small liver grafts could regenerate even in xenogeneic animals without hyper-acute rejection. Our results suggest that liver grafts regenerated in a xenogeneic environment such as an in vivo bioreactor are potentially suitable as transplantable liver grafts for humans.

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CONFLICT OF INTEREST

The authors of this manuscript have no conflict of interest to disclose as described by the *Xenotransplantation*.

AUTHOR CONTRIBUTION

Yuki Masano and Shintaro Yagi participated in research design, performance of the research, data analysis, and writing of the manuscript. Yosuke Miyachi and Shinya Okumura participated in performance of the research and data analysis. Hironori Haga participated in evaluation and writing of the histopathological section. Toshimi Kaido, Eiji Kobayashi, and Shinji Uemoto participated in research design and the writing of the manuscript.

ORCID

Yuki Masano  <https://orcid.org/0000-0003-0048-794X>

Shintaro Yagi  <https://orcid.org/0000-0001-7465-5761>

Eiji Kobayashi  <https://orcid.org/0000-0001-8617-3778>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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