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**Construction of Liver Tissue in vivo with Preparative Partial Hepatic Irradiation and Growth Stimulus: Investigations of Less Invasive Techniques and Progenitor Cells.**

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## Abstract

**Background:** The selective proliferation of transplanted hepatocytes by providing a growth stimulus, such as partial hepatectomy or hepatocyte growth factor, concomitant with hepatic irradiation (HIR) that can suppress proliferation of host hepatocytes was reported. We have conducted experiments which focus on I) less invasive and clinically applicable techniques and II) progenitor cells.

**Materials and methods:** I) Dipeptidyl-peptidase IV (DPPIV)-F344 or jaundiced Gunn rats received partial HIR (only 30% of whole liver) and portal vein branch ligation (PVBL) of one lobe, followed by intrasplenic hepatocyte transplantation at  $1 \times 10^7$ .

II) After partial HIR+PVBL, two kinds of progenitor cells were transplanted, i.e., small hepatocytes (SHs) or adipose derived mesenchymal stem cells (ADMSCs).

**Results:** I) Sixteen weeks later, the donor cells constituted more than 70% of the hepatocytes of the irradiated lobe, showing connexin 32, PCK-1 and glycogen storage. Moreover, the serum bilirubin level decreased significantly in jaundiced Gunn rats and remained at this level throughout the 24-week experimental period.

II): SHs grew more quickly than hepatocytes, and after 8 weeks, around 40% of host hepatocytes were replaced with transplanted SHs. 2) Although donor cells were engrafted after 8 weeks, their proliferation was not observed.

**Conclusion:** HIR can be given to a selective lobe of liver and is a low-invasive and effective means for transplanted hepatocytes to proliferate combined with PVBL. Even a

smaller number of SHs can construct liver tissue with their prevailing proliferative ability.

Key words: hepatocyte transplantation, hepatic irradiation, portal vein branch ligation, small hepatocyte, adipose derived mesenchymal stem cell

## Introduction

Hepatocyte transplantation (HT) has been performed in both animal experiments and in patients with hepatic inherited disorders (1-4). Because the number of hepatocytes transplanted safely at one time is limited due to the occurrence of portal hypertension, transplanted hepatocytes need to proliferate in the host liver to completely correct the underlying disorder (5, 6). Selective proliferation of transplanted hepatocytes by providing a growth stimulus by partial hepatectomy of the host liver, combined with hepatic irradiation (HIR) of the whole liver that can suppress proliferation of host hepatocytes, and consequently, almost the whole liver would be constructed with donor hepatocytes (7). Moreover, jaundiced Gunn rats of Crigler-Najjar syndrome type I model, which lack hepatic uridinediphosphoglucuronate glucuronosyltransferase (UGT1A1) activity, had UGT1A1 protein and enzymatic activity in the liver, and normalization of serum bilirubin levels (8). Although these results are very attractive, hepatectomy is too invasive to be carried out in patients. Instead of hepatectomy, Fas-mediated apoptosis of host hepatocytes (9) or administration of a recombinant adenovirus vector expressing hepatocyte growth factor (HGF) (10, 11) has been successful in inducing massive repopulation of transplanted hepatocytes. However, these strategies appear difficult to implement clinically because of potential toxicity.

Irradiation-induced apoptosis of hepatocytes and sinusoidal endothelial cells within 24 hours after HIR improved by one week (12). Chronically, radiation-induced liver disease (RILD), characterized by histopathologic features of veno-occlusive disease, could occur within several months after hepatic radiation therapy, and the patient, with irradiation of an extended liver volume, would show fatigue, weight gain, hepatomegaly, and ascites (13). Recently, Zhou et al. reported single liver lobe repopulation with transplanted hepatocytes cured jaundice in Gunn rats using preparative regional HIR, in order to reduce the susceptibility to RILD (14). They injected an adenovirus vector expressing HGF to provide a proliferative stimulus. Previously, as potential clinically applicable methods, Eguchi et al. demonstrated that regenerative stimulus by portal vein branch ligation (PVBL) induced proliferation of transplanted hepatocytes and enhanced the therapeutic efficacy of HT in hyperlipidemic rabbits (15). In clinical settings, a portal vein occlusion can be made by embolization with radiological intervention. This is often used as a preparation to elicit liver hypertrophy in the nonembolized lobes with liver atrophy in the embolized lobes in the patient who has a scheduled extended hepatectomy (16). We addressed the partial HIR of single lobe combined with PVBL, as preparation for the induction of selective proliferation of transplanted hepatocytes.

Another essential obstacle to clinical HT is the shortage of donor hepatocytes. In

the present study, we investigated the utility of small hepatocytes (SHs), which are known as progenitor cells of hepatocytes in the liver, and have a significant ability to proliferate both in vitro (17) and in vivo (18) in rat. In addition, we tried stem cell transplantation derived from adipose tissue. Adipose derived mesenchymal stem cells (ADMSCs) have advantages in terms of minimal ethical issues and easy accessibility compared with other kinds of stem cells, such as bone marrow-derived or ES cells.

## Materials and Methods

### Animals

Male wild-type F344 rats and dipeptidyl peptidase (DPP) IV deficient (DPPI-) F344 rats weighing 200-250 g were obtained commercially (Japan SLC, Inc., Hamamatsu, Japan and Charles River Laboratories Japan, Inc., Yokohama, Japan, respectively). Both jaundiced Gunn/Slc-j/j and Gunn/Slc-j/ - (non-jaundiced) were purchased from Japan SLC, Inc. The animals were housed in a temperature-controlled environment with a 12-hr light/dark cycle and had free access to standard rat chow and water. All experiments were performed according to the Guidelines for Animal Experimentation at Nagasaki University.

### D) Experiment to assess the efficacy of preparative partial HIR+PVBL

### Isolation of hepatocytes

The cells were isolated with a modified collagenase perfusion method using male wild-type F344 rats or non-jaundiced Gunn/Slc-j<sup>-</sup> rats, as originally described by Berry and Friend (19). After liver dissociation, the cells were filtered through a 70- $\mu$  m Cell Strainer (Becton, Dickinson and Company, NJ, USA) and washed twice at 50 $\times$ g for 2 min each. Cell viability was determined by trypan blue dye exclusion. Hepatocytes above 80% viability were used for transplantation.

### Partial hepatic irradiation and portal vein branch ligation

Anesthesia was induced by ether inhalation and was maintained by the intraperitoneal injection of pentobarbital sodium with recipient animals (DPPIV-F344 or jaundiced Gunn/Slc-j/j rats) and a transverse abdominal incision was made. For partial hepatic irradiation (HIR) to only the left lateral lobe at 50 Gy, lead shields (2 mm thick) were placed to protect the other hepatic lobes and other abdominal organs. A X-ray irradiation device (ISOVOLT TITAN 320; GE Inspection Technologies Ltd., Tokyo, Japan) was used (320 kVP, 5 mA, and 0.5 mm Cu + 0.5 mm Al filtration at dose rate 0.87 Gy/min). Immediately after partial HIR, the portal vein supplying 20% of the liver was ligated to induce growth stimulus (Fig. 1). Thereafter, the abdomen was closed.

### Hepatocyte transplantation

One to 3 days after the preparation, fresh hepatocytes were injected intrasplenically. Hepatocytes at  $1 \times 10^7$  from wild-type F344 rats or non-jaundiced Gunn/Slc-j<sup>-/-</sup> rats were injected in either the anesthetized recipient DPPIV-F344 rats or the jaundiced Gunn rats, respectively.

### Experimental design

#### DPPIV rat experiment

Six rats underwent HT after partial HIR+PVBL. Two rats were killed at 4 and 16 weeks after HT, respectively, and samples were collected for DPPIV histochemistry, PAS stain, and immunohistochemistry.

#### Gunn rat experiment

The rats were divided into three groups according to preparations (six for the non-HT group; only HIR+PVBL without HT, six for the PVBL group; HT following only PVBL, and eight for HIR+PVBL group; HT following HIR+PVBL). Serum was collected from all the rats weekly until 24 weeks after HT to measure the serum bilirubin levels.

#### Identification of transplanted cells by DPPIV histochemistry

DPPIV enzyme activity was detected as previously described (12). Briefly, 5- $\mu$  m thick cryostat sections of the liver were fixed in chloroform and acetone for 10 min at 4° C. The sections were incubated for 30 min at 37° C in a solution containing 0.4 mg/ml glycyl-L-proline-4-methoxy-2-naphthylamide and 1 mg/ml Fast Blue B salt in TMS buffer (pH 6.5). The proportion of repopulated areas by DPPIV positive donor cells to the total hepatic areas in each section was determined using the Photoshop CS5 software program (Adobe Systems Inc., CA, USA).

#### Serum bilirubin concentrations

The serum bilirubin concentrations were determined by enzymatic methods at SRL, Inc. (Tokyo, Japan).

#### Detection of glycogen storage by PAS stain

Tissue glycogen was determined by incubation in 1% aqueous periodic acid for 5 min and Schiff's reagent for another 15 min with cryostat sections fixed with cold methanol (20).

#### Structural and functional investigation by immunohistochemistry

To investigate whether repopulated donor hepatocytes had liver-specific structure

and function, cryostat sections fixed with cold acetone were stained using the mouse monoclonal anti-connexin 32 antibody (Abcam plc., Cambridge, UK) and the rabbit polyclonal anti-PCK-1 antibody (Abcam plc). The antibody binding was then visualized using an EnVision/HRP (DAKO Japan corp., Tokyo, Japan).

#### Proliferation cell nuclear antigen staining

Proliferation cell nuclear antigen (PCNA) staining was performed to detect DNA synthesis as a marker of hepatocellular mitosis in response to the preparative treatments. Other four DPPIV-F344 rats underwent HIR+PVBL, and 2 rats were killed on days 1 and 2 after the procedures, respectively. Immunohistochemical staining was performed using the mouse monoclonal anti-PCNA antibody (DAKO Japan corp.) and an EnVision/HRP with formalin-fixed paraffin-embedded sections.

#### Serum hepatocyte growth factor levels after HIR+PVBL

To investigate the changes in serum hepatocyte growth factor (HGF) levels, we performed HIR+PVBL without HT in the additional five DPPIV-F344 rats. Serum specimens were collected 1, 3, 7, 14, 21, and 28 days after surgery. The serum levels of HGF were measured using a Quantikine ELISA Mouse/Rat HGF Immunoassay kit (R&D Systems, Inc., MN, USA).

## II) Experiments for progenitor cell transplantation

### Fractions of parenchymal hepatocytes and small hepatocytes

Fractions of parenchymal hepatocytes (PHs) and SHs from wild-type F344 rat liver were obtained as reported by Katayama et al. (18) with some modifications. The pellet obtained by collagenase perfusion and centrifugation at 50×g was additionally centrifuged through 45% Percoll at 50×g for 24 min. The pellet thus obtained was further centrifuged at 50×g for 1 min, and the pellet and supernatant were used as a fraction of PHs and SHs, respectively.

### Isolation of ADMSCs

ADMSCs were isolated from rat peritoneal adipose tissue specimens obtained from wild-type F344 rats as reported previously with some modifications (21). The tissue was rinsed with cold PBS (Life Technologies Japan Co., Tokyo, Japan.) and minced into small fragments with a blade. The fragments were digested with 0.1 % collagenase type I (Life Technologies Japan Co.) in PBS for 60 min at 37° C with vigorous shaking. The top lipid layer was removed and the remaining digest was filtered through a 70- $\mu$ m Cell Strainer and centrifuged at 220×g for 10 min. The pellet was treated with 160 mM NH<sub>4</sub>Cl for 10 min to lyse

the red blood cells. The remaining cells were suspended in Dulbecco's modified Eagle's medium (Life Technologies Japan Co.) supplemented with 10% fetal bovine serum (Life Technologies Japan Co.) and plated at a density of  $1 \times 10^6$  cells in a 10-cm dish. The culture media were changed thrice weekly. The cells were maintained at subconfluent levels and passaged using trypsin/EDTA (Life Technologies Japan Co.).

#### Experimental design

Following HIR+PVBL, SHs or PHs ( $2 \times 10^6$ ), or ADMSCs of P2 to P4 ( $5 \times 10^6$ ) were transplanted to four DPPIV-F344 rats, respectively. They were then sacrificed 8 weeks after HT for DPPIV histochemistry.

#### Statistical analysis

The results are expressed as the mean  $\pm$  standard deviation (SD). Mann-Whitney's U test was used for the comparison of 2 groups. Data were analyzed using the analysis of variance (ANOVA) for multiple comparisons. A p-value of less than 0.05 was considered to be statistically significant.

#### Results

##### Repopulation of transplanted hepatocytes in the irradiated lobes

After HT following preparative partial HIR+PVBL, transplanted hepatocytes grew and, subsequently, showed massive repopulation only in the irradiated lobes after 16 weeks, but did not display a similar behavior in the non-irradiated lobes (Fig. 2A). In addition, immunohistochemical and PAS stains demonstrated expression of connexin 32, PCK-1, and glycogen storage in the repopulated hepatocytes, similar to the native liver (Figure. 2B, C, and D).

#### Changes in the serum bilirubin concentrations

Although the rats in the PVBL group showed significant, but transient decrease in serum bilirubin concentrations at 4 weeks after transplantation, the level recovered to preoperative value at 7 weeks and remained constant during the observation period (24 weeks). In the HIR+PVBL group rats, serum bilirubin levels were reduced by 59.1% at 4 weeks after transplantation and kept this level for the remaining period of the observation (Fig. 3). The animals in the non-HT group did not show any significant changes in their bilirubin levels.

#### Regenerative response after partial HIR+PVBL

DNA synthesis in proliferative hepatocytes was examined using PCNA staining. While in the non-irradiated liver lobe, many cells were PCNA-positive at 2 days after HIR+PVBL; in contrast, very few hepatocytes were PCNA-positive in the

irradiated lobe (Fig. 4). To investigate the relevance of serum HGF with repopulation of transplanted hepatocytes, we measured its concentrations over time after HIR+PVBL by ELISA. Unexpectedly, the serum HGF levels did not increase for at least 4 weeks (Fig. 5).

#### Engraftment and repopulation of SHs and ADMSCs

After 8 weeks of transplantation, a massive hepatic repopulation was acquired in the irradiated lobes with SHs, even with a small number of cells being transplanted initially (Fig. 6A). The transplanted SHs proliferated more rapidly in the irradiated recipient's liver compared with the PHs (38.5% vs. 7.2%,  $p < 0.038$ ) (Fig. 6E). In addition, the repopulated SHs showed storage of glycogen (Fig. 6B). Although ADMSCs seemed to be engrafted in the recipient's liver, no massive proliferation was obtained (Fig. 6C, D).

#### Discussion

HT has been studied as an alternative method to liver transplantation in both the laboratory and clinical setting. However, it has not been prevalent as a standard therapeutic option, mainly because of the limited number of hepatocytes at one time and the shortage of transplantable primary hepatocytes (4, 22). To overcome these hurdles, the methods for growth of transplanted hepatocytes in

the recipient, or the development or availability of substitute for primary hepatocytes, such as xenogeneic hepatocytes, fetal hepatocytes, and hepatic stem cells/progenitor cells have been explored.

The transplanted hepatocytes must grow over host hepatocytes to achieve repopulation in the host liver. Portal vein embolization (PVE) is often used in the patient with a scheduled extended hepatectomy, as a preoperative intervention to elicit liver hypertrophy in the nonembolized lobes (16). PVE is not accompanied by a lack of major tissue loss and we expected that this intervention would be a less invasive substitution for a partial hepatectomy to induce cell repopulation. In this study, we ligated a branch of the portal vein, supplying only one lobe, instead of PVE, because the animals were too small to carry out embolization. We performed partial HIR for 30% of the whole liver in order to induce minimal hepatic injury due to irradiation. As a result, a massive repopulation was obtained only in the irradiated nonligated lobe, but the therapeutic effect was significant, as shown by a reduction in the serum bilirubin levels. The serum bilirubin has been reported to decrease due to auxiliary partial liver transplantation with 12% of the liver in Gunn rat (23), thus suggesting that HIR in even smaller regions of the liver could be sufficient in our model. Preparative PVBL alone, as a control, led to just a transient reduction in bilirubin. There have been some reports in which therapeutic effect by HT with preparative HIR

alone was transient and minimum (8, 9). PVBL could provide a selective growth stimulus to transplanted hepatocytes in combination with HIR, resulted in massive proliferation, and significant and continuous therapeutic effects. Furthermore, the hepatocytes proliferated while retaining normal structure and function, as demonstrated by positive staining of connexin32, PCK-1, and glycogen.

PCNA staining showed that PVBL induced a regenerative stimulus to hepatocytes. We measured the levels of serum HGF, which is known as a strong stimulator for hepatocytes growth. Although supplying HGF via an adenovirus vector was demonstrated to induce proliferation of transplanted hepatocytes (10, 11, 14), PVBL did not increase the levels of HGF, as observed in the clinical PVE study (16). Liver regeneration is regulated by an orchestrated and complex response, involving not only HGF, but also various kinds of mediators. Further studies are needed to clarify the mechanism of proliferation.

Because of a shortage of transplantable primary hepatocytes, we analyzed the ability in engraftment and proliferation of substitutive cells with the partial HIR+PVBL model. SHs are known as progenitor cells in normal liver. As we expected, SHs proliferated in shorter periods than PHs did, due to the hepatic function of glycogen storage. Although, in the present study, we did not assess either the characteristics of repopulated SHs or the therapeutic effects of

SH transplantation, Shibata and Hirata et al. demonstrated the therapeutic effect of transplantation with preparative partial hepatectomy + HIR in analbuminemic rats, comparable to mature hepatocytes (24). SHs are in the non-parenchymal fraction in the liver (17, 18, 24). In most clinical cases, hepatocytes for transplantation must be isolated from cadaver livers that were judged unsuitable for liver transplantation, i.e., macrosteatosis due to nonviral cirrhosis. These livers often have critical problems in the isolation of hepatocytes, such as susceptibility to damage caused by ischemia, which is the major reason for poor viability of cells and which prevents high quality of hepatocytes from being obtained (25). SHs might be useful for additional sources of cells as well as for the alteration of PHs, especially in the marginal livers.

ADMSCs have been demonstrated to differentiate into various types of cells in vitro, including cells with hepatocyte-like forms and functions by adding some humoral factors (26). We transplanted undifferentiated ADMSCs from peritoneal adipose tissue after HIR+PVBL, and did not obtain repopulation, though some cells were engrafted. In the reported study, hepatocyte-like cells differentiated from ADMSCs in vitro, proliferated in the recipient liver with preparation of retrorsine, which blocks the recipient hepatocyte cell cycle, in conjunction with hepatectomy; but, undifferentiated ADMSCs did not (26, 27). In our partial HIR+PVBL model, differentiation in vitro should be required to obtain

repopulation by ADMSCs.

In summary, PVBL, which is applicable as PTE in the clinical setting, was efficient for transplanted hepatocytes to proliferate in combination with HIR. Only partial HIR to small parts of liver should be enough to cure some hepatic metabolic disorders. With this preparative method, SHs proliferated more quickly than PHs and could be a potential source for HT. ADMSCs should be transplanted after differentiation in vitro, and the therapeutic effects on disorders should be evaluated.

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Figure legends

Fig. 1. Partial hepatic irradiation (HIR) and portal vein branch ligation (PVBL)

Fig. 2. DPPIV histochemistry showed massive hepatic repopulation of donor hepatocytes (red) only in the irradiated lobes 16 weeks after HT with preparative HIR and a growth stimulus (PVBL) (A). Repopulated donor hepatocytes expressed connexin 32 (B) and PCK-1 (C), and stored glycogen (D).

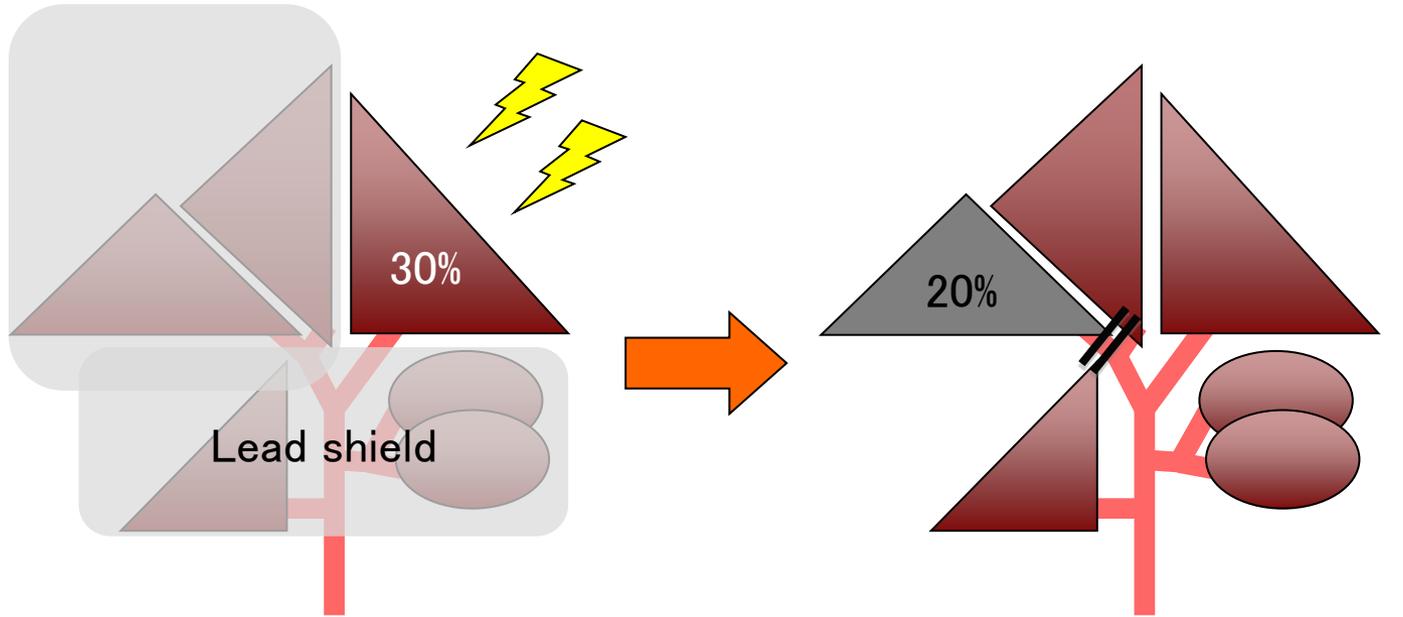
Fig. 3. Serum bilirubin concentrations after hepatocyte transplantation. Only the rats in the HIR+PVBL group (diamonds, n=8) showed a continuously significant decline in serum bilirubin concentrations compared with the other two groups (circles; the non-HT group p=0.002, and triangles; the PVBL group p=0.002, Mann-Whitney's *U* test, n=6 each).

Fig. 4. PCNA staining after partial HIR+PVBL. While many PCNA-positive hepatocytes were seen in the non-irradiated lobe 48 hours after operation, positive cells were rare in the irradiated lobe.

Fig. 5. Serum HGF after PVBL+partial HIR (ELISA). After PVBL+partial HIR, the serum HGF levels did not show significant fluctuations over time (ANOVA, n=3-5 each).

Fig. 6. Recipient livers after 8 weeks of transplantation. Massive hepatic repopulation was acquired after SH transplantation in the irradiated lobes (A, DPPIV histochemistry) and repopulated SHs showed storage of glycogen (B, PAS stain). Although ADMSCs seemed to be engrafted in the recipient liver, repopulation was not acquired (C and D, DPPIV histochemistry). E shows that the ratio of repopulated areas after 8 weeks of transplantation in the irradiated lobes. The transplanted SHs proliferated more rapidly than PHs ( $p < 0.038$ , Mann-Whitney's U test, n=4 each).

Fig. 1



Partial hepatic irradiation (HIR)  
to only the left lateral lobe

Ligation of the portal vein  
supplying 20% of the liver  
to induce growth stimulus

Fig. 2

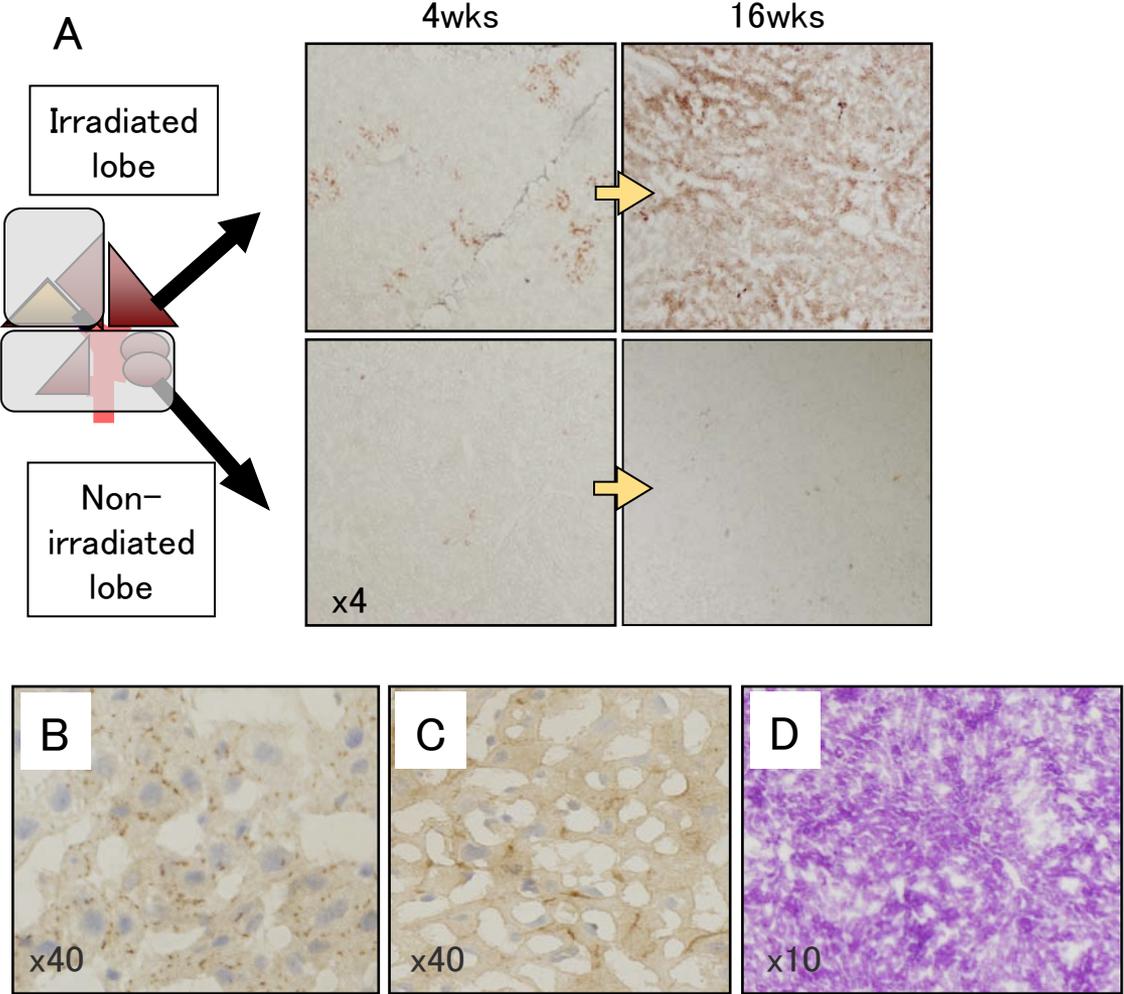


Fig. 3

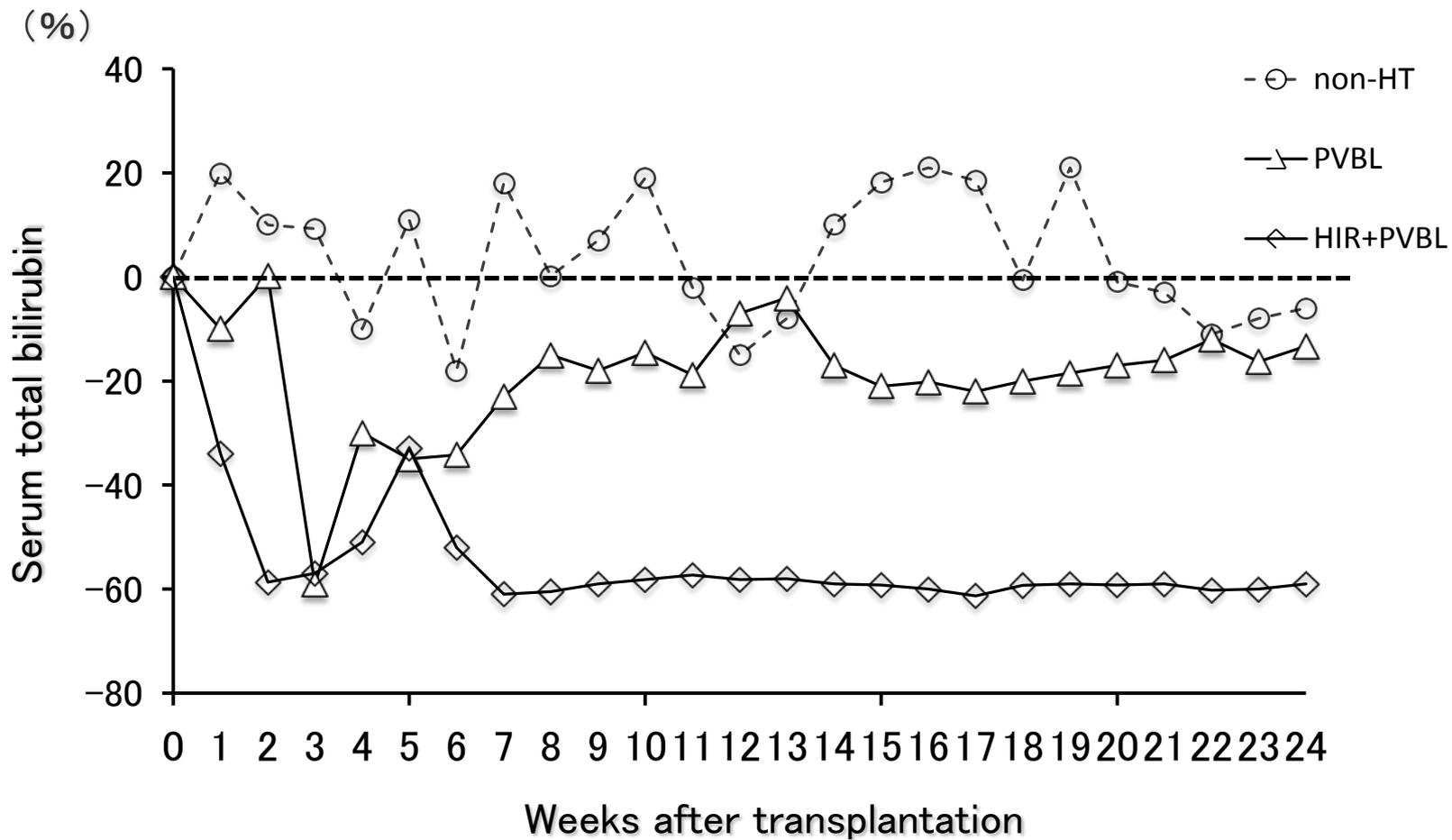


Fig. 4

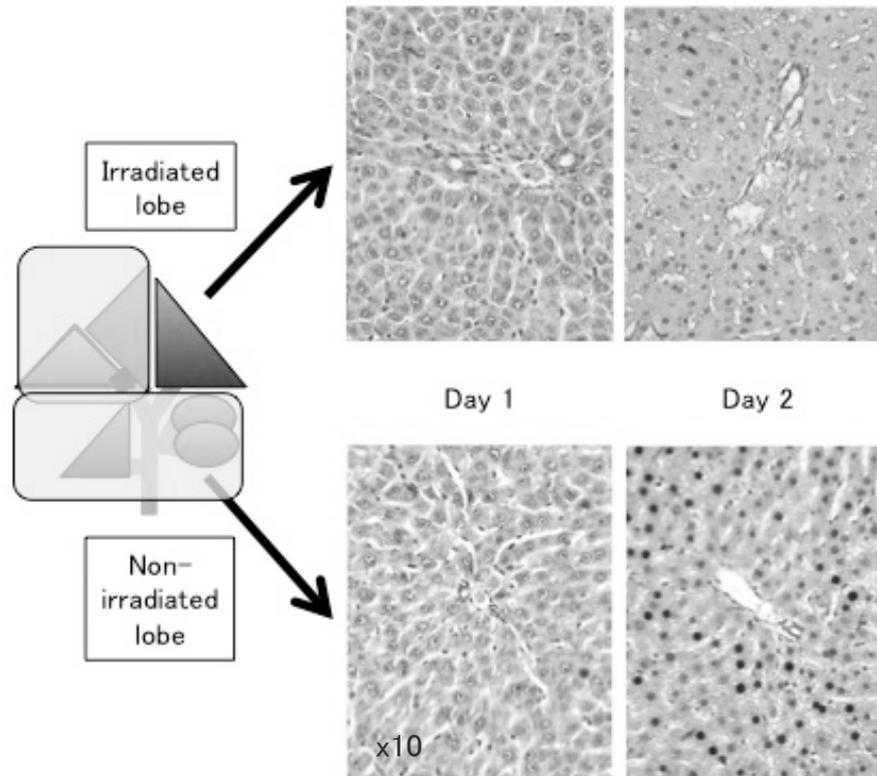


Fig. 5

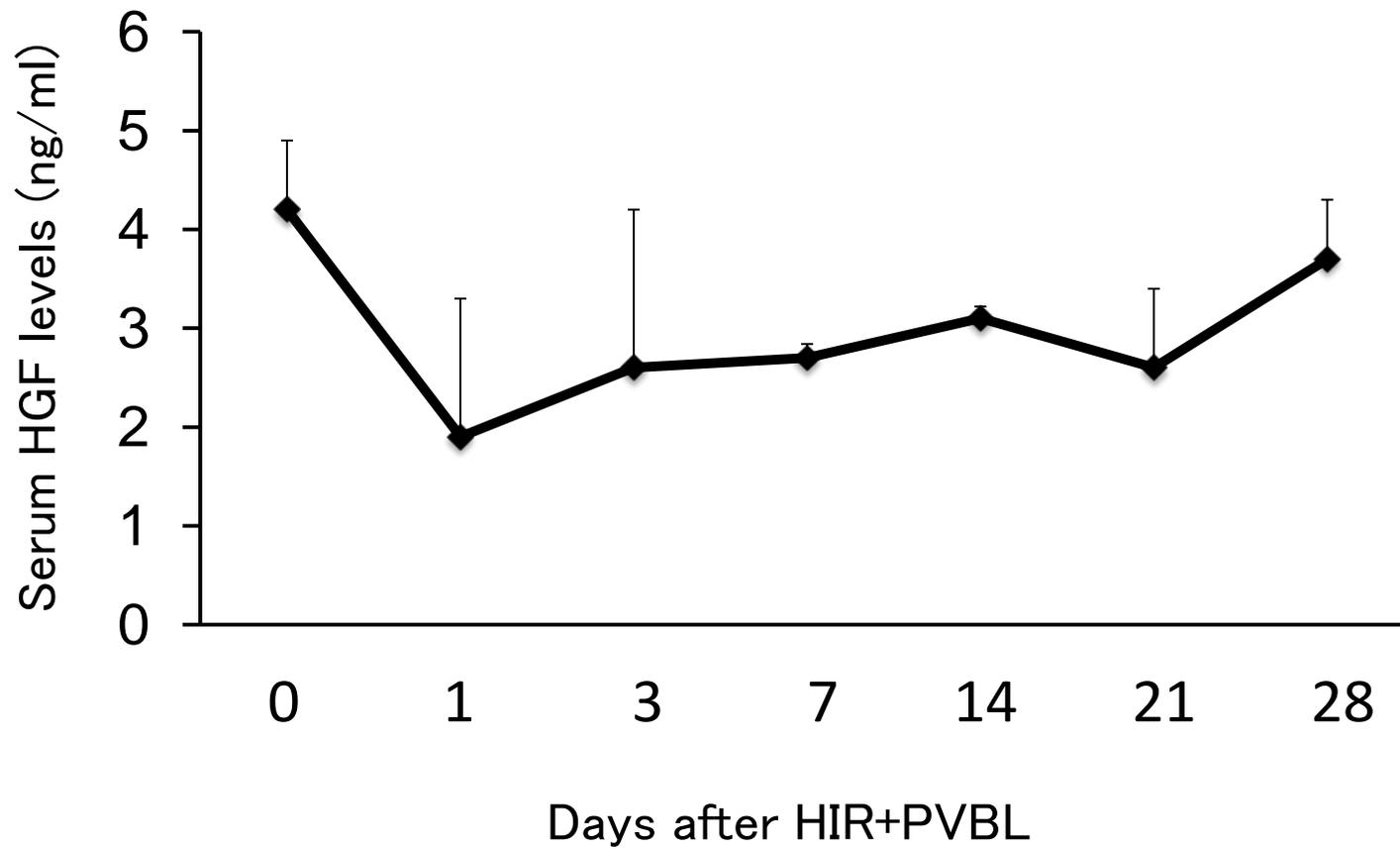


Fig. 6

