# Construction and Transplantation of an Engineered Hepatic Tissue Using a Polyaminourethane-Coated Nonwoven Polytetrafluoroethylene Fabric

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**Background.** Acute liver failure (ALF) is a serious condition that has a high mortality rate. Construction of an efficient culture and transplantation engineering system of hepatic tissue is an important approach to treat patients suffering from ALF to provide short-term hepatic support until the damaged liver spontaneously recovers or a donor liver becomes available for transplantation. Here, we evaluate the construction and transplantation of an engineered hepatic tissue (EHT) using primary isolated hepatocytes cultured onto polyaminourethane (PAU)-coated, nonwoven polytetrafluoroethylene (PTFE) fabric.

**Methods.** The isolated hepatocytes cultured onto PAU-coated PTFE fabric were able to adhere and spread over the individual fibers of the net and formed hepatic clusters after 3 days, such clusters revealed Gap junctions and well-developed bile canaliculi.

**Results.** When PAU-coated PTFE was utilized, ammonia-, and diazepam- metabolizing capacities and albumin production ability were significantly increased compared with collagen control. To test the function of this hepatic tissue in vivo, we transplanted a nonwoven PAU-coated PTFE fabric inoculated with one million hepatocytes on the surface of the spleen of Balb/c mice suffering from ALF induced by 90% hepatectomy, and found that this EHT prolonged the survival of liver failure-induced mice without adverse effects. Ultrastructure analyses showed good attachment of the cells on the surface of PTFE fabric and strong albumin expression seven days after the newly formed hepatic tissue was transplanted.

**Conclusion.** We have here demonstrated the efficient construction and transplantation of hepatic tissue using primary hepatocytes and PAU-coated PTFE fabric.

**Keywords:** Tissue engineering, Hepatocyte, Transplantation, Acute liver failure.

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Fulminant hepatic failure is a life-threatening condition that is treated by transplantation of the liver when a donor organ can be found. Because the natural history of acute failure varies so widely, even transient hepatic failure, when severe, must be treated by transplantation and lifelong immune suppression (*1*, *2*). However, the shortage of donor livers, high costs, and the risks associated with major surgery in those most severely ill limit the use of this form of therapy (*2*). Therefore, treatment of hepatic failure could be dramatically improved by development of methods for temporary hepatic support. Cleansing of hepatic toxins from the blood by charcoal hemofiltration, plasma exchange (*3*), and more recently albumin dialysis improves some parameters of hepatic function, but improved survival of patients with acute liver failure has been difficult to document (*4*, *5*).

Therefore, use of a bioartifical liver (BAL) has greater theoretic potential as a minimally intrusive therapy, as incorporated hepatocytes might provide additional metabolic capacity and physiologically active molecules important for recovery of native hepatic function (*6*). However, BALs require connection to the circulatory system, causing platelet consumption, hemodynamic instability, and the increased risk of bleeding associated with the need to use anticoagulants (*6*, *7*).

Most of the currently available BALs utilize porcine hepatocytes due to severe shortage of human livers for hepatocyte isolation. Bioincompatibility of humans and porcines and fear of transmission of unknown viruses are major hurdles (*8–10*). Researchers have been exploring alternative sources of human hepatocytes, such as immortalized human hepatocytes (*11*) and stem cell-derived hepatocytes (*12–17*).

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More investigation is required before clinical trials can be considered. We have currently developed a new type of bioartificial device that allows a three-dimensional cell culture based on a polyaminourethane (PAU)-coated, nonwoven polytetrafluoroethylene (PTFE) fabric previously developed by our laboratory to control blood glucose levels in diabetic porcines (*18*).

In this study, we have applied PAU-coated PTFE fabric for a scaffold of primary human, porcine, and mouse hepatocytes in the process of hepatic tissue construction in order to maximize the use and functional capacity of discarded livers. We also transplanted this engineered hepatic tissue (EHT) on the surface of the spleen in mice with 90% hepatectomized induced-liver failure; this new system of hepatocyte transplantation resulted in 60% animal survival.

## MATERIALS AND METHODS

### Isolation and Culture of Human, Porcine, and Mouse Hepatocytes

We obtained the consent of human subjects used in the current studies, and the present experiments were approved by the Institutional Ethics Committee. The livers of four different human donors that were used for these experiments were discarded for liver transplantation because of excessive fat content (moderate to severe macrosteatosis ≥30%) and focal ischemia. The livers were transported from National Disease Research Interchange (NDRI) to Japan, which took about 40 hr. The lateral segment of the livers was removed at Human Animal Bridge (HAB) Research Institute (Chiba, Japan), transported to Okayama using University of Wisconsin solution of 4°C for six hr, weighting 80–100 g then isolated with a four-step retrograde dispase/collagenase perfusion method as previously reported (*19*). The mean yield obtained from four human livers was $4.2\times10^6\pm1.45\times10^6$ viable cells/gram liver tissue. This procedure was performed for porcine hepatocytes isolation as well. All procedures performed on the pigs and mice were approved by the Okayama University Institutional Animal Care and Use Committee and thus within the guidelines of laboratory animals. Under general anesthesia, pigs (JA West, Okayama, Japan) weighting 20 kg underwent upper middle incision and the left lateral lobes, weighting 80 g, were surgically removed for hepatocyte isolation. And mouse primary hepatocytes were isolated from Balb/c mice (Nippon CLEA, Tokyo Japan) weighing 25 g by a two-step collagenase digestion method, as previously reported (*20*). Cell viability was assessed by a trypan blue dye exclusion method in all cases. In the hepatocyte isolation and culture experiments, the isolated hepatocytes of which viabilities were more than 90% were used in all cases. In all groups, hepatocytes were seeded onto PAU-coated PTFE fabric (kindly provided by Kuraray Medical, Japan) or collagen type IV (BD Biosciences, San Jose, CA) cultured with Williams' E solution (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma), $1\times10^7$ mol/L insulin (GibcoBRL), 25 ng/L EGF (Sigma), $1\times10^6$ mol/L dexamethasone (Sigma), $1\times10^5$ U/L penicillin, and 100 mg/L streptomycin (Sigma). The effect of deleted variant of hepatocyte growth factor (HGF; 100 ng/ml) (kindly provided by Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan) was also evaluated in hepatocyte cultures using PAU-PTFE or collagen type IV. In the following experiments, hepatocyte functions were measured per unit of time per mg of cellular protein at each of time points. The data were analyzed from four independent experiments in human and mouse hepatocyte cultures and from five different porcine hepatocyte cultures and each of the experiments had three samples. Cellular protein concentrations were determined by Multiscan JX (Thermo Electron Co., Yokohama, Japan) (Fig. 1).

### Morphological Assessment

During the time of culture, morphological appearance of EHT cells was observed using a phase contrast microscope (Olympus CK40-SL Japan). On days 1 and 14, the cells inoculated onto unwoven PTFE cloth coated with PAU were subjected to microstructural analysis using a scanning electron microscope (SEM) (Hitachi S-2300, Hitachi Co. Ltd., Tokyo, Japan) and transitional electron microscope (TEM) (Hitachi H-7100, Hitachi Co. Ltd.). For SEM, the samples were washed with phosphate-buffered saline (PBS) followed by fixation with 2% glutaraldehyde for two hr at 37°C, and gently washed with PBS. The samples were then postfixed with osmium tetraoxide for two hr and dehydration was accomplished using a graded series of ethanol (50%, 60%, 70%, 80%, 90%, and 99%). The samples were then dried at critical point for two hr in absolute alcohol and mounted on an aluminum stub and sputter-coated with gold before viewing under SEM. For TEM, the cells were fixed, first in 2.5% glutaraldehyde in 0.1 M phosphate buffer, and then in 1.0% OsO4 in 0.1 M phosphate buffer (pH 7.2). The samples were dehydrated through graded concentrations of ethanol and embedded in Epon, as previously reported (*21*). Ultrathin sections of the samples were double-stained with uranyl and

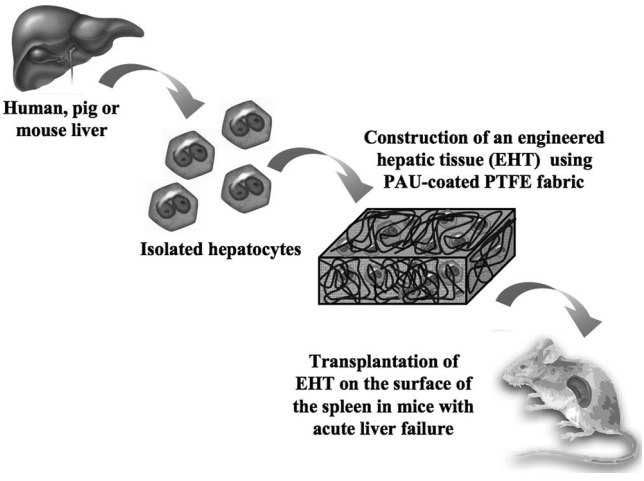


**FIGURE 1.** Schematic representation of the strategy for the construction and transplantation of engineered hepatic tissue. Human, porcine, and mouse hepatocytes were isolated and cultured onto polyaminourethane-coated nonwoven polytetrafluoroethylene fabric (PAU-coated PTFE) to construct an engineered hepatic tissue (EHT). The hepatocytes were examined for two weeks in culture using PAU-coated PTFE or collagen. Such hepatocytes seeded onto PAU-coated PTFE to form EHT were transplanted on the surface of the spleen of 90% hepatectomized mice.

observed under TEM. Ten different areas were randomly chosen and examined.

### Assay for Mitochondrial Function

In all, 100,000 hepatocytes were inoculated into each well of a 24-well plate (BD Biosciences) and the mitochondrial function per unit cells was measured over time by assay using 0.5 mg/ml of 3-(4.5-dimethyl-thiazole-2-yl)-2.5-diphenyl tetrazolium bromide (MTT) (Sigma) as previously reported (*22*). MTT values for all species of hepatocytes were expressed as optical density units (OD) during the time of evaluation.

### Evaluation of Metabolic and Synthetic Capacities of Primary Hepatocytes

At different time points of culture, hepatocytes cultured on collagen or unwoven PTFE were subjected to metabolic and synthetic tests. Ammonium sulfate (0.56 mM) and diazepam (1 $\mu$g/mL) were added to individual wells of 12-well plates and the amount of each substrate remaining in the media after culture for four hr was measured. The ammonia concentration was determined using a Fuji Dri-Chem slide (Fuji Co., Tokyo, Japan) and concentration of diazepam was measured by SRL (Tokyo, Japan). Four hours after ammonia loading, 10 $\mu$m of culture medium was collected for urea synthesis, as previously reported (*23*). Albumin secretion into the culture medium for 24 hr was measured by an albumin enzyme-linked immunosorbent assay (ELISA) kit (ALBUWELL II; Exocell, Philadelphia, PA) specific for human and mouse albumin secretion into the culture medium for 24 hr was evaluated using a mouse albumin ELISA kit (Shibayagi; Gunma, Japan) and we used albumin enzyme-linked immunoabsorbent assay quantitation kit (Bethyl, Laboratories, Inc., Montgomery, TX) for porcine albumin.

### Transplantation Experiments

To examine the facility to transplant this EHT containing primary hepatocytes on mice with liver failure, we performed in vivo transplantation experiments. We performed in vivo transplantation of EHT containing the hepatocytes of three different species (human hepatocytes, porcine hepatocytes, and mouse hepatocytes) on balb/c mice (n=10 each). First, the hepatocytes ($1\times10^6$) were inoculated on the non-woven cloth made of PTFE (10×5 mm) coated with PAU having cell-adhesive properties, which were placed on the bottom of a well of 12-well culture plates and cultured for 72 hr. All the following procedures performed on the mice were approved by the Okayama University Institutional Animal Care and Use Committee and thus within the guidelines of laboratory animals. Balb/c mice (Nippon CLEA Co.) weighing 25 g were performed 90% hepatectomy to induce acute liver failure (ALF) and simultaneously PTFE non-woven cloth containing hepatocytes were transplanted on the surface of the spleen of the mice. Just before transplantation, the splenic capsule was peeled off to facilitate the blood supply to the hepatocytes on the PTFE fabric as previously reported (*22*). The experimental groups were divided into the following five groups: Group 1, transplantation of PAU-PTFE fabric containing $1\times10^6$ hepatocytes (EHT) (n=10); Group 2, transplantation of PAU-PTFE fabric $1\times10^6$ mouse embryonic fibroblasts (MEF) (n=10); Group 3, transplantation of PAU-PTFE fabric only without hepatocytes (n=10); Group 4, transplantation of PAU-uncoated PTFE fabric only without hepatocytes (n=10) and Group 5, transplantation of $1\times10^6$ hepatocytes without PAU-PTFE fabric (n=10). One week after EHT transplantation, splenectomy was performed in the surviving mice for the histological examinations. Then, the rest of the animals were followed up to posttransplant 30 days. To prevent dehydration of the experimental animals, subcutaneous injection of 2 mL of saline was conducted on day one and on day two after 90% hepatectomy.

### Histological Analyses

Autopsies were performed at the time of death or sacrifice. The liver and spleen containing EHT specimens obtained from the mice were fixed with 20% formalin, embedded in paraffin, and processed for staining with hematoxylin and eosin. Additional samples were incubated with polyclonal rabbit antimouse albumin (Cedarlane, Hornby, ON, Canada) followed by Cy2- or Cy3-labeled secondary antibody (Amersham Biosciences, Piscataway, NJ). Samples were observed under a confocal laser scanning microscope (LSM510, Carl Zeiss, Germany).

### Statistical Analyses

Mean values are presented with standard deviations (SDs). A two-tailed Student's *t* test was used to calculate the significance of difference in mean values. The Kaplan-Meier method was used to calculate the survival data, and their significance was determined by the Mann-Whitney *U* test. A *P* value $<0.05$ was considered statistically significant.

## RESULTS

### PAU-Coated PTFE Cultured Hepatocytes Formed Hepatic Spheroids and Maintained Viability

The hepatocytes of all species were uniformly distributed over the PTFE cloth and cells began to form anchored spherical multicell aggregates 72 hr after plating (Fig. 2A). Notably, in the study using SEM, PAU-coated PTFE human hepatocytes showed organized spherical aggregates (100 $\mu$m of diameter; Fig. 2B), which were consistent with the findings observed in phase contrast microscopy. Transmission electron microscope (TEM) revealed that the cells cultured on PAU-coated PTFE showed Gap-junction between the cells (Fig. 2C) and well-developed bile canaliculi (BC; Fig. 2D). The photographs presented are representative of all the experiments performed. In an MTT assay for viability of the hepatocytes during the time, absorbance of an MTT reagent was compared among the different species to evaluate mitochondrial function of the hepatocytes according to the optical density (OD) units. PAU-coated PTFE fabric-applied cultures maintained significantly better mitochondrial function of human, porcine, and mouse hepatocytes than conventional collagen type IV cultures. The supplementation of dHGF (100 ng/ml) significantly improved the parameter in the hepatocyte cultures of all of three different species (PAU-coated PTFE and collagen substrate).

The same phenomenon was observed in all cases (Fig. 2E–G). The results suggested that PAU-coated PTFE promoted the viability of human, porcine, and mouse hepatocytes in vitro (Fig. 2E–G). The addition of dHGF improved the viability of human hepatocytes by 0.07 OD on day seven

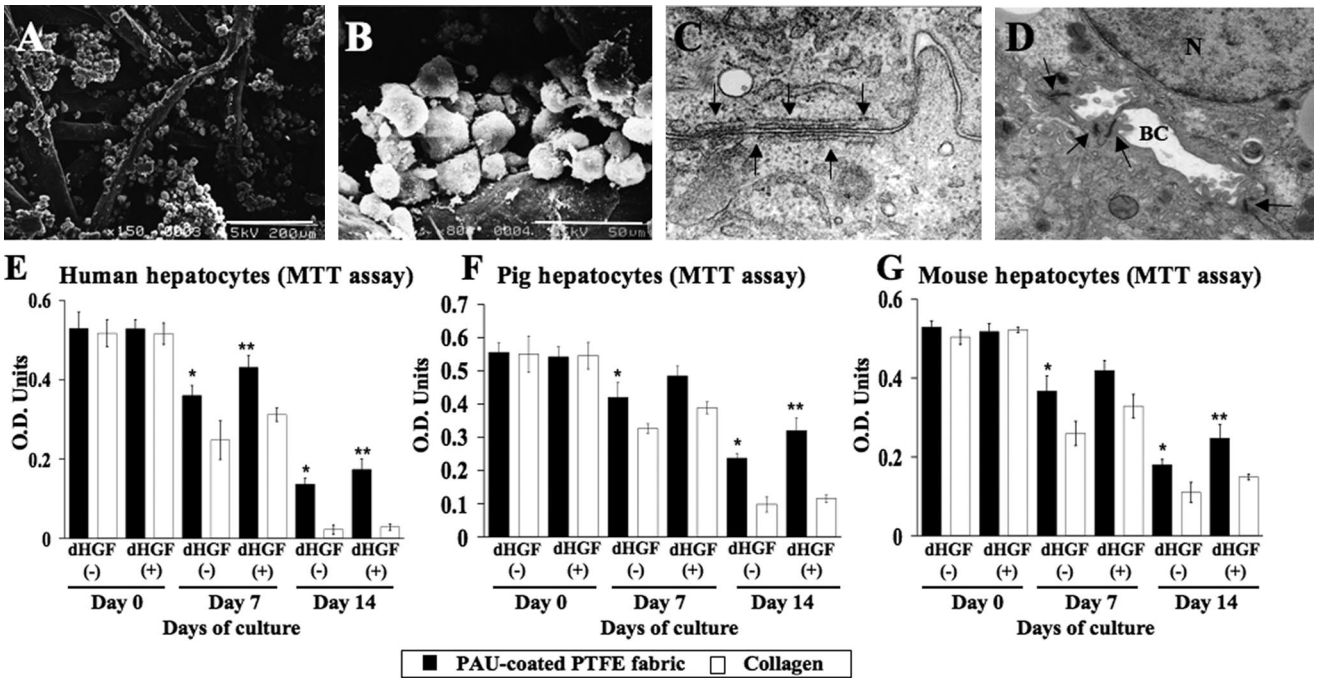


**FIGURE 2.** Morphology and viability of human, porcine, and mouse hepatocytes cultured onto PAU-coated PTFE. (A) The hepatocytes attached favorably on the surface of nonwoven PTFE fabric coated with PAU. The hepatocytes were uniformly distributed over the PTFE fabric. Bar=200 micrometer. (B) After 72 hours of culture the hepatocytes started forming hepatic-like clusters in a three-dimensional platform provided by PTFE fabric. Bar=50 micrometer. (C) TEM showed that such hepatocytes demonstrated gap-junction between the cells and (D) well-developed bile caniculi (C: original magnification, ×30,000; D: original magnification, ×15,000). Human (E), porcine (F), and mouse (G) hepatocytes cultured onto PAU-PTFE fabric revealed a significant maintenance of mitochondrial function evaluated by MTT assay indicating better viability during the time. The supplementation of dHGF (100 ng/ml) improved viability and mitochondrial function of the hepatocytes cultured on PAU-coated PTFE fabric. *$P<0.05$ for PAU-PTFE vs. collagen. **$P<0.05$ for dHGF(+) vs. dHGF(−), respectively. The data were analyzed from four independent experiments in human and mouse hepatocyte cultures and from five different porcine hepatocyte cultures and each of the experiments had three samples.

and 0.04 OD on day 14, porcine hepatocytes by 0.06 OD on day seven and by 0.09 OD on day 14, and mouse hepatocytes by 0.05 OD on day seven and by 0.06 OD on day 14 in PAU-PTFE cultures. These data were obtained from four independent experiments in human and mouse hepatocyte cultures and from five different porcine hepatocyte cultures.

**Human Hepatocytes**

On day 0: PAU-PTFE without dHGF, 0.50±0.04 OD; PAU-PTFE with dHGF, 0.52±0.02 OD; collagen without dHGF, 0.50±0.03 OD; collagen with dHGF, 0.51±0.03 OD. On day seven: PAU-PTFE without dHGF, 0.36±0.02 OD; PAU-PTFE with dHGF, 0.43±0.03 OD; collagen without dHGF, 0.24±0.04 OD; collagen with dHGF, 0.31±0.01 OD. On day 14: PAU-PTFE without dHGF, 0.13±0.01 OD; PAU-PTFE with dHGF, 0.17±0.02 OD; collagen without dHGF, 0.02±0.01 OD; collagen with dHGF, 0.02±0.01 OD (Fig. 2E).

**Porcine Hepatocytes**

On day 0: PAU-PTFE without dHGF, 0.50±0.02 OD; PAU-PTFE with dHGF, 0.54±0.03 OD; collagen without dHGF, 0.50±0.05 OD; collagen with dHGF, 0.54±0.04 OD. On day seven: PAU-PTFE without dHGF, 0.42±0.04 OD; PAU-PTFE with dHGF, 0.48±0.03 OD; collagen without dHGF, 0.30±0.01 OD; collagen with dHGF, 0.38±0.02 OD. On day 14: PAU-PTFE without dHGF, 0.23±0.01 OD; PAU-PTFE with dHGF, 0.32±0.038 OD; collagen without dHGF, 0.09±0.02 OD; collagen with dHGF, 0.11±0.01 OD (Fig. 2F).

**Mouse Hepatocytes**

On day 0: PAU-PTFE without dHGF, 0.52±0.01 OD; PAU-PTFE with dHGF, 0.51±0.02 OD; collagen without dHGF, 0.50±0.01 OD; collagen with dHGF, 0.52±0.01 OD. On day seven: PAU-PTFE without dHGF, 0.36±0.03 OD; PAU-PTFE with dHGF, 0.4±0.02 OD; collagen without dHGF, 0.25±0.01 OD; collagen without dHGF, 0.32±0.03 OD. On day 14: PAU-PTFE without dHGF, 0.18±0.01 OD units; PAU-PTFE with dHGF, 0.18±0.01 OD; collagen without dHGF, 0.10±0.02 OD; collagen with dHGF, 0.14±0.01 OD (Fig. 2G).

## PAU-Coated PTFE-Cultured Hepatocytes Metabolized Ammonia and Diazepam

Metabolic rates of both ammonia and diazepam in human, porcine, and mouse hepatocyte cultures per four hours per mg of cellular protein were comparatively analyzed. Significantly higher metabolic capacities of ammonia were observed in PAU-coated PTFE-cultured hepatocytes than those cultured in collagen. The supplementation of dHGF (100 ng/ml) significantly improved ammonia-metabolizing capacities in both PAU-PTFE and collagen cultures.

The results suggested that PAU-coated PTFE was better than the conventional collagen culture in terms of metabolic

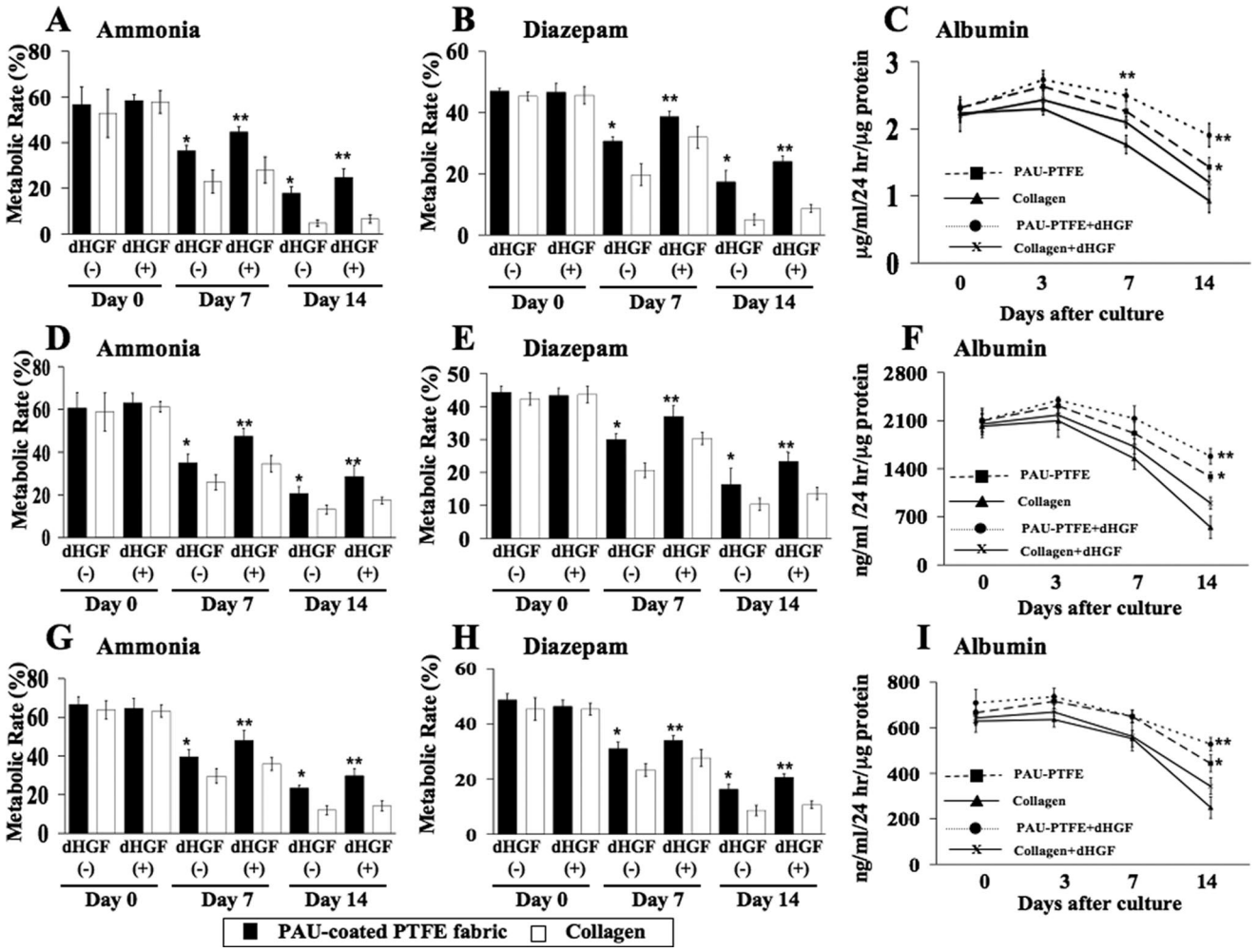


**FIGURE 3.** Functional capacities of human, porcine, and mouse hepatocytes. Application of PTFE fabric coated with PAU in hepatocyte culture significantly enhanced ammonia-metabolizing activity four hours after loading ammonium sulfate in the culture medium at the final concentration of 0.56 mM, ammonia metabolic rates of the hepatocytes per mg of cellular protein were comparatively analyzed of (A) human, (D) porcine, and (G) mouse hepatocytes. After adding diazepam (1 g/mL) in the culture medium, respectively, metabolic rates were compared four hours. The (B) human, (E) porcine, and (H) mouse hepatocytes cultured onto PTFE fabric significantly metabolized diazepam per mg of cellular protein compared to the collagen cultured hepatocytes. Although significantly better production of albumin was observed in the (C) human, (G) porcine, and (I) mouse hepatocytes (per mg of cellular protein) cultured onto PAU-PTFE fabric, the ability reduced gradually on day seven even PAU-PTFE-cultured hepatocytes. The supplementation of dHGF (100 ng/mL) significantly improved ammonia- and diazepam-metabolizing capacities of the mouse, porcine, and human hepatocytes cultured on PAU-coated PTFE fabric. *$P<0.05$ for PAU-PTFE vs. collagen. **$P<0.05$ for dHGF(+) vs. dHGF(−), respectively. These parameters were compared per microgram cell. The data were analyzed from four independent experiments in human and mouse hepatocyte cultures and from five different porcine hepatocyte cultures and each of the experiments had three samples.

ability of ammonia and diazepam, but when dHGF was added in both groups ammonia metabolism of human hepatocytes was increased by 8.2% on day seven and by 7.0% on day 14, porcine hepatocytes by 12.4% on day seven and by 8.0% on day 14, and mouse hepatocytes by 8.4% on day seven and by 6.4% on day 14; and diazepam metabolism of human hepatocytes was increased by 8.0% on day seven and by 6.7% on day 14, porcine hepatocytes by 7.0% on day seven and by 7.0% on day 14, and mouse hepatocytes by 3.0% on day seven and by 4.3% on day 14 in PAU-PTFE cultures. The values were compared per microgram cell. Data are means+SD.

**Ammonia**

*Human Hepatocytes*

On day 0: PAU-PTFE without dHGF, 56.6±7.7%; PAU-PTFE with dHGF, 58.4±2.5%; collagen without dHGF, 52.8±10.5%; collagen with dHGF, 57.8±5.0%. On day seven: PAU-PTFE without dHGF, 34.2±4.9%; PAU-PTFE with dHGF, 44.6±2.2%; collagen without dHGF, 23.8±6.3%; collagen with dHGF, 28.0±5.7%. On day 14: PAU-PTFE without dHGF, 17.8±2.8%; PAU-PTFE with dHGF, 24.8±3.7%; collagen without dHGF, 7.8±2.1%; collagen with dHGF, 6.6±1.8%.

*Porcine Hepatocytes*

On day 0: PAU-PTFE without dHGF, 60.6±7.1%; PAU-PTFE with dHGF, 63.0±4.5%; collagen without dHGF, 58.8±8.8%; collagen with dHGF, 61.2±2.4%. On day seven: PAU-PTFE without dHGF, 35.0±4%; PAU-PTFE with dHGF, 47.4±3.6%; collagen without dHGF, 25.8±3.5%; collagen with dHGF, 34.6±3.7%. On day 14: PAU-PTFE without dHGF, 20.6±3.2%; PAU-PTFE with dHGF, 28.6±5.1%; collagen without dHGF, 13.2±2.0%; collagen with dHGF, 17.4±1.5%.

*Mouse Hepatocytes*

On day 0: PAU-PTFE without dHGF, 66.6±3.9%; PAU-PTFE with dHGF, 64.6±5.0%; collagen without dHGF, 63.8±4.8%; collagen with dHGF, 63.2±3.0%. On day seven: PAU-PTFE without dHGF, 39.6±3.6%; PAU-PTFE with dHGF, 48.0±3.3%; collagen without dHGF, 29.6±3.6%; collagen with dHGF, 35.8±3.3%. On day 14: PAU-PTFE without dHGF, 23.4±1.5%; PAU-PTFE with dHGF, 29.8±3.6%; collagen without dHGF, 12.0±2.4%, collagen with dHGF, 14.2±2.6% (Fig. 3A, D, G).

**Diazepam**

Metabolism of diazepam was significantly higher in PAU-coated PTFE cultured hepatocytes than the hepatocytes cultured with collagen.

**Human Hepatocytes**

On day 0: PAU-PTFE without dHGF, 47±0.8%; PAU-PTFE with dHGF, 46.6±2.8%; collagen without dHGF, 45.3±1.3%; collagen with dHGF, 45.6±2.8%. On day seven: PAU-PTFE without dHGF, 30.6±1.3%; PAU-PTFE with dHGF, 38.6±1.8%; collagen without dHGF, 19.6±3.6%; collagen with dHGF, 23.6±3.3%. On day 14: PAU-PTFE without dHGF, 17.3±3.7%; PAU-PTFE with dHGF, 24.0±1.7%; collagen without dHGF, 5.0±1.7%; collagen with dHGF, 6.3±2.2%.

**Porcine Hepatocytes**

On day 0: PAU-PTFE without dHGF, 44.3±1.8%; PAU-PTFE with dHGF, 43.3±2.2%; collagen without dHGF, 42.3±1.8%; collagen with dHGF, 43.6±2.5%. On day seven: PAU-PTFE without dHGF, 30±1.7%; PAU-PTFE with dHGF, 37.0±3.2%; collagen without dHGF, 20.6±2.2%; collagen with dHGF, 25.3±3.7%. On day 14: PAU-PTFE without dHGF, 16.3±4.9%; PAU-PTFE with dHGF, 21.3±2.7%; collagen without dHGF, 10.3±1.8%; collagen with dHGF, 12.3±2.7%.

**Mouse Hepatocytes**

On day 0: PAU-PTFE without dHGF, 48.6±2.2%; PAU-PTFE with dHGF, 46.3±2.2%; collagen without dHGF, 45.3±4.1%; collagen with dHGF, 45.3±2.2%. On day seven: PAU-PTFE without dHGF, 31±2.3%; PAU-PTFE with dHGF, 36.0±1.7%; collagen without dHGF, 23.3±2.2%; collagen with dHGF, 26.0±1.7%. On day 14: PAU-PTFE without dHGF, 16.3±1.8%; PAU-PTFE with dHGF, 19.6±1.8%; collagen without dHGF, 8.6±1.8%; collagen with dHGF, 10.3±1.3% (Fig. 3B, E, H).

### PAU-Coated PTFE-Cultured Hepatocytes Showed Higher Albumin Production

The daily albumin production of the human, porcine, and mouse hepatocytes per mg of cellular protein was compared for 14 days along the groups. Albumin production was significantly maintained in the PAU-coated PTFE-cultured hepatocytes compared to the collagen-treated hepatocytes (Fig. 3C, F, I), but showed a slight decrease on day 14. In contrast, the collagen-treated hepatocytes produced no albumin on day 14. When dHGF was supplemented into the culture medium, albumin production of the hepatocytes in both PAU-coated PTFE and collagen cultures improved significantly. Data are means+SD.

### Transplantation of an Engineered Hepatic Tissue (EHT) Prolonged the Survival in Liver Failure–Induced Mice

We evaluated the effect of transplantation EHT on the survival of Balb/c mice suffering from ALF induced by 90% hepatectomy. The 90% hepatectomized mice treated with PAU-PTFE fabric and MEF (G2), PAU-PTFE only (G3), PAU-uncoated PTFE (G4), or hepatocytes ($1\times10^6$) only (G5) showed hypoglycemia. Such animals revealed hyperammonemia and they developed encephalopathy (Fig. 4A–C). However, treatment of 90% hepatectomized mice with transplantation of engineered hepatic tissue (G1), EHT, containing $1\times10^6$ hepatocytes inoculated between PAU-coated PTFE fabrics significantly improved blood levels of glucose and ammonia (Fig. 4A, C). EHT treatment also reversed hepatic encephalopathy (Fig. 4B). After EHT transplantation, a 30-day survival rate of the mice was 60% for EHT transplant, and 0% for PAU-PTFE containing MEF transplant, PAU-PTFE alone transplant, PAU-uncoated PTFE transplant, and hepatocytes only, respectively (Fig. 4D). Notably, all of the mice in negative controls (G2, G3, G4, and G5) died of ALF within four days after 90% hepatectomy. The data presented in this study were those obtained using mouse hepatocytes, but similar data were obtained from both porcine and human hepatocyte EHT transplant.

### Histological Examination

All of the 90% hepatectomized mice (G2, G3, G4, and G5) without EHT transplant died of ALF, demonstrating jaundice and hemorrhage which confirmed by the histological examination of the liver of massive necrosis and hemorrhage (Fig. 5A). In contrast, surviving mice (G1) with EHT transplant showed almost normal structure of the liver on day 30 (Fig. 5B) when they were sacrificed for histological examinations. Appropriate attachment of the PAU-coated nonwoven PTEF fabric was observed on the surface of the spleen (Fig. 5C, E). Immunofluorescent study for albumin in the spleen removed on day seven from the mice with EHT transplant demonstrated the strong expression of albumin of the hepatocytes between PTFE fabrics for at least seven days (Fig. 5D, F) indicated by a green signal, then the expression decreased and disappeared after 14 days. We found viable hepatocytes with trabecular arrangement on day seven (Fig. 5E), which was confirmed by albumin-positive staining (Fig. 5F) in the EHT-treated mice. These findings were consistent with the results obtained from in vitro experiments and suggested that the EHT transplanted bridged the animals to completely recover from ALF.

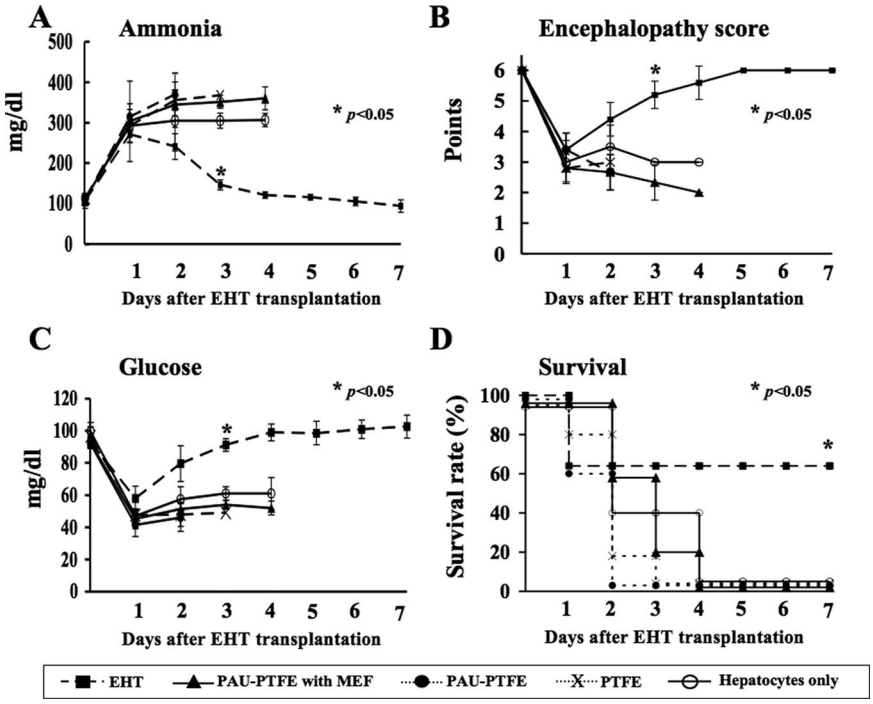


**FIGURE 4.** Ammonia, hepatic encephalopathy, blood glucose, and survival of hepatectomized mice after EHT therapy. We evaluated the effect of transplantation of EHT on the surface of the spleen containing mouse hepatocytes on BALB/C mice that were hepatectomized (90% liver removal). (A) Blood ammonia levels, (B) encephalopathy scores, (C) blood glucose levels, and (D) survival were determined for seven days. Sixty percentage of the 30-day survival rate was achieved in the mice with EHT transplant (G1, n=10). In contrast, control mice with transplantation of PAU-PTFE containing mouse embryonic fibroblast (MEF) (G2, n=10), PAU-PTFE fabric only (G3, n=10), PAU-uncoated PTFE only (G4, n=10) and hepatocytes only (G5, n=10) died within four days due to liver failure. *$P<0.05$ for G1 vs. G2, G3, G4, and G5.

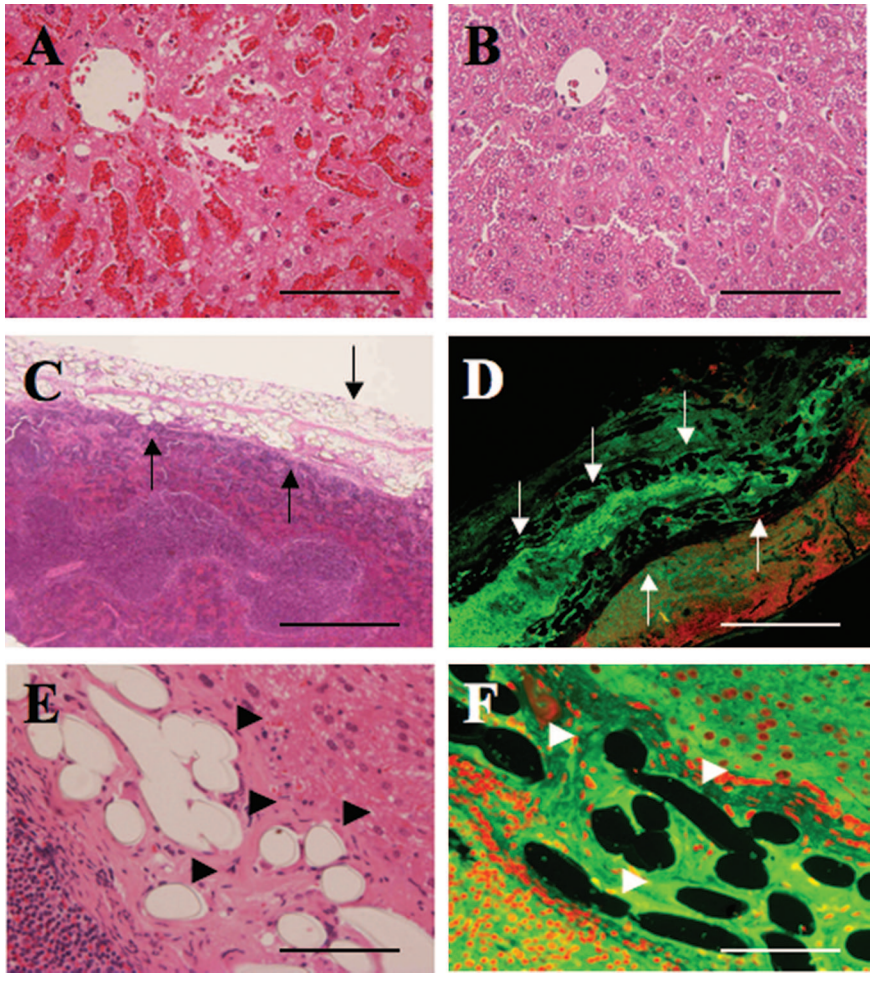


**FIGURE 5.** Histological findings of ALF mice after EHT transplantation. (A) The liver specimen obtained from control mice that died of ALF demonstrated massive liver necrosis and hemorrhage (original magnification, ×100). (B) The liver of the mice regenerated and showed the normal structure when sacrificed at the posttransplant day 30 (original magnification, ×100). (C) In the spleen removed on day seven after EHT transplant, nonwoven PTFE fabric containing mouse hepatocytes was identified on the surface of the spleen, indicated by black arrows and white arrows (D) on the immunofluorescent study to detect albumin expression indicated by green signal (original magnification, ×40). (E) Trabecular arrangement of hepatocytes contained into the PTFE fabric (indicated by black head arrows), which were positive for albumin staining (F), indicated by a green signal, and PAU-PTFE fabric indicated by white head arrows was observed (B, C, F, G: bars=100 μm; D, E: bars=500 μm).

## DISCUSSION

Normal human hepatocytes are an ideal source for hepatocyte transplantation (*24*); however, the shortage of donor livers severely limits the use of normal human hepatocytes. In addition, once human hepatocytes are available from discarded livers that are not suitable for organ transplantation, primary isolated hepatocytes cannot be cultured for long periods in vitro (*25*). To overcome these problems, other cell sources have been investigated as a potential alternative to hepatocytes like immortal hepatocytes (*11*), porcine hepatocytes (*26*), and embryonic stem cells (*16*). In this study, we have applied a PAU-coated PTFE to construct and transplant an engineered hepatic tissue that in vitro can maintain the function of human, porcine or mouse hepatocytes. This material previously developed for bioartificial pancreas by our group is able to provide a three-dimensional atmosphere and a good attachment circumstance to the cells. Such an environment can maintain cell-cell contact and such interactions in hepatocyte culture coordinately modulates liver transcription factors and allows the cultured hepatocytes to perform sophisticated functions (*27*).

We have established a procedure to develop a functional artificial hepatic tissue that can be used as a stage for cell-based therapies. This procedure using PAU-PTFE supported not only the viability of the hepatocytes in vitro but also the engraftment of transplanted hepatocytes resulting in engineering a hepatic tissue. This new-engineered hepatic tissue possesses the ability to rescue animals suffering from ALF and expressed albumin similar to the native liver. This clearly demonstrates that the EHT on the surface of the spleen was integrated into the systemic circulation between the newly generated hepatic tissue and other organs.

Although PAU-PTFE-based hepatocyte culture was significantly better than the conventional collagen culture, we encountered a downward trend in ammonia and diazepam metabolism and albumin production of the hepatocytes in vitro. To overcome this problem, we utilized a deleted variant of HGF (dHGF), because HGF is one of the most important key factors to facilitate liver regeneration after experimental hepatectomy (*28*). We found that supplementation of dHGF improved mitochondrial function by 0.04 to 0.09 O.D. units (Fig. 2E–G), ammonia metabolism by 6.4 to 8.0% (Fig. 3A, D, G) and diazepam metabolism by 4.3 to 7.0% (Fig. 3B, E, H) of the mouse, porcine, and human hepatocytes cultured with PAU-PTFE fabric at 14 days of culture. Additional factors that might induce the decrease of hepatocyte functions in vitro would be the lack of liver nonparenchymal cells for cell-cell interactions, genomic instability, and apoptosis of the cells. Experiments are now ongoing to establish the functional in vitro hepatocyte culture system using coculture of hepatocytes with liver nonparenchymal cells and the supplementation of antiapoptotic molecules in conjunction with PAU-PTFE fabric.

The spleen is one of the most attractive sites for transplanting cells since it is readily accessible, allows large numbers of cells and a splenectomy is a relatively safe procedure if complications or extraction of EHT is required. However, the main problem in transplanting cells directly into the spleen or by injecting the cells into the splenic artery has been the insufficient engraftment of hepatocytes, short-term survival, and cell migration to other organs (*29*, *30*). The present results, as well as our previous experience (*22*), clearly demonstrate the hepatocytes could be efficiently engrafted and survived without the risk of cell migration. In SCID mice assay, we also observed no migration of the hepatocytes, even mouse hepatocytes, into the splenic parenchyma or other organs (liver, stomach, and lung) by albumin immunostaining using species-specific antibodies (data not shown). These observations clear demonstrated that PTFE fabric is safe to prevent cellular migration and it has now prompted us to develop an implantable bioartificial liver device consisted of PTFE fabric in which genetically modified hepatocytes or embryonic stem cell-derived hepatocytes can be transplanted. The EHT was also appropriately structured with the PAU-coated PTFE fabric, which incorporated a platform for neovascularization of the EHT and was shown to be biocompatible. It is one of the important issues to evaluate cytotoxicity of PAU-PTFE fabric. We have so far found no adverse effects or cytotoxicity of PAU-PTFE material in the cultures of human, monkey (*22*), porcine, and mouse hepatocytes and human and porcine islets (*18*).

The EHT was easy to manipulate surgically. An additional advantage is that this EHT can be easily extracted, which helps to avoid occurrences of unwanted side effects. To determine if the established approach for hepatic tissue engineering was applicable using hepatocytes from different species, similar experiments were conducted with human and porcine hepatocytes. Significant high parameters in vitro were obtained when PAU-coated PTFE was used, and histological analysis of in vivo experiments revealed healthy and functional hepatocytes in the EHT transplanted on the surface of the spleen (data not shown). Taken together, hepatic tissue can be effectively engineered using PAU-coated PTFE in an ectopic site using this transplantation approach, whereby the tissue can provide transient hepatic function similarly to the normal liver while the damaged liver recovers.

In summary, the present study demonstrates a method to construct a functional engineered hepatic tissue using PAU-coated PTFE as a platform for hepatocyte transplantation of any species. Because of the simplicity of our approach, such an intervention could represent a possibility for future application in tissue-based therapies.

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