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Transplantation of a fetal liver cell-loaded hyaluronic acid sponge onto the mesentery recovers a Wilson's disease model rat

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An auxiliary liver represents a promising alternative for liver transplantation. The use of a large amount of mature hepatocytes, however, despite their high function, is limited in a clinical setting. Here, we propose a novel transplantation system that dramatically improved a diseased animal by incorporating fetal liver cells (FLCs) as a cell source, the mesentery as a transplantation site and a hyaluronic acid (HA) sponge as a cell scaffold. We transplanted wild-type Long Evans Agouti rat FLCs embedded in HA sponges onto the mesentery of Long Evans Cinnamon (LEC) rats, an animal model for Wilson's disease. The FLC-loaded HA sponges successfully grafted and consequently prevented jaundice. Accordingly, the treated animals showed a significant reduction in blood copper concentration, which consequently led to significant decreases in serum total bilirubin and direct bilirubin, and to a significant increase in albumin productivity. Furthermore, haematoxylin and eosin staining of the host livers demonstrated that fibrosis at the periportal area was moderated in the treated animals. In conclusion, we transplanted FLC-loaded HA sponges onto the mesenteric blood vessels, leading to thick, liver-like tissue possessing blood vessels, and the liver tissue engineered thus exhibited a remarkable therapeutic effect on the copper metabolism deficiency of LEC rats.

Keywords: Fetal liver cell/heterotopic liver tissue engineering/hyaluronic acid sponge/intramesenteric transplantation/Wilson's disease.

Abbreviations: ALB, albumin; DBIL, direct bilirubin; ECM, extracellular matrix; ES, embryonic stem; FLC, fetal liver cell; HA, hyaluronic acid; HE, haematoxylin and eosin; LEA, Long Evans Agouti; LEC, Long Evans Cinnamon; TBIL, total bilirubin; WD, Wilson's disease.

The only treatment for end-stage liver diseases is liver transplantation. Its application, however, has been limited due to the shortage of donors. Various studies have reported on extracorporeal bioartificial liver treatment (*1*), but these temporary procedures are, fundamentally, unable to completely compensate for the donor shortage. Another therapeutic approach is hepatocyte transplantation. This procedure, however, is hampered by the low liver-engraftment rate and survival of transplanted hepatocytes (*2*): repopulation of only 0.5–1% of the liver following transplantation of 20 million cells in the rat or 2 million cells in the mouse liver (*3*, *4*), which can be increased to ~5% by transplanting cells repeatedly. However, with this magnitude of liver reconstitution, correction of metabolic deficiency might be incomplete (*5*).

To partly overcome the shortcoming of the infusion of hepatocyte suspensions, heterotopic liver tissue engineering has been experimentally investigated by transplanting hepatocytes or hepatocyte-scaffold composites into extra-hepatic sites. Uyama *et al.* (*6*) reported that enzyme-deficient Gunn rats that received 1×10^8 and 4×10^8 hepatocytes (in total equivalent to whole rat liver) from normal Wistar rats showed a significantly greater decrease in total serum bilirubin compared with the concurrent control Gunn rats. This report strongly indicates that a large mass of hepatocytes is necessary for correcting metabolic deficiencies in the animals. In general, however, isolated hepatocytes largely lack transplantation quality and quantity because they are available only from cadaveric donor livers and are cryopreserved before use (*7*). Another difficulty in their use is their low proliferative capacity after transplantation, leading to only low-density liver tissues (*8*). It is crucial, therefore, to attempt to obtain transplantable cells from other sources, such as various stem cells or liver progenitor cells that have a higher proliferative capacity (*7*, *9*). Heterotopic transplantation of composites made up of such cells and scaffolds might allow the reconstruction of a large mass of liver-like tissues. However, contrary to the intensive studies on transplantation of adult hepatocytes, little has been studied on heterotopic liver tissue engineering using liver progenitor cells.

Another issue of great importance in heterotopic liver tissue engineering is the supply of vasculature and hepatotrophic factors to the transplanted grafts. Several heterotopic transplantation sites have been investigated to vascularize the transplanted composites by fully utilizing the *in vivo* regenerative capability: the mesentery, omentum, and subcutaneous space (*10*, *11*). In the body, diffusion of oxygen, nutrients and waste products allows only cells within 100–200 μm from the nearest capillary to survive (*12*). The simplest

approach to overcome this limitation is to engineer a thin liver tissue and transplant it onto a well-vascularized site. Accordingly, a well-vascularized site with a large area such as the mesentery or subcutaneous space is required for engineering a heterotopic liver tissue with a sufficient mass. An especially attractive transplantation site is the mesentery in that it provides a transplanted graft with not only oxygen but also hepatotrophic factors via the mesenteric blood vessels that interconnect the small intestine and the liver (*13*, *14*).

In this report, we investigated therapeutic potency of fetal liver cell (FLC)-loaded hyaluronic acid (HA) sponges transplanted onto the mesentery of recipient animals. We selected FLCs of the wild-type Long Evans Agouti (LEA) rat as a model of liver progenitor cells because FLCs are thought to have a highly efficient maturation capacity and a relatively high proliferative capacity (*15*). As a disease model, Long Evans Cinnamon (LEC) rats, a widely used model animal for Wilson's disease (WD), which is an autosomal recessive disorder characterized by defective hepatic copper transport that results in the accumulation of copper in the liver, kidney and central nervous system, was used (*16*–*19*). Here, we employed an HA sponge for a cell-embedded module, since we previously reported an effective support of an HA sponge for a long-term *in vitro* culture of mouse embryonic stem (ES) cell-derived hepatic progenitor cells (*20*). We investigated whether FLC-loaded HA sponges onto the mesentery would effectively support the engineered liver tissue and exhibit a therapeutic effect.

Materials and Methods

HA scaffolds and their conditioning for cell culture

As a scaffold for FLCs, we used a 1.0-mm-thick photo-cross-linked HA sponge that included cinnamic acid as a cross-linking material (Seikagaku Corp., Tokyo, Japan) (Fig. 1A). The sponge matrix has a unique structure composed of an HA layer with a porous structure that has a pore size of 60 μm in diameter. Prior to cell inoculation, the HA sponge sheet was cut into strips ($10 \times 2\,mm^2$) and soaked in hepatocyte culture medium (HCM: Takara, Kyoto, Japan) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, USA) (Fig. 1B), leading to expansion due to water absorption ($\sim 12.5 \times 2.5 \times 1.3\,mm^3$).

Isolation of LEA rat FLCs and their inoculation into HA scaffolds

LEA rat FLCs were isolated from embryonic livers of pregnant LEA rats on Day 15.5 of gestation by the collagenase digestion method described elsewhere (*15*). Harvested LEA rat FLCs were inoculated into HA sponge strips at $2–4 \times 10^6$ cells/piece and cultured for four days at 37°C in a 5% CO_2 incubator prior to transplantation. Meanwhile, a small portion of the cell suspension was applied to a collagen-coated 12-well plate (Asahi Technoglass, Funabashi, Japan) to confirm the morphology of the isolated FLCs. The medium was changed 1 and 3 days after inoculation.

Used animal model of WD

We used the LEC rat as an animal model of WD. In this model, the excessive deposition of copper leads to hepatic, neuropsychiatric and other clinical manifestations. A wide variety of mutations in the P-type copper transporting adenosine triphosphatase (ATPase; *ATP7B*) gene are responsible for defective hepatic copper excretion in WD (*17*). LEC rats develop many clinical and biochemical features of WD, including liver copper accumulation, as a result of a deletion in the rat *Atp7b* gene that shares 85% homology with the human *Atp7b* gene (*18*, *19*).

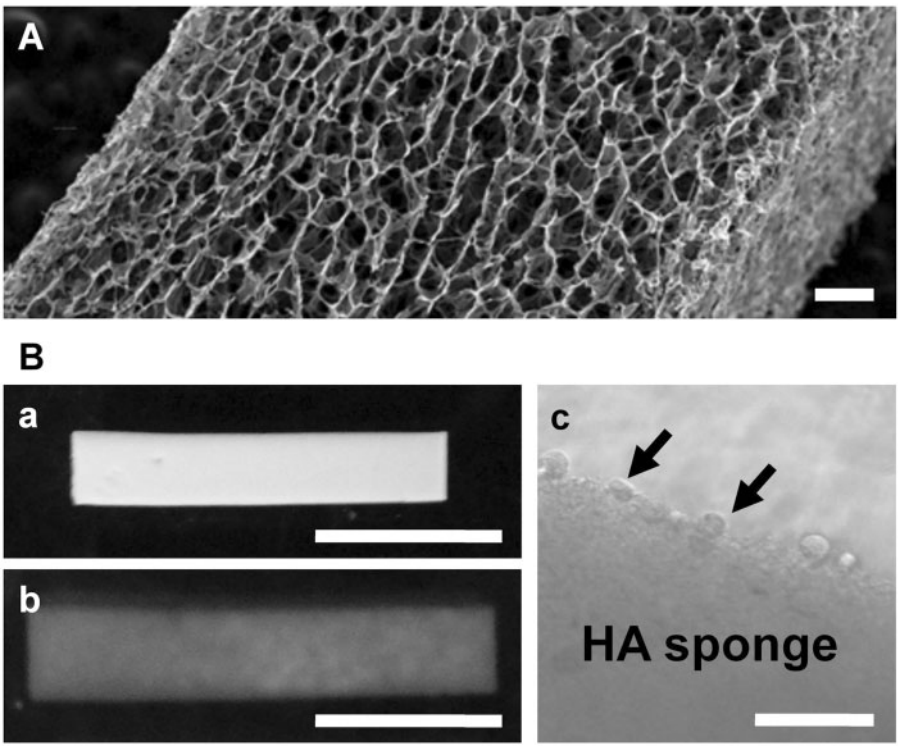

Fig. 1 Aspects of HA sponge. (A) Scanning electron microphotograph of HA sponge. Scale bar: 100 μm. (B) (a) HA sponge sheet cut into a 10 mm × 2 mm sized strip. Scale bar: 500 mm. (b) The same HA sponge shown in (a) restored in HCM and loaded with LEA rat FLCs. The photographic scale is equal to that of (a). Expansion of HA sponge due to water absorption can be observed. Scale bar: 500 mm. (c) Magnified view of the FLC-loaded HA sponge strip (b). Cells were observed on the surface of HA sponge (arrows). Scale bar: 100 μm.

Transplantation of LEA rat FLC-loaded HA sponges

FLC-loaded HA sponges were transplanted to male LEC rats (239–357 g) (provided by Kyoto University, Kyoto, Japan). The surgical procedure is detailed in Fig. 2. In summary, two FLC-loaded strips were attached to the blood vessel, one on the top surface, the other on the reverse surface of the mesentery, using instant adhesive (Aron Alpha, Konishi Company, Osaka, Japan) so that the longer side of the scaffold laid parallel to the blood flow. In the control LEC rat group, the small intestine was pulled out, put back without any treatment, and the abdomen was closed. After transplantation, the animals were fed a conditioned diet (Oriental Yeast Co. Ltd., Tokyo, Japan) containing 10 times more concentrated copper (100 ppm) than a general diet. The animals were sacrificed 3 weeks after transplantation.

Biochemical analyses of rat sera

Three weeks after transplantation, the abdomens of the LEC rats were opened under ether anaesthesia and blood samples were collected from the hearts with a 26-G needle (Terumo, Tokyo, Japan). Serum was separated, frozen immediately and stored at −80°C until analysed. To determine the levels of serum total bilirubin (TBIL), direct bilirubin (DBIL), albumin (ALB) and ammonia (NH_3), 10 µl of sera were applied onto DRI-CHEM slides (Fuji, Tokyo, Japan) and measured using DRI-CHEM 3500 (Fuji). The serum copper (Cu) level was measured by SRL Co. (Tokyo, Japan).

Histochemical analysis by haematoxylin and eosin staining of transplanted grafts and the host livers

FLC-loaded HA scaffolds were resected from the animals 3 weeks after transplantation. After removal of blood and surrounding adipose tissue with surgical scissors in phosphate-buffered saline (PBS) (Sigma, St Louis, USA), the grafts were fixed in 4% paraformaldehyde (PFA) (Sigma) in PBS. Host livers were harvested from the transplanted and control LEC rats. After being washed in PBS, the livers were fixed in 4% PFA in PBS. HE staining was performed by SRL Co.

Statistical analysis

The results are given as the mean ± SD. Statistical analyses were conducted using the Student's *t*-test. A $P<0.05$ was considered significant.

Results

Morphology of the isolated LEA rat FLCs and histology of the transplanted FLC-loaded HA sponges

We confirmed that plated cells isolated from E15.5 LEA rats showed the typical morphology of FLCs: epithelial, with a high nucleus/cytoplasm ratio, and smaller sized compared to mature hepatocytes (Fig. 3A). Isolated FLCs that were inoculated were mainly immobilized only on the surfaces of the HA sponges due to the small pore size of the scaffold (Fig. 1C). However, the graft excised 3 weeks after transplantation showed significant expansion and hepatocyte-like morphology inside the scaffolds, which is distinguishable across the mesentery (Fig. 3B and C). Furthermore, blood vessels and blood cells were observed among the hepatocyte-like cells inside the scaffolds. This indicates that the mesenteric blood vessels neovascularized the grafts. Concomitantly, FLCs were found even more than 200 µm from the HA sponge surface (Fig. 3B). These results demonstrated that the combination of highly proliferative FLCs and three-dimensional (3D) macroporous HA sponges, providing their subsequent growth upon implantation, enabled successful engraftment and further reorganization of the tissue precursor onto the mesentery of the recipient LEC rats.

Therapeutic effects of LEA rat FLC-loaded HA sponges on recipient LEC rats

Two weeks after being fed a conditioned diet, the control LEC rats showed severe jaundice, typically on their tails, ears and limbs, as already reported by Yamashita *et al.* (*21*) (Fig. 4A and B). In contrast,

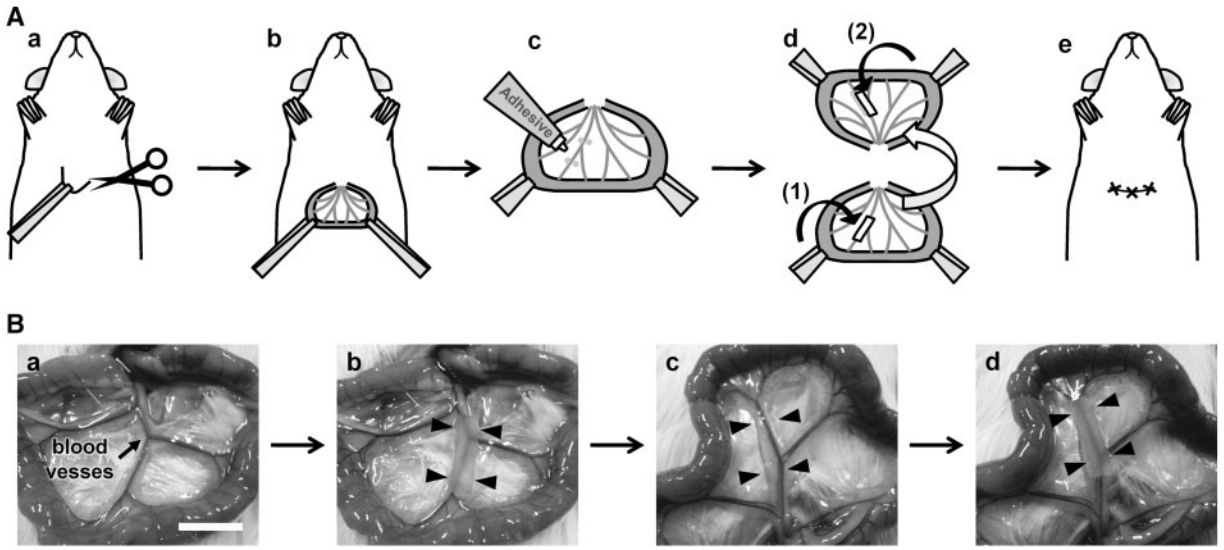

Fig. 2 Schematic view of surgical procedure in FLC-loaded HA sponge transplantation. (A) Summary of the whole surgical procedure. (a) The abdomen of a recipient LEC rat was opened with surgical scissors under ether anaesthesia, and (b) small intestine was pulled out with tweezers. (c) After a small drop of adhesive was applied on each corner surrounding the mesenteric blood vessel, (d) (*1*) an FLC-loaded HA sponge strip was attached onto the blood vessel, (*2*) subsequently the mesentery was folded back and another cell-loaded strip was attached. (e) Finally the abdomen was closed with a skin stapler (Natsume Seisakusho Co. Ltd, Tokyo, Japan). (B) Details of the attachment process of HA sponges. Scale bar: 1 cm. (a) The mesentery was pulled out from the abdomen. (b) An HA sponge strip was attached on the mesenteric blood vessel using adhesive (arrowheads). (c) The mesentery was folded back. The attached HA sponge strip can be seen on the underside (arrowheads). (d) Another FLC-loaded strip was attached (arrowheads).

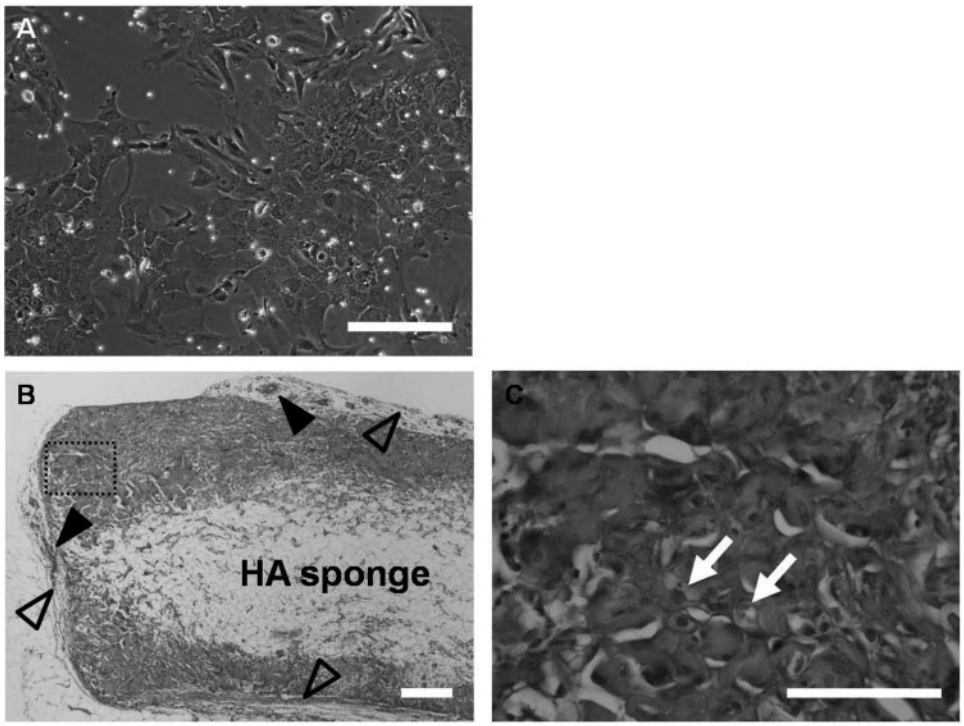

Fig. 3 Morphology of isolated FLCs and histology of the transplanted graft. (A) LEA rat FLCs on D 2 after plating on a collagen-coated 12-well plate. Scale bar: 200 μm. (B) FLC-loaded HA sponge scaffold excised 3 weeks after transplantation. Open arrowheads indicate the mesentery. Filled arrowheads indicate red mesenteric blood vessels surrounding the graft. Scale bar: 200 μm. (C) Higher magnification of the dotted region in (B). Arrows indicate red blood cells. Scale bar: 100 μm.

the rats transplanted with FLC-loaded HA sponges showed no jaundice within 3 weeks after transplantation (Fig. 4C and D).

Accordingly, the treated LEC rats showed a significant reduction in blood Cu concentration compared to the control LEC rats (Fig. 5A). Furthermore, we measured the serum concentration of several liver injury markers, and observed a significant decrease in serum TBIL and DBIL, and a significant increase in ALB productivity in the treated LEC rats as compared to the control LEC rats (Fig. 5B–D). Concomitantly, we observed a tendency towards reduction of NH_3 blood concentration in the treated LEC rats (Fig. 5E).

Histological analysis of the host liver of transplanted and control LEC rats

To evaluate whether the transplanted FLC-loaded HA sponges relieved the injury to the host livers, we investigated the injury status there by HE staining, using the transplanted and control LEC rats. The host liver of the control LEC rat showed fibrosis at the periportal area and extensive nuclear and cellular enlargement, typical histological features of damaged LEC rats (Fig. 6A and B) (*22*, *23*). In contrast, the host liver of the treated LEC rats showed a normal morphology (Fig. 6C and D).

Discussion

In this study, we investigated whether a heterotopically engineered liver tissue using FLCs immobilized in a 3D scaffold made from natural extracellular matrix (ECM) rather than adult hepatocytes has a therapeutic effect on a diseased animal, and noted that wild type LEA rat FLCs when embedded in HA sponges and transplanted on the recipient's mesenteric blood vessels, dramatically restored the liver function of WD model LEC rats. The metabolic deficiency in the LEC rats was remarkably corrected by this transplantation system, which indicates the possibility of the current method being expanded to various metabolic liver dysfunctions. Interestingly, our results demonstrated that the heterotopically engineered liver tissues suppressed host liver injury. These findings are of great importance because they demonstrate the possibility of utilizing liver progenitor cells rather than adult hepatocytes in liver tissue engineering.

Three weeks after transplantation, the FLCs formed a densely packed structure in the HA sponges. FLCs are regarded as a feasible source of liver progenitor cells because they have a highly efficient capacity to differentiate to hepatocytes and biliary epithelial cells (*24*, *25*), and a highly proliferative capacity both *in vitro* (*26*, *27*) and *in vivo* (*8*, *25*, *28*) compared to adult hepatocytes. Oren *et al.* (*8*) performed tissue heterografting of whole rat fetal livers rather than adult liver slices, and showed that FLCs could differentiate into hepatocytes and bile duct epithelium and that their repopulation capacity was better than that of adult hepatocytes. These reports led us to the hypothesis that proliferative and bipotential FLCs might construct a more complex and well-organized liver tissue than adult hepatocytes would, if an appropriate growth space is supplied.

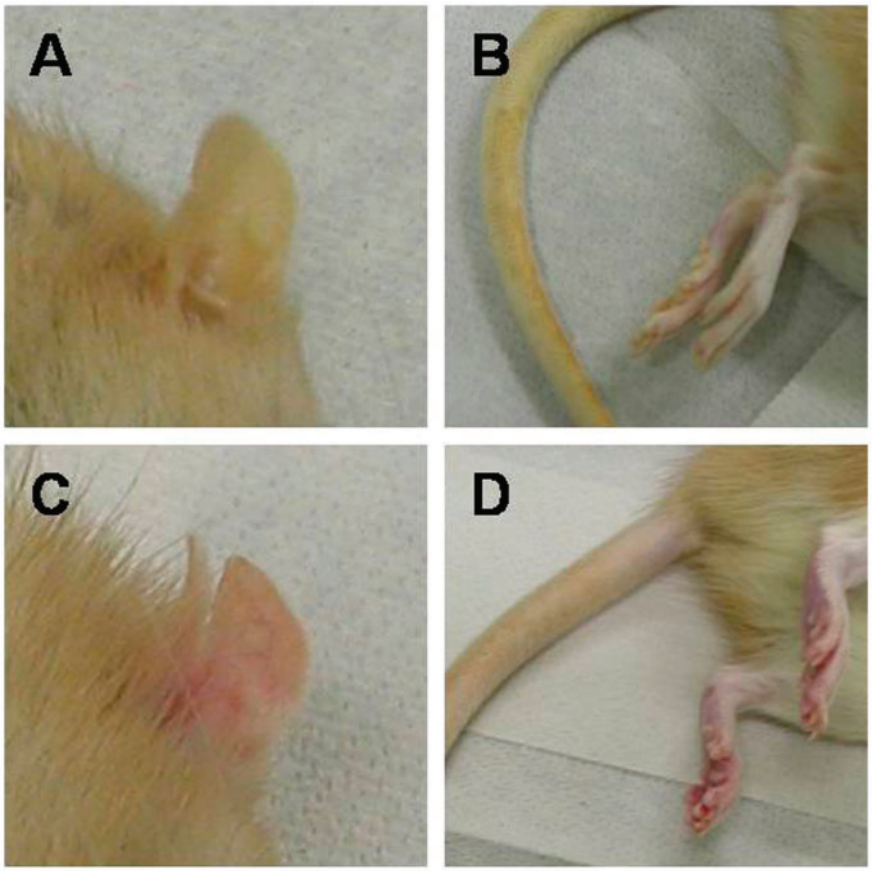

Fig. 4 Aspects of LEC rats 3 weeks after being fed a conditioned diet. Jaundice was observed in control LEC rats, typically on their ears (A), tails and limbs (B). In contrast, no jaundice was observed in treated LEC rats at least within 3 weeks after transplantation (C, D).

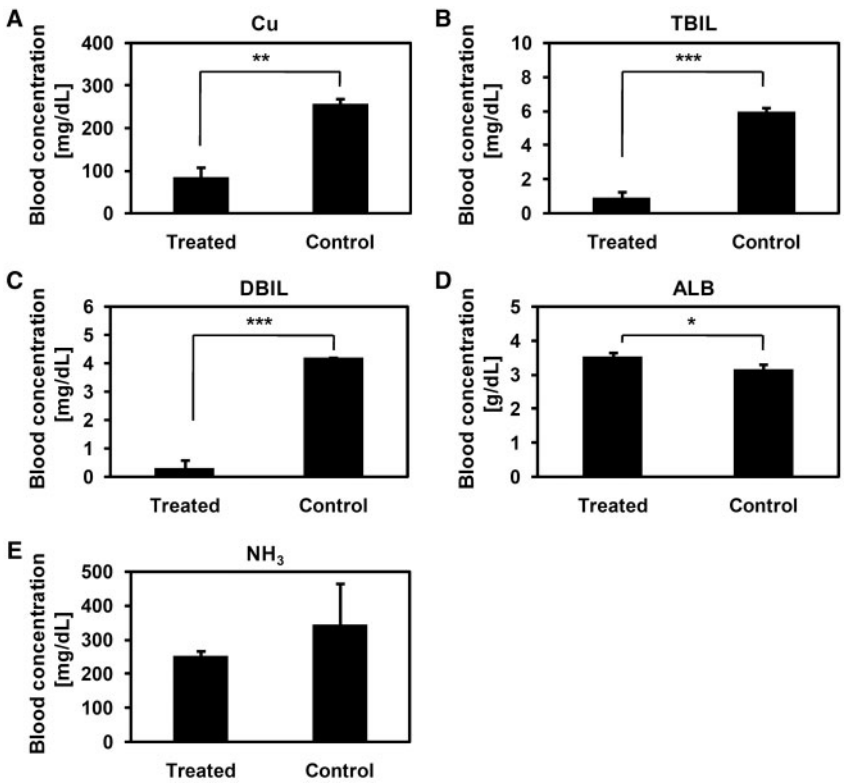

Fig. 5 Biochemical analysis of several liver injury markers. Concentration of Cu, TBIL, DBIL, ALB and NH_3 in blood serum of sacrificed LEC rats. Data are presented as the mean ± SD and were analysed by the Student's *t*-test; $n = 3$–4 for treated animals and 2–3 for control animals. ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

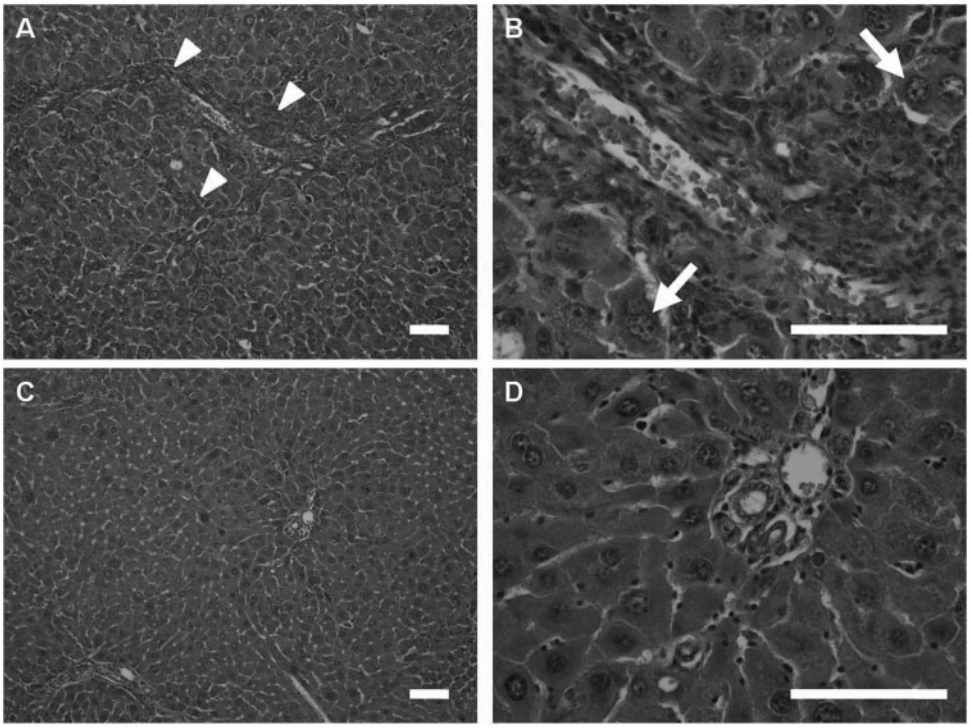

Fig. 6 Histology of the host livers of control and transplanted LEC rats 3 weeks after transplantation. (A) Histology of the host liver of a control LEC rat. Fibrosis was observed at periportal areas (arrowheads). (B) Higher magnification of (A). Extensive nuclear and cellular enlargement was observed in spots (arrows). (C) Histology of the host liver of a transplanted LEC rat. (D) Higher magnification of (C). Scale bar: 100 μm.

In liver tissue engineering, cells are coaxed to grow on ECM scaffolds that provide physical and chemical cues to guide cell differentiation and assembly into 3D tissues. In the liver, HA is present in the matrix of embryonic and fetal tissues and near the presumptive stem cell compartment, the canals of Hering, located in zone 1 of adult livers (*29*), but is not in association with the mature parenchymal cells. Therefore, HA is a candidate matrix component for 3D scaffolds for liver progenitor cells. In fact, several studies, besides ours (*20*), have demonstrated the suitability of HA for cultures of liver progenitor cells including FLCs (*29–32*).

Another remarkable finding is that host liver injury was restored by transplanted FLC-loaded HA sponges. Since host livers lack excretion of hepatic copper into the bile, we surmise that the uptake of copper could be ascribed to heterotopically transplanted grafts, leading to copper accumulation there. This directs us to address the issue of the elimination of the bile produced by an engineered liver tissue. Previous reports on heterotopic liver tissue engineering indicated that final bile clearance was dependent on the native liver (*33–35*). However, it would be necessary to explore the bile excretion system independent of the native liver in treating such a serious liver disease that cannot compensate for bile excretion. Thus, bile clearance is now our next problem to be solved in improvement of engineered liver tissues.

The mesentery has also been reported to be an appropriate site for FLC transplantation (*14*, *36*). However, there are no reports on a therapeutic effect of heterotopic liver tissue engineering using FLCs on the mesentery, presumably because of their poor hepatic functions. In this study we assessed this issue using an HA sponge as the ECM scaffold, and demonstrated the feasibility of this transplantation method. Although HA sponges were not fully occupied by the FLCs 3 weeks after transplantation, the thickness of the cellular region inside the grafts exceeded 200 μm on average (Fig. 3B). This indicated that mesentery-derived neovascularization in the HA sponges promoted, at least in part, FLCs engraftment and proliferation. Given that the embedded FLCs are allowed within 250–500 μm from the surface of the HA sponges and that the formed tissue gives the same cell density, the whole mass of the cellular region is calculated as ~0.21–0.35 cm^3/strip, that is, ~0.42–0.7 cm^3 for each recipient rat, equivalent to ~4–7% in volume of an entire rat liver mass (*37*). Improvements in promoting vascularization of the grafts need to be made, but it is noteworthy and greatly promising that even such a small mass of engineered liver tissue showed a remarkable therapeutic effect on inherited enzyme deficiency in the rat. This is in agreement with a previous report by Park *et al.* (*38*) demonstrating that intrasplenic transplantation of normal hepatocytes in LEC rats prevents fulminant hepatitis and reduces chronic inflammation. Furthermore, several other reports also showed that genetic disorders such as haemophilia or metabolic liver enzyme deficiencies can be restored by hepatocyte transplantation or transplantation of a small mass of engineered liver tissue (*34*, *35*, *39–41*).

However, to restore the loss of homeostasis caused by end-stage liver diseases, such as liver cancer, much larger mass of liver tissue is required. Our results point

to the great promise of our method for future clinical use because the mesentery provides further capacity for a transplantable area, as can be seen in Fig. 2B. An area of at least $30 \times 30\,cm^2$ can be anticipated to be available for transplantation on the human mesentery (*42*). Furthermore, recent progress in methods in engineering thick liver tissues (*43–47*) and vascularizing the engineered liver tissues (*11*, *12*) is remarkable. In general, to sustain human homeostasis, a tentative goal might be the obtaining of a mass sufficient to sustain the human body (20–30% mass of the whole liver, $300–500\,cm^3$) (*11*). If it is possible to engineer a liver tissue possessing 3–4 mm thickness, which is more than 10 times thicker than the current result and would accordingly necessitate vascularization, then engineering a liver tissue with $\sim 300\,cm^3$ can be realized. Moreover, much is expected from the use of human ES cells, induced pluripotent stem cells, or mesenchymal stem cells, and from vigorous development of technology to induce the differentiation of such stem cells into cells possessing hepatic functions (*48*). Therefore, integration of these new methods, including ours, could ultimately make it possible for liver tissue engineering to come to fruition.

In conclusion, by transplanting FLC-loaded HA sponges onto the mesenteric blood vessels of an animal model for WD, we established a novel transplantation system which exhibited a dramatic therapeutic effect. The results, in concert with recent vigorous development of technologies to engineer thick liver tissues, to vascularize engineered tissues, and to expand various stem cells and induce them to functional hepatocytes, hold out the great promise of our method for future clinical use.

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Conflict of interest

None declared.

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