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Decellularized Liver Matrix as a Carrier for Transplantation of Human Fetal and Primary Hepatocytes in Mice

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Abstract

Transplantation of primary hepatocytes has been shown to augment the function of damaged liver and to “bridge” patients to liver transplantation. However, primary hepatocytes often have low levels of engraftment and short survival after transplantation. To explore the potential benefits of using decellularized liver extracellular matrix (DLM) as a carrier for hepatocyte transplantation, DLM from the whole mouse liver was generated. Immortalized human fetal hepatocytes (FH-hTERT) or primary human hepatocytes were infused into DLM, which was then implanted into the omentum of immuno-deficient NOD/SCID/IL2r γ ^{-/-} or NOD/SCID/MPS VII mice. The removal of endogenous cellular components and the preservation of the extracellular matrix proteins and vasculature were demonstrated in the resulting DLM. Bioluminescent imaging revealed that FH-hTERT transduced with a lentiviral vector expressing firefly luciferase survived in the DLM for 8 weeks after peritoneal implantation; whereas, the luciferase signal from FH-hTERT rapidly declined in control mice 3–4 weeks after transplantation via splenic injection or with omental implantation after Matrigel encapsulation. Furthermore, primary human hepatocytes reconstituted in the DLM not only survived 6 weeks after transplantation, but also maintained their function, as demonstrated by mRNA levels of albumin and cytochrome P450 subtypes (CYP3A4, CYP2C9 and CYP1A1) similar to freshly isolated human primary hepatocytes. In contrast, when human primary hepatocytes were transplanted into mice via splenic injection, they failed to express CYP3A4, although they expressed albumin. In conclusion, decellularized liver extracellular matrix provides an excellent environment for long-term survival and maintenance of hepatocyte phenotype after transplantation.

Keywords

Bioluminescent image; Cell transplantation; Decellularization; Extracellular matrix

Liver transplantation is the only established treatment for patients with acute liver failure, end-stage liver disease, and inherited liver-based metabolic disorders. However, the scarcity

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of donor livers means that many patients on the waiting list will never receive a liver transplantation and many more are never listed. The complexity of liver function makes it impossible to use only mechanical devices to provide temporary support, as has been employed for cardiac and renal failure. Extracorporeal liver support devices require viable hepatocytes for many functions; moreover, primary hepatocyte transplantation procedures cause less morbidity and mortality than whole organ transplantation, and they could provide a sufficient cell mass to correct inherited metabolic deficiencies (1). Furthermore, we and others have demonstrated previously that transplantation of immortalized human fetal and neonatal hepatocytes in immunodeficient NOD-SCID mice via splenic injection allows the cells to migrate to the liver and mature in their liver-specific function (2,3).

However, hepatocyte transplantation is still far from a routine practice in the treatment of liver diseases. For example, many hepatocytes die shortly after transplantation and the survival and proliferation rates of transplanted primary or fetal hepatocytes in experimental animal liver are often low even if prior liver injury was induced in the recipient mice (4). Additionally, only a limited number of hepatocytes or liver progenitor cells can be transplanted by the widely accepted methods of injection via the portal vein or spleen. Thus, transplanted cells are incapable of correcting any metabolic abnormalities or to rescuing fulminant liver failure unless they have a proliferative advantage over the recipient hepatocytes.

Primary hepatocytes lose their typical morphology and function in culture within a few days via dedifferentiation or epithelial mesenchymal transition (5,6). This underscores the importance of the liver microenvironment in maintaining hepatocyte function. The extracellular matrix (ECM) not only provides a scaffold to house cells in liver tissue, but it also regulates adhesion, migration, differentiation, proliferation and survival of cells, as well as the interactions among different cell types (7). Recent advances in organ and tissue decellularization make it possible to obtain tissue-specific extracellular matrix from whole organs by perfusion of the organ with various detergents (8). Different from the traditional method of decellularization by immersing thin sliced tissues in various solutions for decellularization, the whole organ decellularized matrix maintains entire vascular network beds. These vascular network beds not only provide a convenient route for infusion of desired cell types but also a 3-dimensional environment for the infused cells in contrast to a 2-D environment provided from thin layers of decellularized matrix. Hence, we hypothesized that decellularized whole liver matrix (DLM) might provide an excellent microenvironment and scaffold for hepatocyte transplantation.

In the present study, we explored the feasibility and potential benefits of using decellularized liver extracellular matrix as a carrier for hepatocyte transplantation. Whole mouse livers were decellularized and subsequently reconstituted with human primary hepatocytes or immortalized fetal hepatocytes (FH-hTERT). The resulting cell-reconstituted DLM scaffolds were implanted into the omentum of immuno-deficient mice. We found that FH-hTERT survived longer when reconstituted in the DLM compared to those that were directly transplanted into recipient mice via splenic injection or by omental implantation with Matrigel encapsulation. Primary human hepatocytes reconstituted in the DLM survived and maintained their liver-specific protein expression up to 6 weeks after the implantation of the DLM.

MATERIALS AND METHODS

Cell Culture and Viral Transduction

The use of primary human hepatocytes and immortalized fetal hepatocytes was approved by the Institutional Review Board at the University of California, Davis, and was performed in

accordance with the guidelines for the protection of human subjects. Human fetal hepatocytes (hFH) were procured by Prof. S. Gupta at Albert Einstein College of Medicine, Bronx, New York with the approval of the Institutional Committee of Clinical Investigations. The immortalization of hFH by the reconstitution of the human telomerase gene was successfully achieved by ectopic expression of the telomerase reverse transcriptase using a retrovirus vector as we described previously (3). Immortalized FH-hTERT were cultured in DMEM high glucose (GIBCO) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1% penicillin/streptomycin, 9×10^{-5} M insulin and 5×10^{-6} M hydrocortisone (Sigma-Aldrich Co. St. Louis, MO). Human primary hepatocytes (hPH) were isolated, plated into culture plates as previously described (9), and provided by the Liver Tissue Procurement and Distribution System (LTPADS). Culture medium was changed to complete HCM medium (Lonza, Walkersville, MD) shortly after transfer by LTPADS (5). Cells were transduced with a lentiviral LUX-PGK-EGFP vector encoding the firefly luciferase and green fluorescent protein genes at a multiplicity of infection (MOI) of 20 in the presence of protamine sulfate (8 μ g/ml) (4,10). Seven days after transduction, GFP-positive FH-hTERT, but not hPH, were selected by fluorescence-activated cell sorting (FACS) as described previously (4).

Whole Liver Decellularization and Reconstitution of the Decellularized Liver Matrix with Hepatocytes

All animal experiments were performed in compliance with the NIH Guidelines for experimental animals, and the animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC). The liver perfusion procedure was performed according to a method we described previously (11–13). Briefly, the portal vein was cannulated as an inflow, and the inferior vena cava was cut as an opening of the outflow. Liver perfusion was carried out *in situ* at 37°C and at the speed of 5 ml/minutes. Decellularization was achieved by a method similar to the whole heart decellularization described previously (8) with modifications. Mouse liver was perfused sequentially with heparinized phosphate buffered saline (PBS) (12.5 U heparin/ml) for 15 min, 1% sodium dodecyl sulfate (SDS) for 2 hrs and 1% Triton-X100 for 30 min. Detergents were washed away by perfusion with PBS for additional 3 hrs and medium without FBS for 10 min. In order to visualize the vascular networks, DLM was injected with crystal violet dissolved in 1% low melting agarose via the portal vein. Micrograph images of vasculature in the resulting DLM were taken under a microscope. In order to examine the efficiency of the decellularization procedure, both fresh mouse liver and DLM were minced. DNA content in the liver and DLM was extracted as previously described (14) and quantitated by a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). To reconstitute the resulting DLM, FH-hTERT (2–4 million) or hPH (1–2 million) in 1 ml of medium were infused through a perfusion catheter after the completion of the decellularization procedure.

Transplantation of Hepatocytes in Mouse Models

NOD/SCID/MPS VII mice (15) and NOD/SCID/IL2 $\gamma^{-/-}$ mice (The Jackson Laboratories, Bar Harbor, Maine) were bred at the animal facility of the University of California, Davis. Mice that did not show thymoma or other tumor growth were included for data analysis. After culture for one day, decellularized liver matrix (approximately 0.5×0.5×0.1 cm in size) reconstituted with either FH-hTERT or hPH was implanted into the peritoneal cavity of immunodeficient mice by suturing the DLM into a pocket created by the omentum tissue. Animals were anesthetized with a mouse cocktail consisting of xylazine (5–10 mg/kg) and ketamine (50–100 mg/kg) in PBS by intraperitoneal injection. The middle incision was properly closed by silk suture. The first control group of animals was transplanted with one million human FH-hTERT or primary hepatocytes in 100 μ l medium via splenic injection as we described previously (4). The second control group received transplantation of FH-

hTERT after Matrigel encapsulation (1 million cells in 100 μ l of 25% Matrigel in medium (v/v)) into the omentum by direct injection.

Immunohistochemical and Immunofluorescent Analysis

After decellularization or being harvested from implanted animals, DLM was frozen in optimal cutting temperature embedding medium (Sakura, Torrance, CA) and sectioned in 12 μ m thickness. The DLM sections harvested from NOD/SCID/MPS VII mice were stained for β -glucuronidase (GUSB) activity as described previously (16). For immunostaining, frozen sections were fixed in 4% paraformaldehyde for 20 min, washed with PBS, and permeabilized with 0.2% Triton-X100 in PBS for 30 min. DLM sections were then blocked with 1% bovine serum albumin (BSA) for 1 hour and incubated with primary antibodies for 1–2 hrs. After washing with PBS, DLM sections were incubated with secondary antibodies conjugated with Alexa Fluor 488 (Invitrogen, Carlsbad, CA) for 1 hour. After washing with PBS, DLM sections were mounted with mounting medium containing 4,6-diaminidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). In order to examine cellular components in DLM sections, they were also stained for hematoxylin and eosin routinely. Primary antibodies against laminin and collagen IV were kindly provided by Dr. J. Peters (University of California, Davis), and were used at 1:400 dilution. Primary antibodies against fibronectin was obtained from Calbiochem (EMD, Gibbstown, NJ), and used at 1:200 dilution.

Quantitative Real-time RT-PCR

Fresh mouse liver and recellularized DLM were mechanically minced. Total RNA was isolated using RNeasy kits (Qiagen, Valencia, CA). First strand cDNA was generated using reverse transcriptase (Applied Biosystems, Foster City, CA). cDNA was subsequently subjected to PCR amplification using the ABI 7300 system under default conditions (Applied Biosystems, CA). The primers and probes for the human serum albumin (ALB) and α 1-antitrypsin (AAT) were described previously (3). The primers and probes for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), CYP3A4, CYP2C9 and CYP1A1 were purchased from Applied Biosystems. All samples were assayed in duplicate reactions and the means were normalized by the endogenous human GAPDH mRNA levels, and RNA levels were compared to RNA isolated from primary human hepatocytes right after receiving them from LTPADS, as described previously (3).

Bioluminescent Imaging

Transplanted mice were injected intraperitoneally with D-luciferin potassium salt (150 mg/kg body weight in 100 μ l PBS) and imaged under isofluorane anesthesia with the IVIS 100 Imaging System (Xenogen Corp.) at the Center for Molecular and Genomic Imaging, Department of Biomedical Engineering, UC Davis, for bioluminescent signals the day after transplantation and once a week thereafter (4). Individual mice were imaged for 5 min each time under anesthesia. Bioluminescence intensity was quantified in units of maximum photons per second per centimeter squared per steradian (p/s/cm²/sr) with the Living Imaging@2.50 software.

Statistical Analysis

Bioluminescent intensity was expressed as means $\pm$ SEM, and the data of splenic injection, omentum injection and DLM implantation were analyzed by the one way variance test, followed by Newman-Keuls test for multiple comparisons between any two groups at the corresponding time points. The *in vitro* RT-PCR data were analyzed by unpaired student t test. The *in vivo* RT-PCR data were expressed as a medium value, and the data comparing

DLM implantation with splenic injection were analyzed by signed rank sum test. A *p*-value of less than 0.05 was considered as statistically significant.

RESULTS

Decellularization of Mouse Liver

To create whole liver decellularized extracellular matrix, we perfused mouse liver *in situ* with a series of detergent solutions as previously described for rat heart decellularization. The removal of cellular components was reflected by the color change of the liver during the perfusion (Fig. 1A). The liver became semi-transparent after perfusion with 1% SDS for 2 hrs and then 1% Triton-X100 for 30 min (Fig. 1B). After subsequent perfusion with PBS for 3 hrs to wash away the remaining detergents, the resulting DLM was removed from the mouse, and cryopreserved and sectioned for further characterization. No significant remains of cellular components in the DLM were evidenced by H&E staining (Fig. 1C) and DAPI staining of these DLM sections (Fig. 1E). Residual DNA content in DLM was only 4% of the normal liver (73 ± 39 $\mu\text{g/g}$ DLM vs. 1750 ± 291 $\mu\text{g/g}$ liver). The vascular network was well preserved in DLM and was easily visualized by injection of crystal violet via the portal vein (Fig. 1D). The preservation of the extracellular matrix (ECM) proteins, such as collagen IV, fibronectin and laminin, in the DLM was verified by positive immunostaining of these ECM components (Fig. 1E). Therefore, our perfusion protocol with a series of detergent solutions effectively removed cellular components while preserving important extracellular matrix proteins, including collagen IV, fibronectin and laminin, as well as the vasculature.

Survival of Immortalized Human Fetal Hepatocytes in DLM in Culture

To assess whether the DLM facilitates the survival of liver cells, we first used FH-hTERT transduced with a lentiviral LUX-PGK-EGFP vector encoding the luciferase gene and the green fluorescent protein (GFP) gene to reconstitute DLM via infusion. The majority of the cells remained within the vascular bed directly after the infusion (Fig. 2A). After culture for 1 week following cell reconstitution, GFP positive cells were still visible in the DLM and migrated into the parenchymal matrix (Fig. 2B&C), suggesting that these reconstituted cells survived in the DLM. This was also shown using FH-hTERT without LUX-PGK-EGFP lentiviral transduction (data not shown). Furthermore, quantitative real-time RT-PCR analysis of human albumin (ALB) and α -antitrypsin (AAT) mRNA levels in the DLM reconstituted with FH-hTERT showed a 2.5 to 3.5-fold increase in the levels of both hepatic-specific genes in comparison to FH-hTERT cultured under standard conditions (Fig. 2D), suggesting that these cells in DLM improved significantly in their hepatic-specific gene expression *in vitro*.

Bioluminescent Imaging of Mice Transplanted with FH-hTERT

Having established that DLM supports the survival of FH-hTERT cells *in vitro*, we next assessed whether the DLM facilitates the survival and function of these cells *in vivo*. The bioluminescent imaging modality offers a non-invasive approach to track the engraftment and repopulation of transplanted cells *in vivo*. To employ this technology, we reconstituted DLM with FH-hTERT after transduction of the lentiviral LUX-PGK-EGFP vector, and then implanted the reconstituted DLM in the omentum of NOD/SCID/IL2 $\gamma^{-/-}$ mice. For comparison, FH-hTERT with lentiviral vector transduction were injected into the spleen because splenic injection is a widely accepted method of hepatocyte transplantation in rodents. In a separate group, lentiviral vector-transduced FH-hTERT were first encapsulated in commercially available Matrigel, and then Matrigel-encapsulated FH-hTERT were injected into the omentum. Bioluminescent imaging of transplanted cells was conducted 1 day after cell transplantation, and once a week thereafter for 8 weeks. Fig. 3A shows

repeated bioluminescent imaging of three representative mice at selected time points with DLM implantation, splenic or omentum injection; while Fig. 3B shows the average bioluminescent intensity of luciferase activity in these three groups of mice. We found that bioluminescent signals rapidly faded in the liver area of mice with splenic injection within 3 weeks and that the bioluminescent signal strength declined to 0.39% of the initial level 37 days after splenic injection. The bioluminescent signal strength in mice receiving the injection of FH-hTERT with Matrigel encapsulation in the omentum declined (0.923%) in a trend similar to that of splenic injection. In contrast, bioluminescent signals declined less rapidly in mice transplanted with cells reconstituted in the DLM up to 8 weeks (2.65%), and statistically significant difference in bioluminescent intensity at several time points exists between the DLM group and the other 2 groups ($p < 0.05$ – 0.001). These data clearly demonstrate that DLM enhanced the survival of immortalized fetal hepatocytes *in vivo*.

Survival of Human Primary Hepatocytes in DLM after Implantation

We next assessed whether DLM is a good carrier for the transplantation of primary human hepatocytes. The DLM was reconstituted with hPH and the resulting scaffolds were implanted into the omentum of NOD/SCID/MPS VII mice. Since these mice were null for the enzyme of β -glucuronidase, which is encoded by the GUSB gene, human hepatocytes with normal GUSB expression can be easily visualized by using the substrate reaction to detect β -glucuronidase enzyme activity. One week after implantation, the implanted DLM was collected for β -glucuronidase staining. β -Glucuronidase-positive cells in red were clearly visible in the DLM (Fig. 4A). A similar experiment was performed using hPH transduced with the lentiviral LUX-PGK-EGFP vector in NOD/SCID/IL2 $\gamma^{-/-}$ mice, a more severely immunodeficient strain. Six weeks after implantation, GFP-positive cells were identified in the DLM under a fluorescent microscope (Fig. 4B). It is also noticeable that GFP-negative mouse cells had migrated into the implanted DLM (Fig. 4B). Therefore, these data clearly demonstrate that the DLM facilitates the survival of human primary hepatocytes *in vivo*.

Function of Primary Human Hepatocytes in the DLM after Implantation

Having established that the DLM facilitates the survival of hPH, we next examined whether hPH maintained their liver-specific function in DLM after being implanted into mice. Human primary hepatocytes were infused into DLM and subsequently the DLM reconstituted with human primary hepatocytes was implanted into the omentum of NOD/SCID/IL2 $\gamma^{-/-}$ mice. Human primary hepatocyte transplantation via splenic injection was used as a control. Six weeks after implantation or transplantation, total RNA was isolated from the implanted DLM or the livers of the mice with splenic injection. Quantitative real-time RT-PCR analysis was carried out using RNA from freshly isolated hPH as a control to evaluate mRNA levels of the liver-specific genes in these samples. Cells in the DLM showed a level of albumin expression comparable to freshly isolated hPH (Fig. 5A). Human primary hepatocytes in mouse liver after splenic injection showed a similar level of albumin gene expression to cells in DLM (Fig. 5A), although their medium albumin expression level was slightly higher than cells in DLM ($p > 0.05$). One of the hepatic-specific functions is to metabolize endogenous substrates and xenobiotics including drugs. The cytochrome P450 family enzymes (CYPs) catalyze the oxidation and transformation of endogenous or exogenous substances. CYP3A4 is the most abundant P450 subtype in the liver. We found that hPHs reconstituted in DLM in 3 out of 4 mice exhibited a high level of CYP3A4 mRNA compared to the freshly isolated hPH (Fig. 5B). In contrast, hPHs after splenic injection did not show any CYP3A4 mRNA (Fig. 5B). Similarly, increased CYP1A1 expression was detected in hPHs reconstituted in DLM in all 4 mice, but it was absent in most of the mice (5 out of 6) with splenic injection (Fig. 5C). The CYP2C9 levels in hPHs reconstituted in DLM were similar to freshly isolated hPHs. hPHs transplanted in mice via splenic injection

showed a detectable CYP2C9 mRNA level in 4 out of 6 mice (Fig. 5D). In summary, these data demonstrate that hPHs reconstituted in the DLM maintained liver-specific gene expression levels at least as high as splenic injection, and that two key markers of hepatocyte maturation, CYP3A4 and CYP1A1, were expressed at significantly higher levels in hPH that had been reconstituted in the decellularized matrix.

DISCUSSION

Decellularized extracellular matrix of blood vessels, cardiac valves, bladder and intestine has been used for facilitating cell transplantation (17–20). An *in vitro* study of using decellularized liver extracellular matrix for hepatocyte culture has been reported (21). It was shown that human hepatocytes cultured between two layers of porcine liver decellularized matrix *in vitro* for 10 days exhibited liver-specific function similar to those cells grown in a Matrigel sandwich (21), and that rat hepatocytes seeded between the sheets of decellularized liver matrix showed good viability and function *in vitro* (22,23). Some of these previous studies employed pieces of decellularized liver matrices, and the decellularized matrix tissue was lyophilized into a powder form, and was rehydrated to generate a gel-like carrier. Our study started with whole liver decellularization and cells were infused into the DLM immediately after decellularization. Our decellularization procedure which employed a much shorter period (6 hrs instead of 3 days) was as effective as a long decellularization protocol in terms of residual DNA content in the DLM (24). At the same time, the structure of DLM was extremely well preserved as demonstrated by full preservation of extracellular matrix and vasculature (Fig. 1). Moreover, our *in vitro* and *in vivo* data clearly demonstrated that the DLM facilitated both survival and function of human primary hepatocytes and fetal hepatocytes for up to 6–8 weeks after implantation as evidenced by bioluminescent imaging, immunohistochemical staining and quantitative RT-PCR assays.

Splenic injection has been widely used as a route for transplantation of hepatocytes in rodents (25). We compared cell survival between using the DLM as a carrier and splenic injection, and found that fetal hepatocytes reconstituted in the DLM survived much longer than those with splenic injection. It appears that fetal hepatocytes migrated to the liver within a few days after splenic injection as demonstrated in our bioluminescent imaging study (data not shown). With this route of cell transplantation, the luciferase signal strength rapidly declined within 3 weeks after cell transplantation, which was similar to the findings we previously reported when NOD-SCID mice were not pre-treated with methylcholanthrene and monocrotaline (4). We added an additional control group by the direct injection of HF-hTERT into the omentum after Matrigel encapsulation. The CCD camera imaging showed a trend of decline in bioluminescent intensity similar to that of splenic injection. In contrast, bioluminescent signal strength from HF-hTERT reconstituted into the DLM was sustained for up to 8 weeks. Presumably, the engraftment of HF-hTERT would be easier in DLM than in mouse liver because there is a vast space available, and intact extracellular matrix components in their original configuration remain after the completion of the decellularization. The result appeared to be better than when Matrigel was used to encapsulate HF-hTERT and encapsulated cells were implanted into the omentum (26). Human primary hepatocytes via either splenic injection or implantation in DLM survived in mice, and expressed liver-specific genes, such as albumin and CYP2C9. Moreover, primary hepatocytes in DLM expressed key mature markers, CYP3A4 and CYP1A1. Our data indicate that DLM is superior to splenic injection for maintaining the function of primary human hepatocytes.

The establishment of a proper vascular system in the reconstituted DLM may be a critical issue for the survival of the transplanted cells. Bioluminescent imaging of FH-hTERT and primary hepatocytes with lentiviral LUX-PGK-EGFP transduction reconstituted in DLM

revealed that the luciferase signals were sustained for a period of 8 weeks after implantation in NOD/SCID/IL2 $\gamma^{-/-}$ mice, a strain of mouse which is to date the most immunodeficient, although the strength of the signals declined after the first week. These data indicate that the reconstituted cells may be able to access some, but not sufficient, blood supply as indicated by the presence of mouse cells in the implanted DLM. We employed small pieces ($0.5 \times 0.5 \times 0.1$ cm³) of reconstituted DLM which were implanted in vascular-rich omentum in our experiments. This may have contributed to the prolonged survival and improved function of primary hepatocytes because the omentum has been a favorable site for engraftment of hepatocyte polymer tissue-engineered constructs in comparison to subcutaneous compartments (26). However, when a larger size of DLM is needed for human cell transplantation, adequate blood supply with existing vasculature will be essential. Infusion of vascular endothelial cells or their precursor cells together with hepatocytes may facilitate the revascularization of the DLM. Linke *et al.* reported that pre-seeding a decellularized porcine jejunal segment with macrovascular endothelial cells before seeding porcine hepatocytes led to the maintenance of liver-specific function for 3 weeks *in vitro* (27). In our previous studies, we demonstrated that human bone marrow or umbilical cord blood-derived precursor endothelial cells or endothelial cells isolated from placenta and other stem cell types rapidly improved vascularization of ischemic tissues (28–30). We are currently investigating the potential benefits of co-seeding hepatocytes with these cells in DLM to promote more rapid and robust revascularization. Another option would be vessel anastomosis to the recipient's systemic or portal circulation (24). Although the recent study reported by Uygun *et al.* demonstrated the feasibility of the transplantation of a re-grown liver lobe from DLM with rat hepatocytes, the duration of the graft survival in rat recipients still requires improvement (24). In the present study we have examined the long-term survival of human hepatocytes in an engineered liver graft.

Our data suggest that DLM is an excellent carrier for transplantation of primary hepatocytes. However, the mechanism underlying this benefit is yet to be investigated. Integrins are major mediators of cell adhesion. ECM components including collagen and fibronectin bind to the RGD domain of integrins, and activate not only focal adhesion molecules but also cell survival signals, for instance, via the phosphoinositol-3, Akt or MAPK signaling pathways (31). In a study by Gupta and colleagues, infusion of collagen or fibronectin-like polymer through the portal vein prior to hepatocyte transplantation enhanced the engraftment of transplanted cells (32), which suggests a crucial role of extracellular matrix components in the integrity and function of transplanted hepatocytes. The decellularized liver matrix with the natural extracellular matrix components in a three-dimensional configuration appears to be responsible for prolonged survival and function of hepatocytes.

In conclusion, the findings in the present study demonstrate that decellularized liver matrix allows human fetal hepatocytes to survive longer than splenic or omentum injection in mice after transplantation. Moreover, the decellularized liver matrix maintains the liver-specific function of primary hepatocytes after implantation. Taken together, these data suggest the possibility that decellularized liver matrix may be developed as an alternative carrier for hepatocyte transplantation, when a large number of viable hepatocytes are required to functionally replace a failing liver.

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List of Abbreviations

AAT	α 1-antitrypsin
ALB	albumin
CYP	cytochrome p450 family
DAPI	4,6-diaminidino-2-phenylindole
DLM	decellularized liver matrix
ECM	extracellular matrix
FH-hTERT	telomerase-immortalized human fetal hepatocytes
GUSB	beta-glucuronidase
NOD/SCID/IL2γ^{-/-}	nonobese diabetic/severe combined immunodeficient/interleukin 2 receptor γ deficient
NOD/SCID/MPS VII	nonobese diabetic/severe combined immunodeficient/ mucopolysaccharidosis type VII
hPH	human primary hepatocytes
RT-PCR	reverse transcriptase polymerase reaction

References

1. Fox IJ, Chowdhury JR, Kaufman SS, Goertzen TC, Chowdhury NR, Warkentin PI, et al. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. *N Engl J Med*. 1998; 338:1422–6. [PubMed: 9580649]
2. Wege H, Chui MS, Le HT, Strom SC, Zern MA. In vitro expansion of human hepatocytes is restricted by telomere-dependent replicative aging. *Cell Transplant*. 2003; 12:897–906. [PubMed: 14763510]
3. Wege H, Le HT, Chui MS, Liu L, Wu J, Giri R, et al. Telomerase reconstitution immortalizes human fetal hepatocytes without disrupting their differentiation potential. *Gastroenterology*. 2003; 124:432–44. [PubMed: 12557149]
4. Choi MS, Catana AM, Wu J, Kim YS, Yoon SJ, Borowsky AD, et al. Use of bioluminescent imaging to assay the transplantation of immortalized human fetal hepatocytes into mice. *Cell Transplant*. 2008; 17:899–909. [PubMed: 19069633]
5. Yamamoto N, Wu J, Zhang Y, Catana AM, Cai H, Strom S, et al. An optimal culture condition maintains human hepatocyte phenotype after long-term culture. *Hepatol Res*. 2006; 35:169–77. [PubMed: 16807086]
6. Copple BL. Hypoxia stimulates hepatocyte epithelial to mesenchymal transition by hypoxia-inducible factor and transforming growth factor-beta-dependent mechanisms. *Liver Int*. 2010; 30:669–82. [PubMed: 20158611]
7. Svegliati-Baroni G, De Minicis S, Marziani M. Hepatic fibrogenesis in response to chronic liver injury: novel insights on the role of cell-to-cell interaction and transition. *Liver Int*. 2008; 28:1052–64. [PubMed: 18783548]
8. Ott HC, Matthiesen TS, Goh SK, Black LD, Kren SM, Netoff TI, et al. Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nat Med*. 2008; 14:213–21. [PubMed: 18193059]

9. Kostrubsky VE, Ramachandran V, Venkataramanan R, Dorko K, Esplen JE, Zhang S, et al. The use of human hepatocyte cultures to study the induction of cytochrome P-450. *Drug Metab Dispos.* 1999; 27:887–94. [PubMed: 10421615]
10. Oertel M, Rosencrantz R, Chen YQ, Thota PN, Sandhu JS, Dabeva MD, et al. Repopulation of rat liver by fetal hepatoblasts and adult hepatocytes transduced ex vivo with lentiviral vectors. *Hepatology.* 2003; 37:994–1005. [PubMed: 12717380]
11. Wu J, Soderbergh H, Karlsson K, Danielsson A. Protective effect of S-adenosyl-L-methionine on bromobenzene- and D-galactosamine-induced toxicity to isolated rat hepatocytes. *Hepatology.* 1996; 23:359–65. [PubMed: 8591864]
12. Wu J, Liu SL, Zhu JL, Norton PA, Nojiri S, Hoek JB, et al. Roles of tissue transglutaminase in ethanol-induced inhibition of hepatocyte proliferation and alpha 1-adrenergic signal transduction. *J Biol Chem.* 2000; 275:22213–9. [PubMed: 10801782]
13. Zhang Y, Venugopal SK, He S, Liu P, Wu J, Zern MA. Ethanol induces apoptosis in hepatocytes by a pathway involving novel protein kinase C isoforms. *Cell Signal.* 2007; 19:2339–50. [PubMed: 17728104]
14. Laird PW, Zijderfeld A, Linders K, Rudnicki MA, Jaenisch R, Berns A. Simplified mammalian DNA isolation procedure. *Nucleic Acids Res.* 1991; 19:4293. [PubMed: 1870982]
15. Zhou P, Hohm S, Olusanya Y, Hess DA, Nolte J. Human progenitor cells with high aldehyde dehydrogenase activity efficiently engraft into damaged liver in a novel model. *Hepatology.* 2009; 49:1992–2000. [PubMed: 19437487]
16. Hofling AA, Devine S, Vogler C, Sands MS. Human CD34+ hematopoietic progenitor cell-directed lentiviral-mediated gene therapy in a xenotransplantation model of lysosomal storage disease. *Mol Ther.* 2004; 9:856–65. [PubMed: 15194052]
17. Schmidt CE, Baier JM. Acellular vascular tissues: natural biomaterials for tissue repair and tissue engineering. *Biomaterials.* 2000; 21:2215–31. [PubMed: 11026628]
18. Schultheiss D, Gabouev AI, Cebotari S, Tudorache I, Walles T, Schlote N, et al. Biological vascularized matrix for bladder tissue engineering: matrix preparation, reseeding technique and short-term implantation in a porcine model. *J Urol.* 2005; 173:276–80. [PubMed: 15592096]
19. El-Hakim A, Marcovich R, Chiu KY, Lee BR, Smith AD. First prize: ureteral segmental replacement revisited. *J Endourol.* 2005; 19:1069–74. [PubMed: 16283842]
20. Badylak SF, Park K, Peppas N, McCabe G, Yoder M. Marrow-derived cells populate scaffolds composed of xenogeneic extracellular matrix. *Exp Hematol.* 2001; 29:1310–8. [PubMed: 11698127]
21. Sellaro TL, Ranade A, Faulk DM, McCabe GP, Dorko K, Badylak SF, et al. Maintenance of human hepatocyte function in vitro by liver-derived extracellular matrix gels. *Tissue Eng Part A.* 2009; 16:1075–82. [PubMed: 19845461]
22. Burra P, Tomat S, Conconi MT, Macchi C, Russo FP, Parnigotto PP, et al. Acellular liver matrix improves the survival and functions of isolated rat hepatocytes cultured in vitro. *Int J Mol Med.* 2004; 14:511–5. [PubMed: 15375576]
23. Lin P, Chan WC, Badylak SF, Bhatia SN. Assessing porcine liver-derived biomatrix for hepatic tissue engineering. *Tissue Eng.* 2004; 10:1046–53. [PubMed: 15363162]
24. Uygun BE, Soto-Gutierrez A, Yagi H, Izamis ML, Guzzardi MA, Shulman C, et al. Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. *Nat Med.* 2010; 16:814–20. [PubMed: 20543851]
25. Benten D, Kumaran V, Joseph B, Schattenberg J, Popov Y, Schuppan D, et al. Hepatocyte transplantation activates hepatic stellate cells with beneficial modulation of cell engraftment in the rat. *Hepatology.* 2005; 42:1072–81. [PubMed: 16250034]
26. Lee H, Cusick RA, Utsunomiya H, Ma PX, Langer R, Vacanti JP. Effect of implantation site on hepatocytes heterotopically transplanted on biodegradable polymer scaffolds. *Tissue Eng.* 2003; 9:1227–32. [PubMed: 14670110]
27. Linke K, Schanz J, Hansmann J, Walles T, Brunner H, Mertsching H. Engineered liver-like tissue on a capillarized matrix for applied research. *Tissue Eng.* 2007; 13:2699–707. [PubMed: 17867928]

28. Capoccia BJ, Robson DL, Levac KD, Maxwell DJ, Hohm SA, Neelamkavil MJ, et al. Revascularization of ischemic limbs after transplantation of human bone marrow cells with high aldehyde dehydrogenase activity. *Blood*. 2009; 113:5340–51. [PubMed: 19324906]
29. Rosova I, Dao M, Capoccia B, Link D, Nolte JA. Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. *Stem Cells*. 2008; 26:2173–82. [PubMed: 18511601]
30. Sondergaard CDH, Maxwell D, Weinheimer C, Rosova I, Creer M, Piwnicka-Worms D, et al. Human cord blood progenitors with high aldehyde dehydrogenase activity improve vascular density in a model of acute myocardial infarction. *J Translat Med*. 2010 in press.
31. Scott MJ, Billiar TR. Beta2-integrin-induced p38 MAPK activation is a key mediator in the CD14/TLR4/MD2-dependent uptake of lipopolysaccharide by hepatocytes. *J Biol Chem*. 2008; 283:29433–46. [PubMed: 18701460]
32. Kumaran V, Joseph B, Benten D, Gupta S. Integrin and extracellular matrix interactions regulate engraftment of transplanted hepatocytes in the rat liver. *Gastroenterology*. 2005; 129:1643–53. [PubMed: 16285962]

Fig. 1. Characterization of the decellularized liver matrix (DLM)

(A) Representative mouse liver images of color changes during an *in situ* decellularization process at 0, 12, 30, 60 and 120 min of perfusion with 1% SDS. (B) DLM harvested from a mouse after the completion of a decellularization procedure. (C) H&E staining of a DLM slice demonstrating no remaining cellular components (100 $\times$). (D). DLM was injected with crystal violet in agarose through the portal vein after the completion of a decellularization procedure for the visualization of remaining vasculature networks (20 $\times$). (E) Mouse liver and the DLM cryosections were immuno-stained with antibodies against the indicated extracellular matrix proteins (fibronectin, laminin and collagen IV in green) and DAPI (blue) in the mouse liver. Please note that there was no DAPI staining in the DLM on the corresponding right panels (200 $\times$).

Fig. 2. FH-hTERT cultured in DLM

FH-hTERT transduced with a lentiviral vector carrying LUX-PGK-EGFP were infused into the DLM after the completion of perfusion (A) and cultured for 7 days (B & C). Fluorescent images were taken at 40 $\times$ (A & B) and 200 $\times$ (C) magnification. (D) Quantitative real-time RT-PCR analysis of ALB and AAT mRNA levels in FH-hTERT reconstituted in DLM cultured for 7 days. ** $p < 0.01$ compared to FH-hTERT cultured in standard conditions (n=3).

Fig. 3. Bioluminescent imaging of FH-hTERT over time after transplantation

After transduction with lentiviral LUX-PGK-EGFP vector and enrichment by FACS, FH-hTERT were either infused into DLM and then implanted into mice or transplanted via splenic or omentum injection. **(A)** Representative bioluminescent images for the same mice over time with three modes of transplantation. **(B)** Bioluminescent signal intensity for the mice with splenic injection (n=5), omentum injection (n=4) or DLM implantation (n=4) at each time point. *, ** and *** correspond to $P < 0.05$, 0.005 and 0.001 respectively in comparison to splenic injection at corresponding time points. Δ and $\Delta\Delta$ correspond to $p < 0.05$ and 0.01 in comparison to omentum injection at corresponding time points. The line indicates minimal signal strength to be imaged.

Fig. 4. DLM facilitates the survival of human primary hepatocytes *in vivo*

(A) GUSB staining (red) of human primary hepatocytes in the DLM 1 week after implantation into NOD/SCID/MPS VII mice. (B) Human primary hepatocytes transduced with the lentiviral LUX-PGK-EGFP vector and reconstituted in DLM were implanted into NOD/SCID/IL2 $\gamma^{-/-}$ mice. The fluorescent image of the harvested DLM was made 6 weeks after implantation. GFP-positive human primary hepatocytes were visualized in green within the DLM.

Fig. 5. Quantitative real-time RT-PCR analysis of mRNA levels of the liver-specific gene ALB (A), CYP3A4 (B), CYP1A1 (C) and CYP2C9 (D) in the livers or DLM implants of transplanted mice 6 weeks after transplantation. Human primary hepatocytes were either reconstituted in DLM or transplanted into in NOD/SCID/IL2 $\gamma^{-/-}$ mice via splenic injection. The median value of each group is indicated with a bar. The number of animals from each group is shown in each plot, and there was no significant statistical difference in gene expression levels between DLM implantation and splenic injection in **B**, **C** and **D**. Expression levels of liver-specific genes were calculated based on that of freshly isolated human primary hepatocytes.