Liver Tissue Engineering at Extrahepatic Sites in Mice as a Potential New Therapy for Genetic Liver Diseases

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Liver tissue engineering using hepatocyte transplantation has been proposed as an alternative to whole-organ transplantation or liver-directed gene therapy to correct various types of hepatic insufficiency. Hepatocytes are not sustained when transplanted under the kidney capsule of syngeneic mice. However, when we transplanted hepatocytes with the extracellular matrix components extracted from Engelbreth-Holm-Swarm cells, hepatocytes survived for at least 140 days and formed small liver tissues. Liver engineering in hemophilia A mice reconstituted 5% to 10% of normal clotting activity, enough to reduce the bleeding time and have a therapeutic benefit. Conversely, the subcutaneous space did not support the persistent survival of hepatocytes with Engelbreth-Holm-Swarm gel matrix. We hypothesized that establishing a local vascular network at the transplantation site would reduce graft loss. To test this idea, we provided a potent angiogenic agent before hepatocyte transplantation into the subcutaneous space. With this procedure, persistent survival was achieved for the length of the experiment (120 days). To establish that these engineered liver tissues also retained their native regeneration potential *in vivo*, we induced two different modes of proliferative stimulus to the naïve liver and confirmed that hepatocytes within the extrahepatic tissues regenerated with activity similar to that of naïve liver. In conclusion, our studies indicate that liver tissues can be engineered and maintained at extrahepatic sites, retain their capacity for regeneration *in vivo*, and used to successfully treat genetic disorders. (HEPATOLOGY 2005; 41:132–140.)

Development of cellular-based therapies, including hepatocyte transplantation and liver tissue engineering, has been attempted to treat different forms of liver diseases as an alternative to liver organ transplantation.[1–4] By transplanting isolated hepatocytes through the portal circulation into the liver, encouraging results were reported in patients with Criglar-Najjar syndrome and a glycogen storage disease.[1,2] However, this approach of cell transplantation through the portal vein is limited in the number of cells that can be transplanted at one time because of potential life-threatening complications.[3,5,6] To increase the utility of hepatocyte-based therapy, it is important to develop a method that allows for the engraftment of a greater number of hepatocytes. In this context, transplanting hepatocytes at an extrahepatic site is attractive because it would provide additional space to maintain a greater number of cells, and with fewer complications.[3,7–9] This type of approach is extremely viable from the standpoint of liver tissue engineering. Researchers have transplanted hepatocytes at several different extrahepatic sites, including the intraperitoneal cavity, pancreas, mesenteric leaves, lung parenchyma, under the kidney capsule, and in the subcutaneous space.[3,5,8,10,11] Irrespective of the sites used, the studies reported inefficient engraftment of hepatocytes and very short-term survival. To overcome this issue, we have recently used an agonistic antibody that stimulates the HGF/cMet pathway to achieve persistent survival of human hepatocytes (>140 days) grafted into mice under the kidney capsule space.[8,12] These successes have encouraged us to establish more clinically feasible methods for liver tissue engineering at

Abbreviations: hAAT, human alpha-1 antitrypsin; EHS, Engelbreth-Holm-Swarm; aFGF, acidic fibroblast growth factor; MS, microspheres; DH, direct hyperplasia; TCPOBOP, 1,4-bis[2-(3,5-dicholoropyridyloxy)] benzene; BrdU, 5-bromo-2-deoxyuridine.

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extrahepatic sites that do not require the administration of additional compounds.

Materials and Methods

Hepatocyte Isolation. Hepatocytes were isolated from 10- to 15-week-old human alpha-1 antitrypsin (hAAT) transgenic mice (hA1AT-FVB/N, kindly provided by Dr. Bumgardner, Ohio State University, Columbus, OH) by *in situ* collagenase perfusion (Collagenase D, Boehringer Mannheim, Indianapolis, IN) of the liver through the inferior vena cava.[3,8] Cells were filtered and hepatocytes were separated from nonparenchymal cells by 3 rounds of low-speed centrifugation at 50*g*. Hepatocytes with viabilities more than 80%, as quantified by trypan blue exclusion, were used in the present studies. Cells were stored at 4°C before transplantation. Hepatocytes also were isolated from C57Bl/6 mice (CLEA Japan Inc., Tokyo, Japan) for the hemophilia mouse study.

Transplantation Procedures. All animal studies used the institutional guidelines set forth by Stanford University and the Nara Medical University Animal Care Committee. In all studies except for the hemophilia A mouse experiments, FVB/N mice (Jackson Laboratories, Bar Harbor, ME) were used as a recipient. Right before the cell transplantation, hA1AT-FVB/N hepatocytes were resuspended (to a ratio of 1×10^7 cells per milliliter) with cold Williams E Medium (Invitrogen Corp., Carlsbad, CA) without serum or with an equal volume of Williams E Medium and cold Engelbreth-Holm-Swarm (EHS) gel (Matrigel, BD Biosciences, Bedford, MA). For under-the-kidney capsule transplantation, a total of 3×10^6 hepatocytes were transplanted by dividing the dose between the two kidney capsule spaces. For subcutaneous transplantation, 6×10^6 hepatocytes were transplanted into the subcutaneous space between the scapulae. Because EHS-gel quickly polymerizes into a 3-dimensional gel at room temperature, all of the procedures were done at 4°C. In some experiments, we placed acidic fibroblast growth factor microspheres (aFGF-MS) (described below) into the subcutaneous space of the back and transplanted hepatocytes at the same location 10 days afterwards.

In the hemophilia mouse study, we used a mouse model of hemophilia A in the C57Bl/6 background, that lack functional factor VIII activity (kindly provided by Dr. Kazazian Jr., University of Pennsylvania, Philadelphia, PA). Hepatocytes were isolated from C57Bl/6 mice as a syngeneic combination. Hepatocytes resuspended with EHS-gel then were transplanted into either the unilateral or the bilateral kidney capsule spaces of factor VIII–deficient hemophilia mice at a ratio of 1.5×10^6 per kidney.[13] To avoid uncontrolled bleeding during surgery, we infused 90 U/kg factor VIII concentrates into the peritoneum (Confact F, Chemo-Sero-Therapeutic Inc., Kumamoto, Japan), 30 minutes before the hepatocyte transplantation or sham operation. The half-life of the infused factor VIII was less than 12 hours.

Measurement of the Mice Factor VIII Activity. The serum factor VIII biological activity was quantitated by the 1-step clotting assay based on the activated partial thrombin time using human FVIII-deficient plasma (bioMerieux Inc., Durham, NC). The advantage of this method over the chromogenic assay in terms of accuracy has been previously described.[14] Pooled human plasma (bioMerieux Inc.) was used as the FVIII activity standard. Before performing the assay, we confirmed that the FVIII activity in the FVIII-KO mouse pooled plasma represented less than 2%, whereas the activity in the normal pooled mice plasma showed higher than 100% of normal human FVIII activity. All standards and samples were measured in duplicate. Mouse tail-clip bleeding time assay was performed by cutting 1 cm from the tip of the mouse tail.[15] The mouse was returned to a separate cage, and the bleeding time was measured. If the mouse did not stop bleeding at 30 minutes, we cauterized the wounds to save the mouse.

Production of Acidic FGF-Microspheres. To provide local release of growth factor into the subcutaneous space, different doses of potent angiogenic agent, aFGF (R&D Systems, Minneapolis, MN, lot # CQ089111) with stabilization by heparin, were incorporated into the microspheres (MS). Bioerodible polyethylene glycol-polylactide-co-glycolide (Polysciences, Warrington, PA) MS were prepared as a modification of previously described techniques.[16,17] An 8:1 ratio of polylactide-co-glycolide (PLGA; 75:25; Polysciences, Warrington, PA) to PEG-8000 (polyethylene glycol, MW 8000, Sigma, St. Louis, MO) was employed with the double emulsion technique to generate MS of final mean diameter 8 to 10 μm. Additionally, a pH buffer of 7.4 was incorporated in all MS preparations as a modification of other techniques to limit local pH changes. MS were prepared to give a continuous mean daily release of 0 ng (saline), 0.0167 ng, 0.167 ng, and 1.67 ng for 14 days in aFGF-MS-G1, aFGF-MS-G2, aFGF-MS-G3, and aFGF-MS-G4, respectively. All of the groups of MS also incorporated 32 units heparin per 5 mg MS. Five micrograms of MS were resuspended with 250 μL Williams E Medium for 8 hours and injected into the subcutaneous space of the back with admixing 250 μL EHS-gel. Ten days later, we removed the subcutaneous tissues for histological analysis of the vascular network or transplanted hepatocytes at the same location.

Enzyme-Linked Immunosorbent Assay. Recipient serum hAAT concentrations were assayed by using antibody against hAAT (Dia Sorin, Stillwater, MN) as described elsewhere.[8,18]

Liver Proliferation Stimulus. Two different modes of liver proliferation were induced at 70 days after hepatocyte transplantation: (1) Hepatectomy group: compensatory regeneration was induced by performing a two-third partial hepatectomy by surgically removing the median and lateral lobes[19,20] under general anesthesia using methoxyflurane (Metafane, Mallinkrodt, IL); and (2) Direct hyperplasia (DH) group: direct hyperplasia mode of hepatocyte proliferation was induced by a single intragastric injection of the primary mitogen 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP, kindly provided by Dr. B. Diwan, National Cancer Institute, Bethesda, MD) at a dose of 3 mg/kg.[19,20] For the analysis of cell cycle proliferation in naïve and engineered livers, we administered 5-bromo-2-deoxyuridine (BrdU) subcutaneously (Sigma, St. Louis, MO) at a dose of 1 mg/d through the use of an osmotic mini-pump (model 2001; Alzet, Palo Alto, CA) for a period of 14 days starting at the same day as giving the regenerative stimulus. Fourteen days later, livers, engineered liver tissues including kidneys and surrounding subcutaneous tissues, and duodenum were removed and processed for histological analysis.

Histological Analysis and Immunohistochemistry. aFGF-MS–treated subcutaneous tissues, livers, engineered liver tissues including kidneys and surrounding subcutaneous tissues, and duodenum (for positive control) were fixed in 10% formalin before embedding into paraffin as previously described.[21] Sections (5 μm) were processed for the following immunohistochemical analysis and hematoxylin & eosin staining. In the aFGF-MS study, vascular numbers were counted at the area surrounding the EHS-gel-plug at 10 randomly selected areas (total of 0.1 mm^2) on the sections stained by hematoxylin & eosin. For immunohistochemistry, after deparaffinization, sections were treated with 3% hydrogen peroxide solution for 20 minutes and then were incubated with 2N HCl for 90 minutes. Nonspecific binding sites were blocked with unconjugated human anti-mouse IgG (Vector Laboratories, Burlingame, CA) and 10% normal goat serum (Vector Laboratories). Sections were incubated overnight at 4°C with mouse anti-BrdU antibody (1:30, Becton Dickinson, San Jose, CA) and rabbit anti-hAAT antibody (1:200, Boehringer Mannheim). After rinses, sections were then incubated for 30 minutes at room temperature with Texas-Red–conjugated goat anti-mouse IgG (1:200, Molecular Probes Inc., Eugene, OR) and FITC-conjugated goat anti-rabbit IgG (1:500, Molecular Probes Inc.). Preparations were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI, 1 μg/mL, Sigma) for 30 minutes, extensively rinsed, mounted, and coverslipped. In each mouse, the BrdU labeling indices of hepatocytes in naïve liver and engineered liver were determined separately by counting a total of 1,000 hepatocytes in 20 to 30 randomly selected liver fields and expressed as a percentage of all positive nuclei. The duodenum was used as a positive control tissue for this experiment.

Statistical Analysis. The significance of differences in the value of hAAT serum between groups were tested by Student *t* test, and differences in the vessel number and BrdU index among groups were tested by a 1-way ANOVA with the use of StatView 5.0 software (SAS Institute Inc., Cary, NC). If a probability *P* value of less than .05 was obtained, the Tukey test was used for comparison for each individual group with the appropriate control.

Results

To engineer liver tissues under the kidney capsule of mice, we isolated hepatocytes from transgenic mice (hA1AT-FVB/N) that express the serum marker protein, human alpha-1 antitrypsin (hAAT) driven by the liver-specific alpha-1 antitrypsin promoter, and transplanted them under the kidney capsule. The maintenance of the transplants *in vivo* was determined by periodic serum measurement of the hAAT transgene product.[4,8,22] The transplanted hepatocytes sharply declined to approximately 30% of the day-3 value by 3 weeks and continuously deteriorated afterward (Fig. 1A). Because extrahepatic sites may lack the proper microenvironment necessary for hepatocyte attachment and differentiation, we studied whether transplanting extracellular matrix components and bound growth factors with the hepatocytes would increase their survival. When we transplanted hepatocytes resuspended in extracellular matrix components extracted from EHS cells, hepatocytes persisted for at least 20 weeks (the length of this experiment; Fig. 1A). Histological examination at week 20 confirmed that the hepatocyte-specific hAAT originated from the transplanted hepatocytes. In addition, these cells showed specific characteristics of differentiated hepatocytes, including periodic acid-Schiff (PAS) staining for glycogen synthesis (Fig. 1B-D). We transplanted the same number of hepatocytes (1.5×10^6 hepatocytes) into two different sites and found that the established kidney capsule hepatocyte transplantation technique resulted in higher survival compared with the hepatocyte transplantation into the liver through the portal vein (Fig. 1E).

To set out to evaluate the liver tissue engineering approach in a clinically relevant model of human disease, we transplanted isogenic wild-type hepatocytes into the kid-

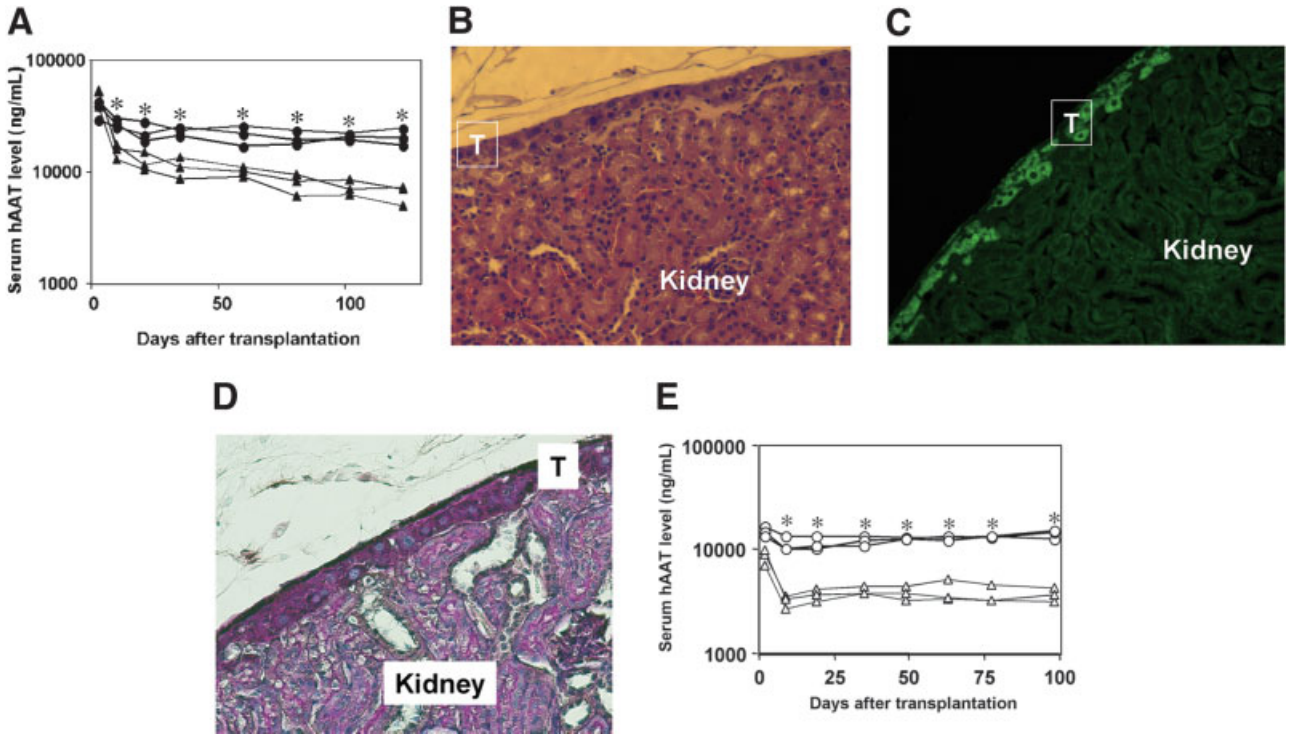

Fig. 1. Engineering extrahepatic liver tissues under the kidney capsule in mice. (A–D) hA1AT-FVB/N mouse hepatocytes were transplanted at day 0. (**closed triangle**, mice received hepatocytes resuspended with WE medium; **closed circle**, mice received hepatocytes resuspended with WE medium plus EHS-gel). (A) Mouse serum hAAT levels after hepatocyte transplantation. $*P < .05$ between WE medium plus EHS-gel group vs. WE medium group at time points of day 9 and thereafter. (B–D) Histological analysis of the transplants at week 20 of mice engrafted hepatocytes resuspended with WE medium plus EHS-gel; hematoxylin-eosin staining (B), hAAT immunostaining (C), or PAS staining (D). Original magnification ×200. T, transplanted hepatocytes. F, Mouse serum hAAT levels after hepatocyte transplantation under the kidney capsule or into the liver through portal vein (**open triangle**, mice received hepatocytes into the liver; **open circle**, mice received hepatocytes resuspended with WE medium plus EHS-gel under the kidney capsule). Each mouse received 1.5×10^6 hepatocytes. $*P < .05$ between groups at time points of day 9 and thereafter.

ney capsule space of factor VIII–deficient hemophilia A mice. Therapeutic levels of factor VIII activity were achieved, reaching approximately 5% and 10% of the normal activity in unilateral and bilateral kidney transplanted groups, respectively (Fig. 2). Hemophilia A mice that underwent a sham operation did not show any detectable serum factor VIII activities after the procedure. In humans, achievement of these levels of factor VIII would convert a patient from a severe form of the disease to a mild one, basically eliminating the bleeding diathesis except in times of substantial trauma. Nevertheless, to establish phenotypic correction, we measured the bleeding time 2 weeks after the transplantation. The liver-treated mice showed similar bleeding times as the control wild-type mouse group and showed significantly shortened bleeding times compared with the sham-treated mice (13.3 ± 2.1, 18.3 ± 2.9, 16.0 ± 2.9, and 30.0 ± 0.1 minutes, in the wild-type control, unilateral transplant, bilateral transplant, and sham-treated groups of Hemophilia A mice, respectively; $n = 3$ in each group; $P < .01$ sham group versus unilateral or bilateral transplant groups).

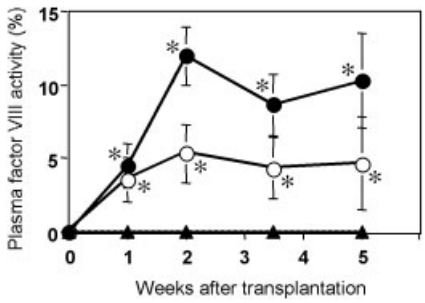

Fig. 2. Plasma factor VIII activities in hemophilia A mice. Isolated C57Bl/6 mouse hepatocytes resuspended with EHS-gel were transplanted under the kidney capsule. Factor VIII–deficient mice received transplants into unilateral (**open circle**), bilateral (**closed circle**) kidney at day 0. Control mice that received a sham operation were used as controls (**closed triangle**).

Because the liver possesses the ability to undergo active proliferation during pathological states, we studied the potential of the engineered liver tissues for proliferation capacity. We induced two distinct pathways of liver proliferation in the mice 70 days after hepatocellular engraftment into the kidney capsule. Compensatory regeneration was induced by performing a two-third partial

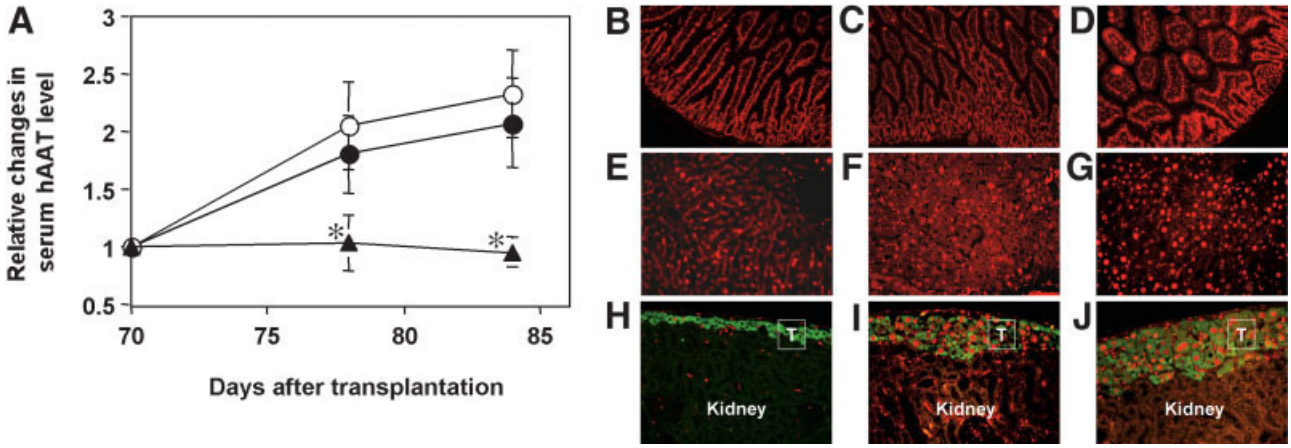

Fig. 3. Proliferation of the naïve liver and liver tissues under the kidney capsule. At day 0, hA1AT-FVB/N mouse hepatocytes resuspended with WE medium plus EHS-gel were transplanted, and at day 70 mice received either two-thirds partial hepatectomy (hepatectomy group) (C, F, I), intragastric injection of TCPOBOP (DH group) (D, G, J), or sham operation as a control (B, E, H). We delivered BrdU continuously for 14 days (days 70–84). (A) Mouse serum hAAT levels after the proliferation stimulus. At days 70, 78, and 84, the serum hAAT levels were measured by enzyme-linked immunosorbent assay, and the relative levels of hAAT were expressed by comparing values with that of at day 70 in each mouse. **Open circle**, mice in the hepatectomy group; **closed circle**, mice in the DH group; **closed triangle**, mice in the control group. *$P < .05$ control group vs. other 2 groups. (B–D) Representative photomicrographs of BrdU immunostaining from duodenum epithelium sections of control group (B), hepatectomy group (C), and DH group (D). BrdU-positive nuclei were colored with Texas red. That all the epithelial cells showed positive staining in their nuclei confirmed that BrdU had continuously been infused. (E–G) Representative photomicrographs of BrdU immunostaining from the naïve liver sections of control group (E), hepatectomy group (F), and DH group (G). (H–J) Representative photomicrographs of BrdU and hAAT co-immunostaining from engrafted hepatocytes in the kidney sections of control group (H), hepatectomy group (I), and DH group (J). hAAT-positive cytoplasm were colored with fluorescein isothiocyanate, and the BrdU-positive nuclei were colored with Texas red. Original magnification, ×100 in B–D and ×200 in E–J.

hepatectomy, and the DH mode of regeneration was induced by intragastric injection of the primary mitogen 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP). To assess cell proliferation status of the naive hepatocytes and grafted hepatocytes, BrdU, a cell cycle marker, was administered for 14 days, and BrdU-positive cells were detected by immunohistochemistry. In the naïve liver, the BrdU labeling indexes were 12.1% ± 2.5%, 95.5% ± 3.3%, and 94.9% ± 2.6% in the non–liver manipulated control (control), hepatectomy, and DH groups, respectively (Fig. 3E-G; Table 1). The weight of the naive livers in the hepatectomy group returned to near normal levels, and that of the DH groups showed a significant increase in liver mass compared with the normal liver (data not shown), both of which were consistent with our previous study.[19] In the engineered liver tissues, hepatocytes showed 12.7% ± 2.1%, 91.9% ± 3.3%, and 88.5% ± 3.7% BrdU labeling in the control, hepatectomy, and DH groups, respectively (Fig. 3H-J; Table 1). At day 84 (2 weeks post–proliferative stimulus), we confirmed that the serum hAAT levels in mice increased to 237% ± 18%, and 205% ± 17% in the hepatectomy and DH groups compared with the pretreatment value at day 70 (Fig. 3A).

We next attempted to engineer liver tissues into the subcutaneous space by transplanting hA1AT-FVB/N

Table 1. Regeneration of the Naïve Livers and Extrahepatic Liver Tissues Engineered at the Kidney and Subcutaneous Space (BrdU Was Delivered for 14 Days After Giving Each Mode of Regeneration Stimulus)

Tissue Engineered Site	Mode of Regeneration Stimulus	BrdU LI of Hepatocytes in the Naïve Liver	BrdU LI of Hepatocytes in the Engineered Liver Tissue
Kidney capsule	Control (3)	12.1 ± 2.5	12.7 ± 2.1
	CR (4)	95.5 ± 3.3*	91.9 ± 3.3*
	DH (4)	94.9 ± 2.6*	88.5 ± 3.7*
Subcutaneous space	Control (3)	12.5 ± 3.4	10.5 ± 3.3
	CR (3)	94.2 ± 4.2*	83.3 ± 4.7*
	DH (3)	93.3 ± 4.9*	75.6 ± 6.7*

NOTE. Seventy days after hepatocyte transplantation, we induced liver regeneration. To detect proliferated cells, we delivered BrdU continuously for 14 days through the Osmotic mini-pump. Numbers of mice examined in parentheses.

Abbreviations: CR, compensatory regeneration induced by performing two-third hepatectomy; DH, direct hyperplasia induced by the injection of TCPOBOP.

*$P < .005$ vs. control group.

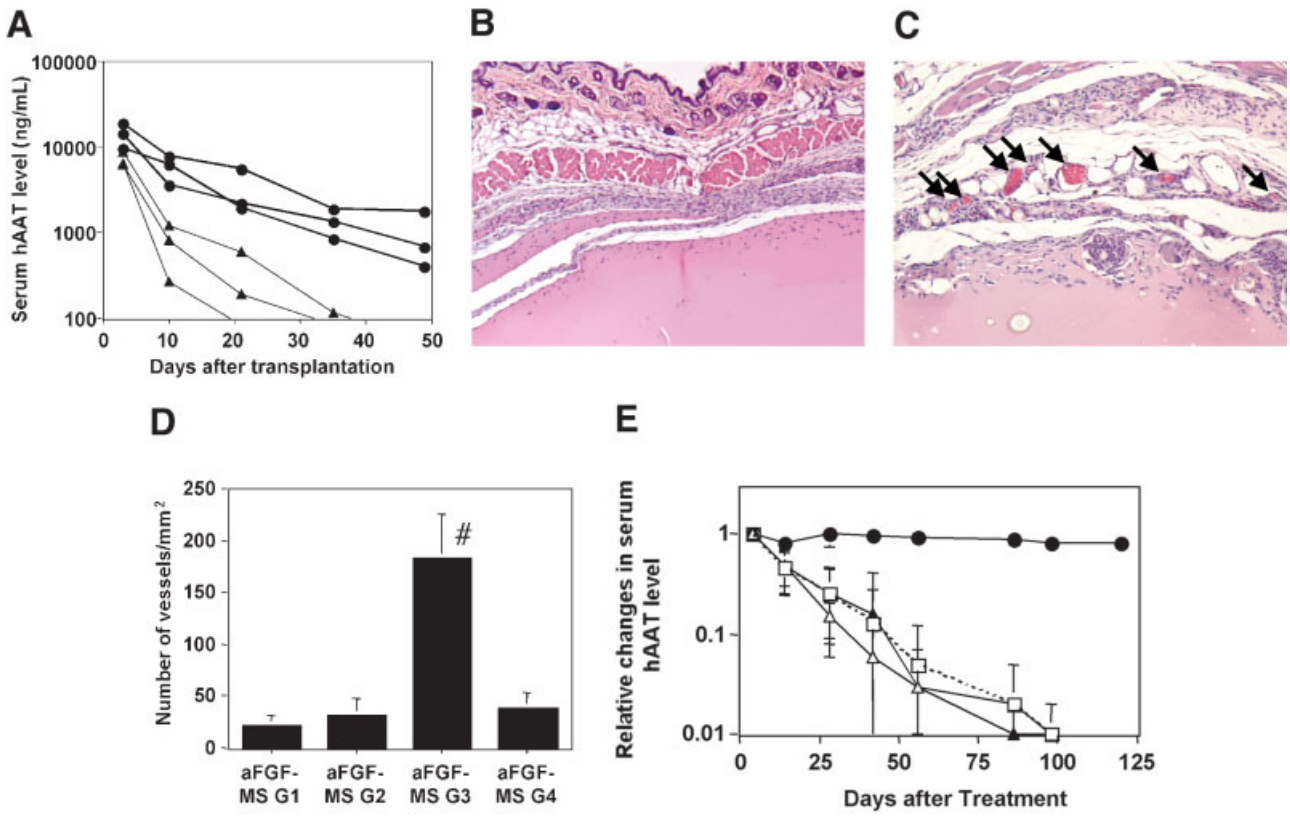

Fig. 4. Engineering extrahepatic liver tissues into the subcutaneous space. (A) Mouse serum hAAT levels after the transplantation. hA1AT-FVB/N mouse hepatocytes were transplanted into the subcutaneous space of the back at day 0 (**closed triangle**, mouse received hepatocytes resuspended with WE medium; **closed circle**, mouse received hepatocytes resuspended with WE medium plus EHS-gel). (B–D) Establishment of vascular network into the subcutaneous space. Microspheres incorporating different doses of aFGF were resuspended with an equal volume of WE medium and EHS-gel. Five micrograms MS were placed into the subcutaneous space of FVB/N mice and analyzed 10 days later. Histological analysis (hematoxylin-eosin staining) of subcutaneous space around the EHS-gel containing either aFGF-MS-G1 (B) or aFGF-MS-G3 (C). **Arrows** denote vessels including blood cells within the channel. (D) Number of vessels into the subcutaneous space around the aFGF microspheres. $^{\#}P < .001$ between aFGF-MS-G3 vs. other groups. (E) Mouse serum hAAT level after transplantation. Different groups of aFGF-MS were placed into the subcutaneous space, and hepatocytes were transplanted at the same location 10 days later. The relative level of hAAT was compared with the value obtained 3 days after the transplantation (**open triangle**, **closed triangle**, **closed circle**, **open square**; aFGF-MS-G1, aFGF-MS-G2, aFGF-MS-G3, aFGF-MS-G4, respectively). Note that aFGF-MS-G3, which induced high level of vascular network (see panel D), showed persistent survival of hepatocytes (**closed circle**).

mouse hepatocytes with or without co-transplanting EHS-gel. Although hepatocytes with the EHS-gel group showed significantly higher survival as compared with the non–EHS-gel group, the transplanted hepatocytes in both of these groups exhibited a sharp drop in survival over time (Fig. 4A). Based on the lack of sufficient vascular support for the transplanted hepatocytes (not shown), we hypothesized that establishing a local vascular network at the transplantation site would allow nutrient and gas transport to the grafts, and this would reduce graft loss.

To do this, we created polylactide-co-glycolide-co-polyethylene glycol MS incorporating different doses of a potent angiogenic agent, aFGF,[23] to provide continuous local delivery (aFGF-MS-G1 to G4). Five micrograms MS (resuspended in EHS-gel) were placed into the subcutaneous space of FVB/N mice, and histological analyses were performed by using subcutaneous tissue samples taken 10 days later. Quantitative increases in local tissue vascularity around the EHS-gel plug were demonstrated in the aFGF-MS-G3–treated animals, with the optimal dosage being a mean daily release of 0.167 ng (Fig. 4B-D). We then placed aFGF-MSs-G3 into the subcutaneous space and transplanted hepatocytes in EHS-gel in the same location 10 days later. When active vascular networks were induced with aFGF-MS-G3, persistent hepatocyte survival was achieved for the duration of this extended experiment (140 days) (Fig. 4E).

To further establish whether the engineered liver tissues in the subcutaneous space also possessed proliferative capacity, we applied the 2 different modes of proliferation (hepatectomy and DH) to mice 70 days after the hepatocyte engraftment by using another set of mice that also had been treated with aFGF-MS-G3. We delivered BrdU for 14 days from the day of the stimulus. Hepatocytes in

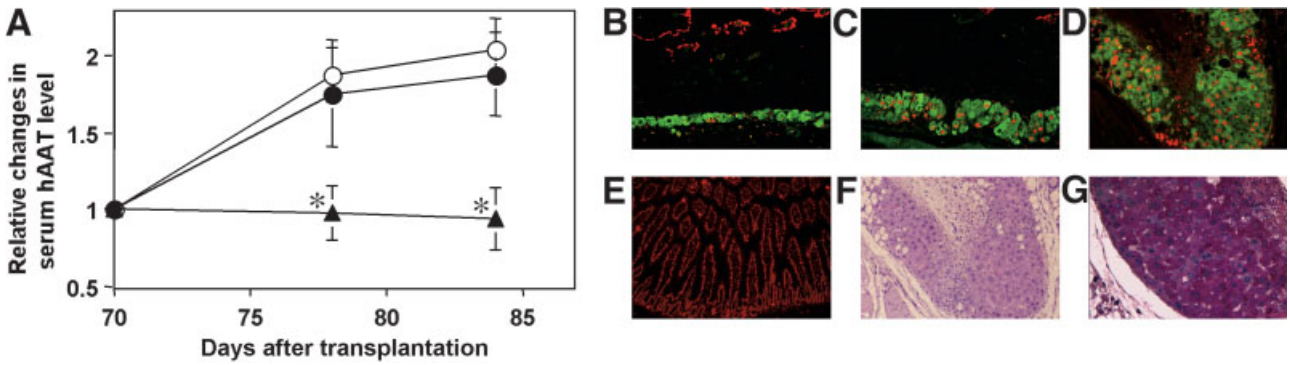

Fig. 5. Proliferation of the naïve liver and liver tissues into the subcutaneous space. Mice received aFGF-MS-G3 into the subcutaneous space and then received hepatocytes resuspended with WE medium plus EHS-gel 10 days later. At day 70, mice received either a two-third partial hepatectomy (hepatectomy group) (C), intragastric injection of TCPOBOP (DH group) (D), or nontreatment as a control (B). We delivered BrdU continuously for 14 days (days 70–84). (A) Mouse serum hAAT levels after the regeneration stimulus. At days 70, 78, and 84, the serum hAAT level was measured by enzyme-linked immunosorbent assay (ELISA), and the relative level of hAAT was expressed by comparing values with that of day 70 in each mouse. **Open circle**, mice in the hepatectomy group; **closed circle**, mice in the DH group; and **closed triangle**, mice in the control group. $*P < .05$ between WE medium plus EHS-gel group vs. WE medium group. (B–D) Representative photomicrographs of BrdU and hAAT co-immunostaining from engrafted hepatocytes in the subcutaneous sections of control group. hAAT-positive cytoplasm were colored with fluorescein isothiocyanate, and the BrdU-positive nuclei are colored with Texas red. (E) Representative photomicrographs of BrdU immunostaining from duodenum epithelium sections of control. BrdU-positive nuclei are colored with Texas red. All the epithelial cells that showed positive staining in their nuclei confirmed that BrdU had continuously been infused to the mice. (F–G) Histological analysis of the engineered liver tissues into the subcutaneous space in the DH group at day 84. HE staining (F) and PAS staining (G). Original magnification, ×100 (E); and ×200 (B–D and F–G).

the engineered liver tissues showed 10.5% ± 3.3%, 93.3% ± 4.7%, and 75.6% ± 6.7% BrdU labeling index in the control, hepatectomy, and DH groups, respectively (Fig. 5B-D; Table 1). Serum hAAT level at day 84 increased to 192% ± 21%, and 180% ± 19% in the hepatectomy and DH groups as compared with their value at day 70, whereas the control group did not show any increase (97.6% ± 5.2%) (Fig. 5A). Histological examination of the graft samples at day 84 confirmed that the hAAT was produced by the transplanted hepatocytes (not shown). After proliferation, the morphology and function of the transplanted cells as differentiated hepatocytes was again confirmed by hematoxylin & eosin (HE) and PAS staining for glycogen. (Fig. 5F-G).

Discussion

The present study shows that hepatocytes can be successfully engrafted and maintained for over 100 days at 2 distinct extrahepatic sites, under the kidney capsule and in the subcutaneous space. The persistency of the grafts at the subcutaneous space was only possible by establishing a vascular network before the transplantation induced by controlled local release of aFGF. We believe the combination of EHS gel and a vascular network provides a complex of matrix proteins and nutrients[24,25] that allows essential support for hepatocellular functions[26] and formation of hepatic organelles,[27] ultimately allowing for persistent generation of a functional extrahepatic organ *in vivo*.

Under-the-kidney capsule space has supported cell engraftment in the experiments of islet cell transplantation.[28] It was suggested that this site allowed rapid establishment of a vascular network that contributed to long-term cell survival. However, hepatocytes alone, grafted under the kidney capsule, have not shown persistent survival.[3,11] Such a difference might be explained by the difference in the nature of the cell types; especially once the hepatocytes are isolated from the liver, cells in culture will undergo a rapid process of de-differentiation leading to loss of function.[29] *In vitro* studies have suggested that providing extracellular matrix components, especially basement membrane proteins, including laminin and type IV collagen, is important for maintaining hepatocyte differentiated function.[26,29] In addition, others have found that EHS-gel has the ability to induce vascularization for a short period.[30] The EHS extracellular matrix gel is rich in basement membrane components, but it also contains a small amount of growth factors, such as epidermal growth factors. In our preliminary experiment, we compared the effect on the transplanted hepatocyte survival of the regular EHS-gel and growth factor reduced EHS-gel. We found there are no differences in these 2 EHS-gel components, suggesting that extracellular matrix components are the predominant contributory factors for the enhanced hepatocyte engraftment (data not shown). These studies clearly demonstrated that providing extracellular matrix components in the transplant setting is important for increasing the engraftment and

stable survival of the hepatocytes, leading to liver tissue engineering. However, further experiments are needed to analyze which of the extracellular matrix element(s) are necessary for facilitating hepatocyte engraftment.

Toward a novel treatment for liver diseases, we then assessed the therapeutic efficacy of our approach for liver tissue engineering in a mouse model of hemophilia A. Hemophilia A is a bleeding disorder, occurring in 1 of approximately 5,000 men who lack the production of functional factor VIII. Protein replacement therapy with infusion of plasma-derived or recombinant factor VIII has been a standard for this disease. Because the liver is a major contributory organ for factor VIII production,[31] recent experience in transplanting whole or part of the liver into hemophilia patients have resulted in curative levels (>50%) of factor VIII production and clotting activity.[32,33] However, raising these levels into the range of 2% to 3% will result in a significant phenotypic improvement in the disease, and this prompted us to establish whether engineering liver tissues would have a therapeutic benefit on hemophilia A.[34] As shown in this study, by engineering liver tissue with 3×10^6 hepatocytes that are equivalent to approx 3% of the naïve liver, we could demonstrate therapeutic efficacy in reconstituting 5% to 10% of normal clotting activity, enough to reduce the bleeding time to near normal values. These findings establish the proof-of-concept that extrahepatic liver tissue engineering is a viable therapy for patients with hepatodeficiency conditions.

The subcutaneous space is a particularly attractive site for cell transplantation and tissue engineering, because it is an easy site to access and manipulate, and it holds a remarkably large capacity for engrafting.[3,7] However, several studies have not been able to achieve persistent survival of hepatocytes transplanted into the subcutaneous space.[8,35] In the previous and present studies, we experienced a significant decrease in cell survival within the first 2 weeks after the transplantation even in the presence of an extracellular matrix component and HGF/cMet stimulation. We hypothesized that a major cause of cell death was insufficient vascularity at the transplantation site. Our strategy, establishing a vascular network using continuous delivery of aFGF before transplantation, clearly demonstrated its importance for persistent survival leading to liver tissue engineering. Other studies have reported that transplanting hepatocytes transfected with vascular endothelial growth factor genes or transplanting hepatocytes with a bFGF-releasing device showed some effects on survival when cells were transplanted into the abdominal cavity.[24,25] However, persistent survival of the hepatocytes was not achieved. In prior strategies, once cells were implanted in the recipient, metabolic needs began immediately, but it took several days for the growth of new blood vessels that would deliver oxygen and nutrients to the grafts.[7] Ready access to the subcutaneous space renders a staged procedure such as that presented here more clinically feasible.

The liver has a number of fascinating properties because of its proliferative capacity[18,36–38] through 2 distinct pathways, compensatory regeneration or direct hyperplasia.[19,20,39] A large number of molecules are involved in the liver proliferation process in both endocrine and paracrine fashions.[36,39] We have previously reported that the activation of HGF/cMet is one of the important pathways for hepatocytes to grow at the extrahepatic sites.[12] Although it has been suggested that shunting the portal circulation to the engrafted hepatocytes (to diverse growth factors from portal to a general circulation) is necessary for their cell proliferation,[40] the present study clearly demonstrates that engineered liver tissues at two different extrahepatic sites could regenerate with similar activity as their naïve livers without the need of portal blood supply. It is also important to note that the onset and completion of the proliferation process of the engineered liver tissues occurred within the same period as naïve liver. These findings, along with the morphological, and enzymatical (PAS staining) analyses, proved that the liver tissues comprising donor hepatocytes were recognized as a part of their own liver *in vivo*.

In summary, the present studies show that liver tissue, which was recognized as a part of the host naïve liver in terms of the proliferation profile, could be engineered at a heterologous site that does not have access to the portal circulation. This approach is further supported by the recent interest in the ability to generate mature hepatocytes from embryonic, hematopoietic, or somatic stem cells as an alternative cell source.[41] These cells could be transplanted in a similar manner, offering new medical therapy for many patients with a myriad of different diseases.[3,34] Taken together, the current work thus can serve as the basis to enable further development of tissue engineering, stem cell work, and molecular medicine approaches to liver disease.

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