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RESEARCH ARTICLE

Functional life-long maintenance of engineered liver tissue in mice following transplantation under the kidney capsule

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Abstract

The ability to engineer biologically active cells and tissue matrices with long-term functional maintenance has been a principal focus for investigators in the field of hepatocyte transplantation and liver tissue engineering. The present study was designed to determine the efficacy and temporal persistence of functional engineered liver tissue following transplantation under the kidney capsule of a normal mouse. Hepatocytes were isolated from human α-1 antitrypsin (hA1AT) transgenic mouse livers. Hepatocytes were subsequently transplanted under the kidney capsule space in combination with extracellular matrix components (Matrigel) for engineering liver tissues. The primary outcome of interest was to assess the level of engineering liver tissue function over the experimental period, which was 450 days. Long-term survival by the engineered liver tissue was confirmed by measuring the serum level of hA1AT in the recipient mice throughout the experimental period. In addition, administration of chemical compounds at day 450 resulted in the ability of the engineered liver tissue to metabolize exogenously circulating compounds and induce drug-metabolizing enzyme production. Moreover, we were able to document that the engineered tissues could retain their native regenerative potential similar to that of naïve livers. Overall, these results demonstrated that liver tissues could be engineered at a heterologous site while stably maintaining its functionality for nearly the life span of a normal mouse. Copyright © 2009 John Wiley & Sons, Ltd.

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1. Introduction

Cell-based therapies based on hepatocyte transplantation or tissue engineering has been a recent area of intense investigation, due to its potential as a new therapeutic modality to treat liver diseases (Fisher and Strom, 2006; Ofosu, 2008; Ohashi *et al*., 2001, 2008; Stephenne *et al*., 2006; Tatsumi *et al*., 2008a, 2008b). Because of its integrated and complex functionality, the hepatocyte has been pivotal in the development of cell-based therapies toward liver diseases. The important therapeutic role of the hepatocyte has been highlighted by the recent successes observed in several types of liver disease using hepatocyte transplantation (Fisher and Strom, 2006; Fox *et al*., 1998; Ohashi *et al*., 2001; Stephenne *et al*.,

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2006; Strom *et al.*, 2006). However, the hepatocyte transplantation procedure can be limited in the number of cells that can be infused at one time (Fox *et al.*, 1998; Ohashi *et al.*, 2001; Stephenne *et al.*, 2006, 2007; Strom *et al.*, 2006) and could prevent the necessary level of therapy that may be required to achieve a particular therapeutic modality. For this reason, an alternative approach has been recently developed using isolated hepatocytes in which the individual cells have been engineering into biologically active tissues, which have the ability to simulate whole-organ liver function in an ectopic site (Griffith and Naughton, 2001; Kuge *et al.*, 2006; Ohashi *et al.*, 2000, 2005a, 2005b, 2005c, 2007; Ohashi, 2008; Yokoyama *et al.*, 2006). Unlike the individual hepatocyte infusion, tissue-engineering technology has not been shown to be limited by cell number, but another major obstacle that needs to be overcome is the ability to maintain viable functional hepatocytes in these ectopic sites for extended periods of time (>6 months). By successfully manipulating these engineered liver tissues to provide extended therapy, a greater number of patients with various liver disorders and diseases could be treated.

As tissue-engineering technology has evolved over the past several years, it has spurred significant interest into the field of liver regenerative medicine (Griffith and Naughton, 2001; Ohashi, 2008). To maximize the efficiency of maintaining viable engineered liver tissue in an ectopic site, such as implantation under the kidney capsule (Kuge *et al.*, 2006; Ohashi *et al.*, 2000, 2005a, 2005b, 2005c), in our laboratory a mixture is created of isolated hepatocytes with extracellular matrix components (matrigel), which are rich in laminin and type IV collagen. The functionality of the engineered liver tissues have been confirmed in our previous short- to medium length studies (i.e. 50–200 days), using parameters that include tissue protein expressions of liver enzymes, viral infectivity, uptake of exogenous chemicals resulting in subsequent metabolism and regenerative growth potential (Kuge *et al.*, 2006; Ohashi *et al.*, 2000, 2005a, 2005b, 2005c).

In the present study, we engineered liver tissues under the kidney capsule in mice and assessed the stability of this ectopic liver system for over 450 days, which is nearly the life-span of a normal mouse. In addition, we also assessed liver-specific functions, such as glycogen synthesis, drug metabolism and regenerative potential, within the engineered liver tissues at the end of the experiment (days 450–464) to document the biological efficacy of these liver tissues after such a prolonged period of time.

2. Materials and methods

2.1. Animals

Transgenic mice expressing human α-1 antitrypsin (hA1AT) under the hepatocyte-specific promoter (hA1AT-FVB/N, H-2^q; kindly provided by Dr Bumgardner, Ohio State University, Columbus, Ohio, USA) at 12–13 weeks of age (Bumgardner *et al.*, 1998) were used as donors for hepatocyte isolation. Wild-type female FVB/N mice (aged 11–12 weeks), which were syngenic to the hA1AT-FVB/N, were used as the recipient animals. All mice were maintained in the Animal Center at Nara Medical University, and all of the mouse experiments were conducted in accordance with the institutional guidelines set forth by Nara Medical University Animal Care Committee. Mice were placed in cages within a temperature-controlled room with a 12 h light/12 h dark cycle as well as *ad libitum* access to food and water.

2.2. Hepatocyte isolation and purification

Hepatocytes were isolated from hA1AT transgenic mice using a modified two-step collagenase perfusion method, as previously described (Ohashi *et al.*, 2005a, 2005b, 2007). Isolated cells were filtered through a nylon mesh membrane and hepatocytes were purified by low-speed centrifugation at $50 \times g$ for 5 min, followed by Percoll (Amersham Biosciences, Uppsala, Sweden) isodensity centrifugation. Cells were resuspended with DMEM medium (Sigma, St. Louis, MO, USA) and the cell viability was determined by Trypan blue exclusion. In the present studies, experiments were conducted only when the hepatocyte viabilities exceeded 90%.

2.3. Liver tissue engineering

Hepatocytes were prepared for engineering liver tissue under the kidney capsule space as previously described (Kuge *et al.*, 2006; Ohashi *et al.*, 2000, 2005a, 2005b, 2007; Yokoyama *et al.*, 2006). In brief, isolated hepatocytes were resuspended with serum-free DMEM with an equal volume of EHS-gel (Matrigel; BD Biosciences, Bedford, MA, USA) to a final ratio of 1.5×10^6 hepatocytes/100 µl. A total of 1.5×10^6 hepatocytes were transplanted under the left kidney capsule space. All the surgical procedures were performed under isofluorane (Forane, Abbott Laboratories, Abott Park, IL, USA).

2.4. Enzyme-linked immunosorbent assay (ELISA)

Functional volumes of the engineered liver tissues were assessed by measuring the recipient serum hA1AT concentrations. Serum samples were periodically obtained from the recipient mice and were assayed by ELISA, using a primary antibody against hA1AT (DiaSorin, Stillwater, MN, USA) and a secondary goat antibody conjugated with horseradish peroxidase (HRP; Research Diagnostics, Flander, NJ, USA), as previously described (Ohashi *et al.*, 2000).

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2.5. Liver regeneration stimulus

At day 450 of the experiments, a liver regenerative stimulus was induced to some of the recipient mice by performing a 70% partial hepatectomy (two-thirds PH) to the naïve liver. In this procedure, we removed the medial and left lateral lobes of the naïve livers as preciously described (Ohashi *et al*., 1996, 2005a, 2007). For analysis of the hepatocyte proliferation status in the naïve and engineered livers, 5-bromo-2′-deoxyuridine (BrdU) was administered at a dose of 1 mg/day, using an osmotic mini-pump (Model 2001; Alzet, Palo Alto, CA, USA). This was initiated on the day of the two-thirds PH and was administered for a period of 14 days until day 463. At this point, the naïve livers, the kidneys containing the engineered liver tissues and the duodenum (as a positive control for BrdU incorporation) were removed and processed for histological analysis. During this 14 day period, serum samples were also obtained at various days (450, 456 and 463) to assess the functional activity of the engineered liver tissues.

2.6. Cytochrome P450 induction

At day 450, some recipients received intraperitoneal injection of phenobarbital (PB; Wako Pure Chemical Industries, Osaka, Japan) or 3-methylcholantree (3-MC; Sigma, St. Louis, MO, USA) for 3 consecutive days (Ohashi *et al*., 2007; Yokoyama *et al*., 2006). The mice were sacrificed 3 h after the last injection. Naïve livers and engineered liver tissues were processed for histological analyses.

2.7. Histological and immunohistochemical analyses

Naïve liver specimens, engineered liver tissues containing neighbouring kidney, and the duodenum of the recipient mice were harvested and fixed in 10% buffered formalin. Specimens were paraffin-embedded and sliced into 5 μm thick sections, which were subsequently processed for haematoxylin and eosin (H&E) staining or immunohistochemical analyses. For the BrdU and hA1AT immunofluorecent co-staining, deparaffinized sections were treated with 2 N HCl for 90 min. Non-specific binding sites were blocked with 10% normal goat serum. The sections were then incubated overnight at 4 °C with mouse anti-BrdU antibody (1 : 30; Becton-Dickinson, San Jose, CA, USA) and rabbit anti-hA1AT antibody (1 : 100; YLEM, Roma, Italy). Alexa-Fluor-488 and Alexa-Fluor-555 (Molecular Probes, Eugene, OR, USA) were used as a secondary reagent. In each mouse, the hepatocytes' BrdU labelling indices in the naïve liver and engineered liver tissues were determined separately by counting a total of 1000 hepatocytes in 20–30 randomly selected liver fields. The BrdU labelling indices were expressed as a percentage of BrdU-positive hepatocytes. For the CYP2B and CYP1A immunostaining, non-specific binding sites were blocked with normal goat (for CYP2B) and normal rabbit (for CYP1A) serum. Tissues were incubated with either rabbit anti-mouse CYP2B antibody (1 : 500; Chemicon International, Temecula, CA, USA) or sheep anti-mouse CYP1A (1 : 500; Chemicon International) at 4 °C overnight. After several washes, the sections were incubated for 30 min at room temperature with ABC solution (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA). Visualization of the immune complexes was performed by incubating with 3,3′-diaminobenzidine (DAB). For the cellular glycogen detection, periodic acid–Schiff (PAS) staining was performed as described previously (Ohashi *et al*., 2007). To confirm the staining specificity of the cellular glycogen, serial sections were pre-treated with salivary amylase for 60 min followed by the same PAS staining procedures.

2.8. Statistical analysis

All the values calculated in the present study were provided as means ± standard deviation (SD). Statistical differences in the values were determined by a Student's *t*-test. A probability value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Stable and persistent viability of the liver tissues engineered under the kidney capsule

Functional activity of the engineered liver tissues was assessed by measuring serum hA1AT levels in the recipient mice. As shown in Figure 1A, B, recipient mice showed stable and persistent serum hA1AT levels in the range 9000–30 000 ng/ml, suggesting that the engineered liver tissues could be viable and stably maintained throughout the 450 days experimental period, which was nearly the life-span of the mice themselves. These mice were generated in two separate experiments, in which each group of mice was generated using a different source of donor hepatocytes to demonstrate the reproducibility of this approach. Histological examination at day 463 confirmed that a thin layer of liver tissue had developed and engrafted within the kidney capsule with a thickness of up to 3 cells in height. The hepatocytes that were found to comprise the engineered liver tissues showed normal healthy morphology in terms of presenting a large and eosinophilic cytoplasm (Figure 1C, D). It is important to note that there was no evidence of tumour formation observed in any of the engineered tissues.

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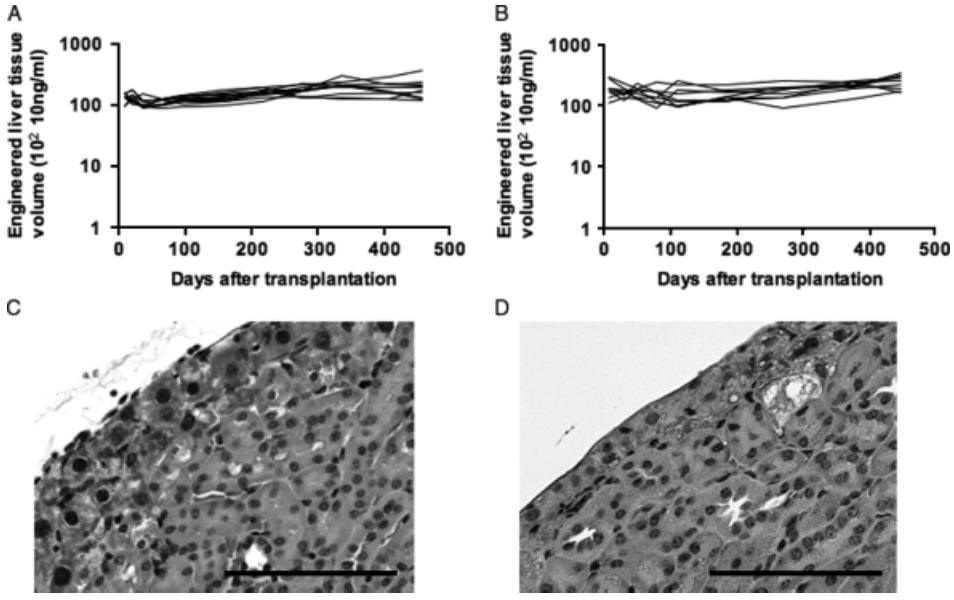

Figure 1. Functional maintenance of the engineered liver tissues under the kidney capsule. (A, B) Functional volume of engineered liver tissues under the kidney capsule was determined by measuring murine serum hAAT levels at various time points up to 450 days. (A) and (B) demonstrate two separate experiments using different donor hepatocytes ($n = 11$ and $n = 9$, respectively). (C) Haematoxylin and eosin staining of the engineered liver tissues are shown at day 453 from the experiment in (A) and (D) from day 464 from experiment in (B). Scale bars = 100 μm

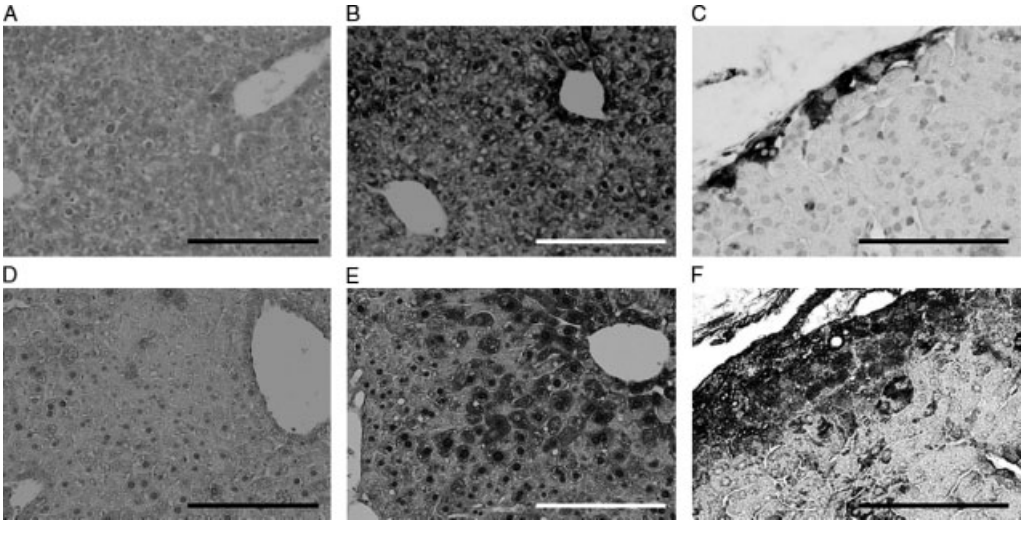

Figure 2. Induction of drug-metabolizing enzymes in naïve mouse livers and engineered liver tissues under the kidney capsule. CYP2B and CYP1A were induced by intraperitoneal injection of PB and 3-MC, respectively. Immunohistochemical staining was performed for CYP2B (A–C) and CYP1A (D–F). (A) Mouse liver without PB treatment. (B) Mouse liver following PB treatment. (C) Engineered liver tissues under kidney capsule following PB treatment. (D) Mouse liver without 3-MC treatment. (E) Mouse liver following 3-MC treatment. (F) Engineered liver tissues under kidney capsule following 3-MC treatment. Scale bars = 100 μm

3.2. Drug-metabolizing functions in engineered liver tissues following long-term implantation under the kidney capsule

To determine whether the engineered liver tissues continued to possess liver-specific drug-metabolizing function after 450 days under the kidney capsule, we inoculated the mice with the engineered liver tissue, using either PB (CYP2B inducer; Waxman and Azaroff, 2006; Yokoyama *et al.*, 2006) or 3-MC (CYP1A inducer; Yokoyama *et al.*, 2006; Zacharova *et al.*, 2003). This enabled us to investigate whether the liver tissue engineered under the kidney capsule long-term after cell transplantation could retain its ability to uptake exogenous compounds as well as induce compound-specific CYP, which would occur in normal livers. As shown in Figure 2, strong immunostaining for CYP2B and CYP1A were found in both the engineered liver tissues and naïve livers. These immunohistochemical findings clearly demonstrate that the engineered liver tissues have retained their ability to take up circulating compounds and have the capability to strongly induce drug-metabolizing

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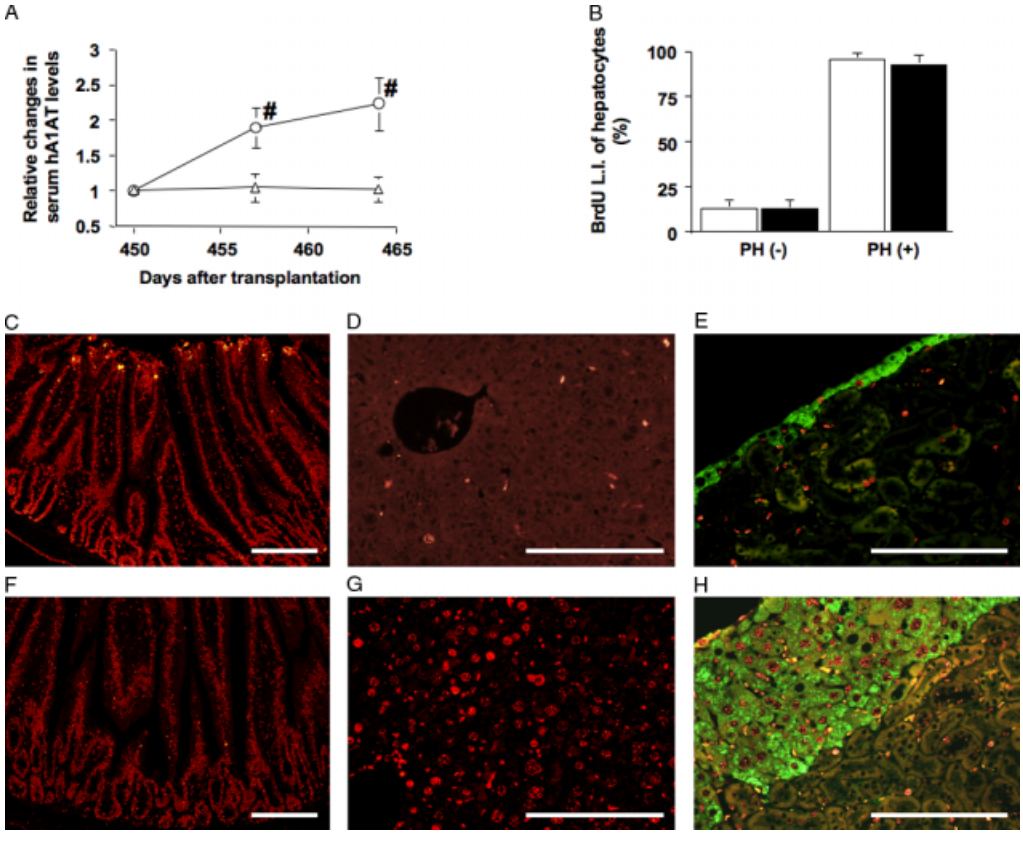

Figure 3. Regenerative ability of the engineered liver tissues within the kidney capsule. We performed sham operation for either control (C–E) or two-thirds PH (F–H) on the recipient mice at day 450. After the surgical procedures, BrdU was continuously delivered through an osmotic minipump for a period of 14 days, starting at day 450, until day 464. (A) Functional activity of the engineered liver tissues was determined by measuring serum hAAT levels by ELISA. At days 450, 457 and 464 the serum was isolated, the hAAT levels were measured and the relative levels of hAAT were calculated by expressing the values relative to day 450. Triangle, mice in sham operation ($n = 5$); circle, mice in two-thirds PH group ($n = 4$). #, $p < 0.05$ between groups. (B) Hepatocyte BrdU labelling index (LI) was examined in naïve livers or engineered liver tissues within the kidney capsule. Representative photomicrographs of BrdU and hAAT co-immunostaining are shown in the duodenum (C, F), naïve liver (D, G) and engineered liver tissues found within the kidney capsule (E, H) from the control, non-PH (C–E) and two-thirds PH (F–H) groups. BrdU-positive nuclei were labelled with Alexa-Fluor-555 and the hAAT-positive cytoplasm from the donor hepatocytes were labelled with Alexa-Fluor-488. Scale bars = 100 μm

enzymes, such as cytochrome P450s, at levels similar to those detected in the naïve livers.

3.3. Regeneration potential of the engineered liver tissues following long-term implantation under the kidney capsule

One of the unique properties of the liver is its ability to regenerate itself following chemical injury or surgical partial removal. Our previous mouse studies demonstrated that engineered liver tissues implanted under the kidney capsule for at least 70 days possessed their full regenerative growth potential following a two-thirds partial hepatectomy (Ohashi *et al.*, 2005a, 2005b). These results led us to investigate whether engineered liver tissues that were implanted under the kidney capsule for a longer period of time, such as 450 days, as performed in the current study, could still retain their ability to regenerate following a proliferative stimulus. To study this effect, we performed two-thirds partial hepatectomy (PH) in the recipient mice at day 450 following hepatocyte transplantation under the kidney capsule; 14 days later (at day 464 of the experiment), the functional activity of the engineered liver tissues was determined by measuring the serum hA1AT levels, and we detected an increase of $223 \pm 37\%$ in the PH mice relative to the levels measured in the non-PH mice (Figure 3).

In addition, we inserted an osmotic minipump in the sham-operated and two-thirds PH recipient mice to administer BrdU, a cell cycle marker, over the 14 day period, starting at day 450, until 464. The BrdU and hA1AT immunofluorescent co-staining of the engineered liver tissues showed significantly greater BrdU-labelling index (LI) in the PH mice ($91.1 \pm 6.4\%$) compared to the control sham-operated mice ($11.1 \pm 5.4\%$). These BrdU LIs were consistent with the values obtained from the naïve livers in the PH and sham-operated mice (Figure 3B). The strong positive signal intensity of hA1AT detected in the engineered liver tissues implanted under the kidney capsule (Figure 3E, H) and the lack of hA1AT-positive cells in all other organs, including the liver (data not shown), confirmed that the serum hA1AT

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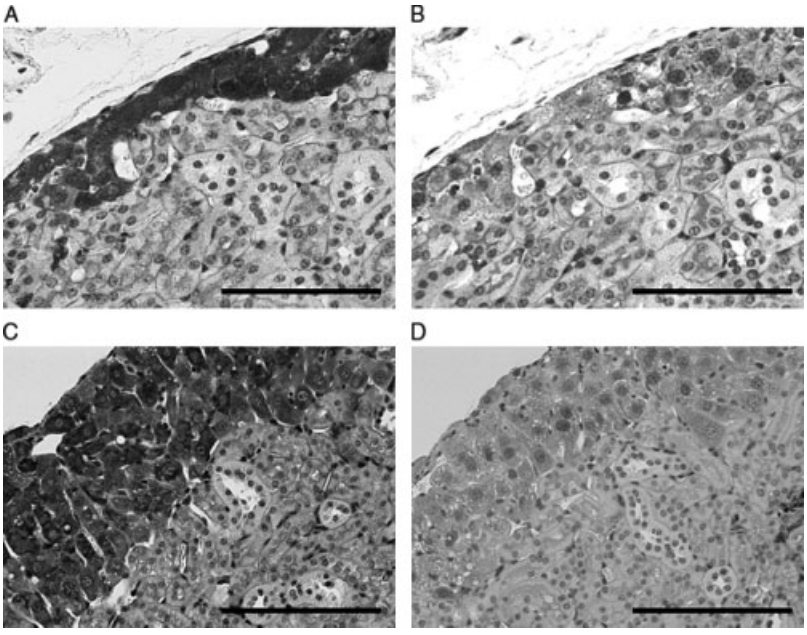

Figure 4. PAS staining of the engineered liver tissues to determine glycogen synthesis. PAS staining was performed to confirm the presence of cellular glycogen in sections that were either non-treated (A, C) or pretreated with salivary amylase (B, D); sections in (A, B) obtained from non-PH mice at day 464, and sections (C, D) obtained from mice that received a two-thirds PH at day 450 and were subsequently sacrificed at day 464. Scale bars = 100 μm

levels were solely originating from the engineered liver tissues.

3.4. Glycogen synthesis in engineered liver tissues following long-term implantation under the kidney capsule

To establish glycogen synthesis, which is another liver function, to the engineered liver tissues implanted under the kidney capsule, we performed PAS staining on sections from sham-operated and two-thirds PH mice at day 464. Both the relatively quiescent hepatocytes in the control mice and the actively proliferating hepatocytes in the two-thirds PH mice showed similarly strong staining for PAS, with the intensity levels being consistent with that observed in the naïve livers (Figure 4). Pretreatment with salivary amylase in the tissue sections significantly diminished the PAS staining, which confirmed that the staining specificity of cellular glycogen in the naïve livers and engineered liver tissues under the kidney capsule.

4. Discussion

The present study achieved life-long stability of *de novo* engineered liver tissues implanted under the kidney capsule. These ectopically transplanted liver tissues were functionally active for at least 450 days, and these bioengineered tissues possessed liver-specific functions in terms of protein expression, drug uptake and metabolism, regenerative growth and glycogen-synthetic capability. At present, we believe that this is the first report to successfully document the ability of bioengineered liver tissues to retain their functionality for the duration observed in this study, i.e. in excess of 450 days.

In our study, we transplanted hepatocytes for the creation of liver tissues under the kidney capsule of mice at 11 weeks of age, and the study was terminated after more than 450 days, since it has been shown that the average life-span of experimental mice is approximately 480 days (Kohn, 1971) and we did not want to risk the loss of the liver tissues and their analyses due to an unscheduled and age-related death. Since we were able to verify stable functional activity throughout the experimental period, we can reasonably speculate that these engineered liver tissues could survive for the duration of the entire life-span of the mouse. At this time, we do not know whether the prolonged ectopic graft survival observed in the present study using small animal models can translate into functional tissues for therapeutic applications in humans. However, the extended 450 day survivability of the engineered liver tissues in the recipient mice is an important advance in the field, and is a necessary proof-of-concept step in the development towards its use as a future clinical application.

Our study examined not only the longevity of the survivability of the engineered liver tissues but also the flexibility of the system to mimic normal liver function. First, the ectopic liver systems were found to have the potential to self-renew hepatocytes, which is a common property of the liver following hepatocellular loss (Fausto, 2001; Michalopoulos, 2007). Previous studies in our laboratory have shown that ~12% of the hepatocytes in the naïve livers entered the cell cycle under normal condition, as measured by BrdU incorporation over a 14 day period (Ohashi *et al.*, 2005a). In the present

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study, we administered BrdU over the same number of days, except at a different time point in the age of the mouse (i.e. days 450–464), and we found that 11.6% of hepatocytes were positive for BrdU in the engineered liver tissues under the kidney capsule. These findings demonstrated that the proliferative rate in the engineered liver systems implanted under the kidney capsule is similar to that of normal livers, and that this level of proliferation is sufficient to maintain these ectopically transplanted tissues over a prolonged period of time.

Another important biological property of the liver is its ability to promote active cellular proliferation in the face of a surgical, necrotic and/or chemical injury (Fausto, 2001; Michalopoulos, 2007) and so it was important to assess whether proliferative stimuli could affect ectopically transplanted liver tissues in a similar manner. In general, normally quiescent hepatocytes rapidly progress into the cell cycle following a proliferative stimulus, which leads to robust cellular proliferation until there is a restoration of functional mass within 14 days. It is important to note that this regenerative proliferation of the liver is mediated by mature hepatocytes and is not attributed to a select population of stem cells (Michalopoulos, 2007). Our results demonstrated a high BrdU labelling index in both the naïve liver and the engineered liver tissues (93.8% and 91.1%, respectively), indicating that the regenerative growth detected in the engineered liver tissue was mediated by mature hepatocytes. The synchronized regeneration events between the two sources of liver cells (i.e. naïve liver and engineered liver tissue) are also highlighted by the significantly increased functional volume by over 200% compared to the pre-PH level, as determined by the measurement of the hA1AT. It has previously been shown that liver regeneration can progress based on the signalling crosstalk between the hepatocytes and non-parenchymal cells (Fausto, 2001; Michalopoulos, 2007). Although isolated hepatocytes were the only cell types used in the generation of the engineered liver tissue, the present data strongly suggests that this type of intercellular crosstalk must have been established and maintained even within our heterotopic tissues.

An additional function that we examined in the engineered liver tissue is its ability to uptake exogenous drugs and subsequently activate enzymes involved in its metabolism, which is a critical property found in normal functioning livers. In previous studies using the primary hepatocyte cell culture system, drug metabolic function would decline in the order of hours or days following the plating of the cells, even though multiple modifications were attempted to optimize the media conditions (Fahl *et al*., 1979; Gomez-Lechon *et al*., 2004). This is in marked contrast to the results in the current study, in which we were able to manipulate the isolated hepatocytes into a tissue sheet and maintain its drug metabolic effects for at least 450 days. It has been reported that the highly porous endothelial linings along the hepatic cord are essential structures for the filtration of circulating particles or compounds from the systemic circulation into the space of Disse. This anatomical feature allows the foreign matter to reach the hepatocytes (Braet *et al*., 2001) for removal from the circulation. Once the compounds are taken up by the hepatocytes, a complex cascade of transcription factors are activated to promote the induction of cytochrome P450 (*CYP450*) gene expression, which are essential intracellular enzymes known for their drug metabolism (Gomez-Lechon *et al*., 2004). Our present findings clearly demonstrated that the engineered liver tissues could simulate normal liver function as a CYP450-inducing and drug-metabolizing system, and it may be possible for these engineered liver tissues not only to metabolize chemical compounds, as shown in this study, but to detoxify endotoxin and ammonia products. These features would be of great importance for the functionality of this liver system, since its therapeutic repertoire would be expanded by enabling its use as a supportive liver-assisting device to eliminate circulating blood waste products in patients suffering from critical liver failure.

In summary, hepatocyte-based therapies have become a viable and alternative therapeutic modality in the treatment of liver diseases, but continued improvement and innovation in this technology would further help in advancing its utilization in clinics (Fox *et al*., 2006; Ofosu, 2008; Ohashi *et al*., 2007; Ohashi, 2008). At present, current technologies in this field using bioartificial liver-assisting devices struggle in their efficacy, due in large part to the inability of the hepatocytes to maintain their biological function within the device (Strain and Neuberger, 2002). Although the current study has not addressed the potential for scalability to larger mammals, our results clearly documented that the *de novo* engineered liver tissues were capable of maintaining their morphology and function even after long-term implantation under the kidney capsule for nearly the lifespan of the mouse. These results increase the likelihood that this technology could contribute as a therapeutic modality for treating liver diseases, and the increased longevity and functionality of the liver tissues could greatly expand the type of liver diseases that could be amenable to this therapeutic approach.

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References

Braet F, Luo D, Spector I, *et al*. 2001; Endothelial and pit cells. In *The Liver: Biology and Pathobiology*, 4th edn, Arias IM, Boyer JL, Chisari FV, *et al*. (eds). Lippincott, Williams & Wilkins: Philadelphia, PA; 437–454.

Bumgardner GL, Li J, Heininger M, *et al*. 1998; *In vivo* immunogenicity of purified allogeneic hepatocytes in a murine hepatocyte transplant model. *Transplantation* **65**: 47–52.

Fahl WE, Michalopoulos G, Sattler GL, *et al*. 1979; Characteristics of microsomal enzyme controls in primary cultures of rat hepatocytes. *Arch Biochem Biophys* **192**: 61–72.

Fausto N. 2001; Liver regeneration. In *The Liver: Biology and Pathobiology*, 4th edn, Arias IM, Boyer JL, Chisari FV, *et al*. (eds). Lippincott, Williams & Wilkins: Philadelphia, PA; 591–610.

Fisher RA, Strom SC. 2006; Human hepatocyte transplantation: worldwide results. *Transplantation* **82**: 441–449.

Fox IJ, Chowdhury JR, Kaufman SS, *et al*. 1998; Treatment of the Crigler–Najjar syndrome type I with hepatocyte transplantation. *N Engl J Med* **338**: 1422–1426.

Fox IJ, Schafer DF, Yannam GR. 2006; Finding a home for cell transplants: location, location, location. *Am J Transpl* **6**: 5–6.

Gomez-Lechon MJ, Donato MT, Castell JV, *et al*. 2004; Human hepatocyte in primary culture: the choice to investigate drug metabolism in man. *Curr Drug Metab* **5**: 443–462.

Griffith JG, Naughton G. 2002; Tissue engineering – current challenges and expanding opportunities. *Science* **295**: 1009–1016.

Kohn RR. 1971; Aging of animals: observations and questions. In *Principles of Mammalian Aging*, Kohn RR (ed.). Prentice-Hall: Englewood Cliffs, NJ; 105–137.

Kuge H, Ohashi K, Yokoyama T, *et al*. 2006; Genetic modification of hepatocytes towards hepatocyte transplantation and liver tissue engineering. *Cell Transpl* **15**: 1–12.

Michalopoulos GK. Liver regeneration. 2007; *J Cell Physiol* **213**: 286–300.

Ofosu FA. 2008; Propagation factor IX-producing hepatocytes for haemophilia B therapy. *Thromb Haemost* **99**: 799–800.

Ohashi K, Tatsumi M, Tsujiuchi T, *et al*. 1996; Enhancement of *N*-nitrosodiethylamine-initiated hepatocarcinogenesis caused by a cholchicine-induced cell cycle disturbance in partially hepatectomized rats. *Cancer Res* **56**: 3474–3479.

Ohashi K, Marion PL, Nakai H, *et al*. 2000; Sustained survival of human hepatocytes in mice: a model for *in vivo* infection with human hepatitis B and hepatitis delta viruses. *Nat Med* **6**: 327–331.

Ohashi K, Park F, Kay MA. 2001; Hepatocyte transplantation: clinical and experimental application. *J Mol Med* **79**: 617–630.

Ohashi K, Waugh JM, Dake MD, *et al*. 2005; Liver tissue engineering at extrahepatic sites in mice as a potential new therapy for genetic liver diseases. *Hepatology* **41**: 132–140.

Ohashi K, Kay MA, Yokoyama T, *et al*. 2005; Stability and repeat regeneration potential of the engineered liver tissues under the kidney capsule in mice. *Cell Transpl* **14**: 621–627.

Ohashi K, Kay MA, Kuge H, *et al*. 2005; Heterotopically transplanted hepatocyte survival depends on extracellular matrix components. *Transpl Proc* **37**: 4587–4588.

Ohashi K, Yokoyama T, Yamato M, *et al*. 2007; Engineering functional two- and three-dimensional liver systems *in vivo* using hepatic tissue sheets. *Nat Med* **13**: 880–885.

Ohashi K. 2008; Liver tissue engineering: the future of liver therapeutics. *Hepatol Res* **38**: S76–87.

Sole K. 2006; New device aids bioengineering of hepatic tissues. *Nature Clin Prac Gastroenterol Hepatol* **3**: 183.

Stephenne X, Najimi M, Sibille C, *et al*. 2006; Sustained engraftment and tissue enzyme activity after liver cell transplantation for argininosuccinate lyase deficiency. *Gastroenterology* **130**: 1317–1323.

Stephenne X, Vosters O, Najimi M, *et al*. 2007; Tissue factor-dependent procoagulant activity of isolated human hepatocytes: relevance to liver cell transplantation. *Liver Transpl* **13**: 599–606.

Strain AJ, Neuberger JM. 2002; A bioartificial liver – state of the art. *Science* **295**: 1005–1007.

Strom SC, Bruzzone P, Cai H, *et al*. 2006; Hepatocyte transplantation: clinical experience and potential for future use. *Cell Transpl* **15**: S105–110.

Tatsumi K, Ohashi K, Kataoka M, *et al*. 2008a; Successful *in vivo* propagation of factor IX-producing hepatocytes in mice: potential for cell-based therapy for haemophilia B. *Thromb Haemost* **99**: 883–891.

Tatsumi K, Ohashi K, Shima M, *et al*. 2008b; Therapeutic effects of hepatocyte transplantation on hemophilia B. *Transplantation* **86**: 167–170.

Waxman DJ, Azaroff L. 2006; Phenobarbital induction of cytochrome P-450 gene expression. *Biochem J* **281**(pt 3): 577–592.

Yokoyama T, Ohashi K, Kuge H, *et al*. 2006; *In vivo* engineering of metabolically active hepatic tissues in a neovascularized subcutaneous cavity. *Am J Transpl* **6**: 50–59.

Zacharova LY, Gulyaeva LF, Lyakhovich VV, *et al*. 2003; Cytochrome P4501A1 and 1A2 gene expression in the liver of 3-methylcholanthrene- and *o*-aminoazotoluene-treated mice: a comparison between PAH-responsive and PAH-nonresponsive strains. *Toxicol Sci* **73**: 108–111.

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