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Therapeutic liver reconstitution with murine cells isolated long after death

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Abstract

Background and Aims—Due to the shortage of donor organs many patients needing liver transplantation cannot receive one. For some liver diseases hepatocyte transplantation could be a viable alternative, but donor cells are currently procured from the same sources as whole organs and thus the supply is severely limited.

Methods—Here, we investigated the possibility of isolating viable hepatocytes for liver cell therapy from the plentiful source of morgue cadavers. In order to determine the utility of this approach, cells were isolated from the livers of non-heart-beating cadaveric mice long after death and transplanted into fumarylacetoacetate hydrolase (Fah) deficient mice, a model for the human metabolic liver disease hereditary tyrosinemia type I and a stringent *in vivo* model for hepatic cell transplantation.

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Results—Surprisingly, complete and therapeutic liver repopulation could be achieved with hepatocytes derived up to 27 hours post-mortem.

Conclusions—Competitive repopulation experiments demonstrated that cadaveric liver cells had a repopulation capacity similar to freshly isolated hepatocytes. Importantly, viable hepatocytes could also be efficiently isolated from cadaveric primate liver (monkey and human). These data provide evidence that non-heart-beating donors could be a suitable source of hepatocytes for much longer time periods than previously thought possible.

Keywords

Fumarylacetoacetate hydrolase (Fah) deficient mice; hereditary tyrosinemia type I; liver disease; hepatocytes; cell transplantation; non-beating heart donor; cadaveric donor; 2-(2-nitro-4-3 trifluoro-methylbenzoyl)-1; 3-cyclohexanedione (NTBC); Rhesus Macaque; human

Background and Aims

Clinical trials have established the feasibility of using hepatocyte transplantation as a treatment for several liver diseases, particularly genetic metabolic diseases and acute liver failure¹. However, this promising therapeutic strategy has been stalled by the lack of quality donor cells since transplantable hepatocytes are isolated only from surgical liver resection specimens or whole organs rejected for transplantation, both very limited in supply. Recently, the generation of hepatocytes via *in vitro* differentiation of human embryonic stem cells has shown promise^{2,3} but transplantation models have shown these hepatocyte-like cells currently to have only limited functionality *in vivo*^{4,5}. Furthermore, safety concerns in regards to the potential tumorigenicity of this cell source remain⁶. It has long been known that death does not result in the immediate demise of all cells within an animal. For example, hematopoietic stem cells are not harmed by the immediate hypoxia following death and can be efficiently isolated 19 hours post mortem⁷⁻⁹. Non-heart-beating donors (NHBDs) have been previously considered as a source of transplantable hepatocytes, but only if the livers were harvested under controlled conditions within 45 minutes after death¹⁰. In the rat, plateable hepatocytes have been isolated for up to 8 hours after death, but were not used for transplantation¹¹.

Here, we therefore wished to determine how long transplantable hepatocytes survive after death. The answer to this question is important because the ability to obtain viable hepatocytes from NHBDs who have died in an uncontrolled setting (morgue cadavers) would establish a much needed, plentiful source of cells for transplantation. Remarkably, we found that hepatic cells isolated up to 27 hours post-mortem could be effectively used for liver cell therapy.

Methods

Mice

All animal procedures were approved by the Institutional Animal Care and Use Committee at OHSU. Rosa26 mice were provided by P. Soriano¹² and *Fah*^{-/-} mice were produced in the Grompe Laboratory¹³. All mice used were in the 129Sv4 background and when possible littermate controls were used.

Mouse liver cell isolation

Mice were sacrificed and either stored without further manipulation at 4°C for a specified time (cadaveric) or perfused under anesthesia while still alive (fresh). Cells were isolated by portal vein perfusion¹⁴, resuspended in DMEM High Glucose 10% FBS, washed twice with

50g centrifugation, followed by 30% Percoll gradient with 100g centrifugation. A minimum of three washes were performed to remove all residual Percoll from the cells.

Transplantation and repopulation

The number of cells transplanted intrasplenically into *Fah*^{-/-} mice¹³ was between 0.18×10^5 and 2.5×10^5 cells/mouse, as previously described¹⁵. After surgery, mice were withdrawn from 2-(2-nitro-4-(3-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) to encourage repopulation, as previously described¹⁶. After six weeks off NTBC with weight stabilization the mice were sacrificed for analysis.

Immunohistochemistry and Xgal staining

Fah immunohistochemistry was done on liver tissue sections using 1:1000 rabbit-*Fah* (Grompe Laboratory) as previously described¹⁵. X-gal staining on liver sections, cells, and whole liver tissue was done as previously described^{17, 18}.

Immunocytochemistry of liver cells

Liver cells were plated on 10% rat tail collagen coated chamber slides. *Fah* and Alb ICC; All antibody incubations were done at room temperature. *Fah*; cells were fixed in methanol 5 seconds, rinsed with acetone, then PBS, treated with 5% donkey serum in PBS with 0.05% Tween (PBS-T) 30 minutes, incubated with 1:1000 rabbit-*Fah* (Grompe Laboratory) 1 hour in PBS-T, and 1:400 donkey-*Fah* rabbit AF 555 (Molecular Probes A31572) for 1 hour. Albumin; cells were fixed in 4% paraformaldehyde 20 minutes, rinsed with PBS, treated with 5% goat serum in PBS-T 30 minutes, incubated with 1:500 rabbit-Albumin (Genetex gtx19196) for 30 minutes, rinsed with PBS-T, and then with goat-*Albumin* rabbit AF 488 (Molecular Probes A11008) for 30 minutes. G6PD and HNF4a ICC; Glucose-6-phosphodiesterase (G6PD; rabbit polyclonal, Bethyl laboratories, Inc, IHC-00007, used at 1:100) and hepatocyte nuclear factor 4-alpha (HNF4a; rabbit polyclonal, Santa Cruz biotechnology, Inc, SC-8987 used at 1:50) on human and mouse hepatocytes was done the following way: Blocking with 5% donkey serum in PBS with 0.05% Triton X-100 for one hour, then incubation with primary antibodies overnight at 4°C, followed by a PBS Triton X-100 wash, 3 times for 10 minutes each, then incubation in 1:300 donkey anti rabbit IgG Cy3 conjugated (Jackson ImmunoResearch laboratories, Inc 711-165-152) for 45 minutes at RT. After washing with PBS 3 times, mounting media with DAPI was added. Normal rabbit IgG (Vector Laboratories, I-1000) at 5 ug/ml and PBS were used as negative controls. Nuclei staining was done with DAPI (Molecular Probes P-36931).

Plasma tyrosine level analysis

Plasma amino acid analysis, to measure tyrosine levels, was performed on a Biochrom 30 HPLC amino acid analyzer. 30-50 µl of plasma was mixed with equal volumes of Seraprep (Cat SP100, Biochrom US) and Lithium Citrate loading buffer (Cat 80-2038-10, Biochrom US). Norleucine was the internal standard. Protein was precipitated by centrifugation at 16,000g for 3 minutes and supernatant was analyzed on a Biochrom 30 HPLC. Amino acid concentrations were determined with EZchrome Elite software.

Fah activity assay

Protein lysates were isolated from frozen liver tissue and the *Fah* activity assay was performed as previously described^{15, 16}.

AAV8-Ttr-Cre vector

An expression cassette composed of a *Ttr* promoter and Cre recombinase was constructed by PCR-amplification from previously described plasmids^{19, 20}. This cassette was used to

replace the H1 promoter in dsAAV-H1²⁰. The dsAAV-H1 backbone is based on the AAV2 genome but contains heterologous packaging signals from AAV2 and AAV4 to prevent inactivating recombination during propagation. For production of AAV8 capsid pseudotyped vector, we transfected A293 cells with the modified dsAAV plasmid, the adenoviral helper plasmid pAd5 and the AAV8 capsid expression plasmid p5E18-VD2/8²¹ using the calcium phosphate method. Virus was collected 72 hours after transfection and concentrated by centrifugation on a density gradient of cesium chloride (Invitrogen). Viral titer was determined by dot blot analysis as described²¹. This virus was injected either retro-orbitally or via the tail vein into Rosa26 reporter mice (R26R)²². One week later cadaveric cells were isolated from these mice for X-gal staining.

qPCR

Genomic DNA was isolated from liver tissue and used for qPCR. Primers; *Fah*-Forward: CCGGGGTTCTCCATCTAGGTC; *Fah*-Reverse: TGGCAACAGCGCATTCTCCTT; *Rosa26*-Forward: CATCAGCCGCTACAGTCAACAG; *Rosa26*-Reverse: CAGCCATGTGCCTTCTTCCGC; Gapdh-Forward: ACGGGAAGCCCATCACCATCTT; Gapdh-Reverse: ACCCTTTTGGCTCCACCCTTCA. Primers specifically recognize genomic DNA, and in the case of *Fah*, only amplify the WT gene (not the mutant). PCR cycles: 95°C 3min; 95°C 15seconds, 67°C 20seconds, 72°C 20seconds (45x).

DNA quantitation was done by determining the cycle threshold for a dilution series of DNA for each primer set. This was graphed linearly and the equation of the line was used to calculate the relationship between cycle threshold and DNA content for each primer set in control and experimental tissue, allowing direct DNA quantitation of *Fah* and *Rosa26* DNA in the samples.

Rhesus Macaque liver cell isolation

All animal procedures were approved by the Institutional Animal Care and Use Committee at the ONPRC/OHSU. Liver lobes were obtained from the Oregon Primate Center and either perfused within 2 hours (fresh isolation) or stored at 4°C for 24 hours before cell isolation. All primates used were adults scheduled for necropsy for reasons unrelated to liver dysfunction. Cell isolation was performed via a modified 3-step collagenase perfusion protocol as previously described²³. In summary, tubing was sewn into lobe vasculature and the following three buffers were delivered via peristaltic pump; Buffer 1: Earle's Balanced Salt Solution (EBSS) without calcium and magnesium, plus 0.5 µmol/L EGTA and 10mM Hepes pH 8; Buffer 2: EBSS with calcium and magnesium, plus 10mM Hepes; Buffer 3: Buffer 2 with 250mg/L Collagenase XI and 50mg/L DNase. The liver lobe was placed in a plastic bag and digested in a 39°C water bath. Following digestion, it was thoroughly chopped with scissors and run through sterile gauze to remove large tissue pieces. The resulting slurry was centrifuged at 100×g 5 minutes at 4°C three consecutive times and resuspended in DMEM HI Glucose (Hyclone) with antibiotics/antimycotics (Cellgro 30-004-CI) and 5% Bovine calf serum, plated and grown overnight at 37°C 5% CO₂. Cell number and viability was determined by trypan blue exclusion.

Human hepatocyte isolation and culture

Human hepatocytes were isolated from surgical liver resections. Buffers 1 and 2 from the Rhesus liver cell preparation were used, see Methods above. For "cadaveric" liver cells, the liver resection was placed in a sterile bag on ice at 4°C for 24 hours. The tissue was then rinsed with Buffer 1 and perfused by placing a Buffer 1 filled tuberculin syringe into vessels that allowed optimal blanching of the tissue. Successful perfusion is visible as tissue blanching when residual blood is pushed out of the tissue. After the tissue was flushed thoroughly it was placed in a dish and finely chopped. After this, all incubations were done

in 50 ml tubes at 38°C in a water bath. The chopped tissue was incubated in Buffer 1 for 15 min, rinsed and digested with Buffer 3 for 15 minutes, followed by successive Buffer 3 incubations in 30 minute increments until the tissue appeared sufficiently digested (minimum 30 minutes and maximum 1 hour). This was followed by further mechanical dissociation and passage through a 100µm and 70µm filter. The cell slurry was centrifuged at 100×g 5 minutes at 4°C three consecutive times, rinsing with DMEM HI Glucose (Hyclone) with antibiotics/antimycotics (Cellgro 30-004-CI) and 5% Bovine calf serum. Cell viability was determined by trypan blue exclusion. 50,000 viable cells were cultured overnight at 37°C 5% CO₂, fixed with 4% PFA for 10 minutes, and washed with PBS 3 times prior to ICC.

Cell viability by trypan blue exclusion

To determine percentage viability, total viable (clear) and dead cells (blue) were counted separately with a hemocytometer, three consecutive times. The average for each was summed and the percentage viability was calculated by dividing the (total number of dead cells)/(total cell number for dead and alive cells). Cell yields for viable cells were calculated by multiplying the following together (average number of viable cells on hemocytometer) × (10⁻⁴) × (the dilution) × (the total volume)."

Statistics

Error bars are shown as ±standard error of the mean (SEM) for a minimum of three replicates. Significance is based on a two-tailed homoscedastic T-test (p<0. 05). Standard deviation was used to describe error involved in comparing viability and total number of cadaveric versus fresh rhesus liver cells.

Results

Viable cells, including mature hepatocytes, survive 24 hours post-mortem in cadaveric liver

We first investigated whether viable liver cells could be isolated 24 hours post-mortem. Donor mice were euthanized and kept intact at 4°C until cell isolation. Initially, we were concerned that the portal vein perfusion needed for efficient liver cell isolation would not be possible after circulatory arrest since blood clots could block the vasculature¹⁴. Standard liver cell isolation methods are typically initiated in live mice so that continued heart beat and blood circulation can be used to efficiently wash out the blood from the liver. Remarkably, we found that efficient liver perfusion was possible in the absence of heartbeat and blood circulation because fibrinolysis takes place several hours after death, digesting blood clots and allowing enzyme access to the vasculature (Figure 1A). Surprisingly, the percentage of viable cells as determined by trypan blue exclusion isolated within 27 hours after death was not significantly different from cells obtained from freshly killed mice (Figure 1B). There was no direct relationship between percentage viability and total viable cell yield. Thus, cell numbers obtained from cadaveric mice were significantly reduced compared to those freshly isolated, while the percentage of viable cells remained similar. The total liver cell yield from 24 hour dead cadaveric mice averaged ~9% of that from freshly killed mice (Figure 1A). The plated cells were mostly binucleated, expressed Fah (Figure 1C) and albumin (Figure 1D), G6PD (Figure 1E) and HNF4a (Figure 1F), all hallmarks of differentiated hepatocytes. Negative controls for the immunocytochemistry were pre-immune sera for Fah (Figure 1C) and secondary antibody only for albumin, glucose 6-phosphate dehydrogenase (G6PD), and hepatocyte nuclear factor-4 alpha (HNF4a) (Figure 1D, E and F, respectively). These results demonstrate that a reasonable total yield of viable liver cells, including mature hepatocytes, could be obtained from refrigerated adult mouse cadavers 24 hours post-mortem.

Cadaveric liver cells are capable of liver engraftment and repopulation

Next, we determined if cells isolated from cadaveric livers are suitable for transplantation and restoration of liver function. To test this, we transplanted these cells into *Fah*^{-/-} mice, a model which allows positive selection of hepatocytes *in vivo*, resulting in therapeutic repopulation of the liver^{13, 16}. To trace the transplanted cells, Rosa26^{+/-} mice ubiquitously expressing the marker beta-galactosidase¹² were used as donors. Remarkably, we found that cadaveric cells efficiently repopulated the livers of the *Fah*^{-/-} recipients (Figure 2A and B). Histology showed repopulation levels exceeding 60% in all cases (data not shown). Not only did cadaveric cells replace the *Fah*-deficient hepatocytes, they were also capable of correcting the abnormal liver function of the recipient mice, as evident from normalization of blood tyrosine, bilirubin and aspartate aminotransferase levels (Figure 2C-E). Successful liver cell therapy was also indicated by weight stabilization of *Fah*^{-/-} mice in the absence of NTBC, a drug required to prevent liver disease in untransplanted mice of this strain (Figure 3A). Moreover, we detected wild-type *Fah* protein (Figure 3B), active *Fah* enzyme (Figure 3C), and wild-type *Fah* DNA (Figure 3D) in recipient *Fah*^{-/-} livers, providing direct evidence for the stable engraftment and hepatocyte-specific function of the transplanted cells. Together, these results show conclusively that cadaveric donor cells were effective in restoring liver function in *Fah*^{-/-} mice.

Effects of post-mortem time and temperature on viability of cadaveric liver cells

Next we used refrigerated donors which had been dead for even longer than 24 hours to determine the maximum time transplantable hepatocytes can be isolated after death (Figure 3E). Liver repopulation was only observed in a single recipient from a donor that had been dead for 27 hours, as evident from the presence of wild-type *Fah* enzyme (Figure 3C) and DNA (Figure 3D) as well as restoration of liver function (Figure 2C-E). None of the mice that received cells isolated 36 and 48 hours post-mortem survived without NTBC (Figure 3E). There was no detectable engraftment in mice that received cells from a donor dead for 36 hours. The liver of these recipient was harvested once death was imminent and PCR detected no wild-type *Fah* DNA (data not shown).

These results show that liver-repopulating capabilities were lost in cadaveric liver cells 27 hours after death.

Cadaver storage temperature has a significant effect on cell viability and transplantability

To further determine whether storage temperature affects the therapeutic efficacy of cadaveric liver cells, we isolated liver cells from mice that were kept at room temperature after death. Complete liver repopulation could be obtained with cells harvested 2 hours post-mortem, but the efficiency dropped off dramatically after that (Figure 3E). Thus, warmer temperature had a marked negative effect on the viability and transplantability of liver cells from non-heart-beating donors.

The repopulation capacity of cadaveric liver cells is not significantly different compared to freshly isolated cells

While these data clearly indicated that cadaveric cells are capable of therapeutic liver repopulation, the relative repopulation capacity of cadaveric cells in direct comparison to fresh cells remained to be determined. For this purpose, we performed competitive repopulation experiments by transplanting *Fah*^{-/-} recipients with cadaveric cells isolated from a Rosa26^{+/-} mouse and freshly isolated wild-type hepatocytes mixed at a 1:1 ratio. As controls, both cadaveric (*Fah*^{+/-}, Rosa26^{+/-}) and fresh (*Fah*^{+/-}) cells were transplanted into separate recipients. Fresh hepatocytes were isolated by initiating the protease perfusion *in situ* while the donor was still alive. High level repopulation with *Fah*-expressing hepatocytes

was found in all three groups (Figure 4A). Additional X-gal staining indicated that the co-transplanted Rosa26^{+/-} cadaveric liver cells and the wild-type hepatocytes contributed about equally to the overall repopulation (Figure 4B and C). To precisely quantify the contribution of cadaveric versus fresh donor cells, we performed qPCR for wild-type *Fah* and *Rosa26* alleles on DNA isolated from recipient livers (Figure 4D). Since the cadaveric donors were hemizygous for the Rosa26 transgene, equal repopulation by cadaveric and fresh donor cells is expected to yield 4 copies of wild-type *Fah* for each copy of *Rosa26* (4:1). The measured ratio from five 1:1 mix transplants averaged 6:1 (± 0.46) indicating that freshly isolated hepatocytes may have a slight repopulation advantage over cadaveric cells. However, this deviation from the expected 4:1 ratio was not statistically significant, indicating that cadaveric liver cells have at least close to normal regenerative capabilities.

Mature hepatocytes are the main cell type responsible for liver repopulation from cadaveric cell sources

While the ability of cadaveric cells to function in transplantation was clear from the above results, the hepatic cell type responsible could be either mature hepatocytes or liver progenitors or stem cells. We hypothesized that liver progenitors were more likely candidates as they are believed to be more resistant to ischemia than hepatocytes²⁴. However, many of the viable cells isolated from cadaveric livers showed features characteristic for mature hepatocytes (Figure 1C and D). To conclusively determine whether mature hepatocytes were at least partially responsible for liver repopulation from post-mortem donors, a genetic strategy was used to mark mature hepatocytes in the cadaveric donor liver prior to cell harvest. For this purpose, Rosa26 reporter (R26R) mice harboring a floxed beta-galactosidase transgene that is activated by Cre-recombinase were used as donors. To limit beta-galactosidase expression to mature hepatocytes, the R26R^{+/-} mice were injected with an adeno-associated virus vector serotype 8 which expresses Cre under the transcriptional control of the hepatocyte-specific transthyretin receptor (*Ttr*) promoter (AAV8-*Ttr-Cre*). While the *Ttr* promoter can be active in other cell types such as embryonic stem cells, the viral delivery system used here labels only mature hepatocytes in the adult liver (Lee et al. manuscript in preparation). As expected, the efficiency and liver tropism of AAV8-*Ttr-Cre* resulted in an entirely blue liver upon X-gal staining (Figure 5A). In contrast, X-gal staining of a toe from an AAV8-*Ttr-Cre*-injected R26R mouse was negative (Figure 5A inset). After genetic marking of mature hepatocytes with AAV8-*Ttr-Cre*, R26R^{+/-} mice were killed, liver cells were isolated 24 hours later, and a sample was plated. The majority of the isolated cells were beta-galactosidase positive and binucleated, a representative cell is shown in (Figure 5B, left panel). The remaining AAV8-*Ttr-Cre* marked R26R^{+/-} cadaveric hepatocytes were transplanted into *Fah*^{-/-} mice. After NTBC withdrawal for a number of weeks the recipient's livers were harvested and the presence of donor cells was determined by whole liver X-gal staining. Gross inspection of a liver lobe from one of these recipients demonstrates successful repopulation with these Xgal labeled cells, seen in Figure 5B (right panel), as blue nodules. Tissue sections demonstrate that most repopulating nodules expressed beta-galactosidase (Figure 5C-left panel) and wild-type *Fah* (Figure 5C-right panel), indicating that they were derived from mature hepatocytes, not liver progenitor cells.

Viable hepatocytes are present 24 hours post-mortem in primate liver

To assure that our findings in mice can be extended to larger animals, we determined whether dead rhesus macaque monkeys can be used as donors of viable liver cells. A liver lobule was removed from an adult non-heart-beating donor 15 minutes after circulatory arrest and kept intact at 4°C. Lobule perfusion and cell isolation were performed 24 hours later. As observed in mice, the number of viable cells isolated from cadaveric liver was about 10% of that compared to immediately perfused liver. Viability was markedly increased in fresh liver (20% dead (± 8)) compared to cadaveric (60% dead (± 17)). Total cell

number isolated from cadaveric liver was 16% (± 2) of that from fresh liver ($n=3$). Although viability and total cell number was lower in cadaveric livers it was still possible to obtain isolates containing a large number of living hepatocytes as evidenced by efficient plating on tissue culture dishes and the presence of binucleated cells that were positive for mature hepatocyte markers, Fah (Figure 6A) and Albumin (Figure 6B).

To determine the clinical relevance of our finding, we then isolated hepatocytes from two matched fresh and “cadaveric” human livers.” In this setting “cadaveric” human livers were liver resection specimens which had been refrigerated for 24 hours before perfusion. Another piece from the same specimen was perfused as soon as possible after surgical removal and designated as “fresh”. Again, viability and total cell numbers were lower in cadaveric cells. Nonetheless, about 10% of the cells were platable, were mostly binucleated, and positive for the mature hepatocyte markers G6PD (Figure 6C) and HNF4a (Figure 6D). This result demonstrates that viable hepatocytes are also present in human livers, even 24 hours after cold ischemia.

Conclusions

It is generally accepted that livers from non-heart-beating cadavers are unsuitable for whole organ transplantation after ischemia for more than one hour, although metabolic resuscitation may be possible if it is initiated within minutes after circulatory arrest^{25, 26}. Similarly, hepatocytes used for *in vitro* studies or cell transplantation are usually isolated from donors with a beating heart, or short-term circulatory arrest²⁶. We wished to determine whether therapeutically useful hepatocytes could also be isolated when death occurs in an uncontrolled environment and when cell isolation is significantly delayed. Here we demonstrate unambiguously that cadaveric mouse livers contain cells useful for transplantation therapy for up to 27 hours post-mortem. The only manipulation required to maintain transplantability for this length of time was simple refrigeration, as routinely performed in humans after death. It was not necessary to perfuse the liver with a preservation solution or use any other special interventions prior to cell isolation. While the cell yield was clearly lower than with freshly dead donors, a large number of viable cells could nonetheless be isolated. Importantly, these cells not only survived prolonged ischemia but maintained their ability to engraft, repopulate, and correct the metabolic liver disease in *Fah*^{-/-} mice. Genetic marking with a *Ttr* driven Cre showed that a large percentage of the repopulating cells were mature hepatocytes. Although *Ttr* is known to be expressed in ES-derived hepatocyte-like cells, fetal liver, and other stem-like cells, the AAV-*Ttr* marking strategy used here labels only hepatocytes and non-parenchymal cells in adult liver (Lee 2010, submitted for publication). Despite this genetic marking experiment, however, we cannot formally rule out a limited additional contribution from liver progenitor cells or other non-hepatocytes. Regardless of the precise nature of the repopulating cell(s) it is clear that they are therapeutically active and capable of restoring liver function. Importantly we were also able to show that viable cells, including hepatocytes, could be readily isolated from cadaveric livers of adult primates. Furthermore, platable hepatocytes could be found in human liver resection specimens after 24 hours of cold ischemia. Thus, it seems likely that most cadaveric human livers also harbor hepatocytes useful for transplantation therapy even after prolonged ischemia and even if death occurred in an uncontrolled setting. While others have shown the presence of viable hepatic progenitors in human cadaveric liver even several days after death, the transplantability of this cell type was not demonstrated²⁷. In contrast, the therapeutic utility of mature hepatocytes is firmly established¹.

Currently, non-beating-heart donor livers are only used in the context of a controlled environment, i. e. when the donor's death can be reliably anticipated. Our data provide the rationale for further exploring the use of cadaveric livers after unanticipated death such as

sudden trauma. Livers from young and otherwise healthy donors are particularly likely to harbor therapeutically useful cells for many hours postmortem. Given the current extreme shortage of sources of transplantable human hepatocytes, the possibility of using this very abundant liver cell source should be explored.

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Figure 1.

Cadaveric mouse liver contains viable hepatocytes. (A) Cell yields from mice that were freshly killed (Fresh) or isolated 24, 27, 36, and 48 hours post-mortem. N=Number of mice measured. (B) Viability of cells isolated at different time points post-mortem; the number of mice tested is in parentheses on the x-axis. C-F; Comparison of hepatic marker expression in fresh and cadaveric cell preparations; immunocytochemistry for Fah in red (C), Albumin in green (D), G6PD in red (E) and HNF4a in red (F) and DAPI nuclear staining (blue). Controls for Fah, pre-immune sera (C), or a secondary antibody only for Alb (D), G6PD (E) and HNF4a (F) were negative.

Figure 2.

Normal liver function in mice repopulated with cadaveric donor cells. (A-B) X-gal staining of tissue sections (A) and whole livers (B) of *Fah*^{-/-} mice transplanted with fresh wild-type (Fresh WT) cells or cadaveric Rosa26. The presence of blue nodules demonstrates successful engraftment by cadaveric hepatocytes in *Fah*^{-/-} mice. (C-E) Correction of hepatic functions after transplantation of cadaveric cells. Untransplanted (UT) controls are shown on the right. Blood levels of tyrosine (C), bilirubin (D), and aspartate aminotransferase (E) in *Fah*^{-/-} mice transplanted with the following cell sources: freshly isolated donor (Fresh WT), 1:1 mix of Fresh:Cadaveric donor, and cadaveric cells alone (24 and 27 hours post-mortem).

Figure 3.

Cadaveric cells were capable of liver engraftment and repopulation. **A**; Representative weight curves after NTBC withdrawal of the recipients. Temporary reinstitution of NTBC treatment shown with gray over-lay; here two mice received fresh (solid lines, square and diamond) and two were transplanted with cadaveric cells (dashed lines, square and triangle). **(B)** Immuno-histochemistry for Fah on liver sections of transplanted *Fah*^{-/-} mice, an Fah positive nodule is encircled in the left panel (Fresh), the cadaveric liver section is completely positive for Fah protein (right panel). **(C)** Fah enzyme activity; UT *Fah*^{-/-} = untransplanted *Fah* mutants (n=2); UT WT= untransplanted wild-type controls (n=3); FRESH = *Fah*^{-/-} mice transplanted with fresh donor cells; 24hr dead = *Fah*^{-/-} mice transplanted with cadaveric cells isolated 24 hours post-mortem (n=3); 27hr dead = isolated 27 hours post-mortem (n=1). **(D)** Fah genomic qPCR to quantitate wild-type DNA in recipient livers. **(E)** Summary of cadaveric cell transplantation results for all time points.

Figure 4.

Competitive repopulation between fresh and cadaveric donor cells. Cadaveric cells were from beta-galactosidase transgenic donors (Rosa26) and fresh cells were from non-transgenic wild-type donors (Fresh WT). (A) Fah immunohistochemistry (brown) demonstrates high levels of repopulation in all treatment groups. (B-C) The presence of cadaveric cells is confirmed by Xgal staining (blue) in tissue sections (B) and whole liver (C). (D) To quantify the relative repopulation levels from fresh and cadaveric cells qPCR for Rosa26 (black) and Fah (gray) was performed on genomic DNA from liver tissue of mice that received populations of fresh (*Fah*^{+/+}) and cadaveric cells (*Fah*^{+/+} Rosa26^{+/+}) in a 1:1 mix (n=5). Untransplanted controls are shown on the right.

Figure 5.

Mature hepatocytes survive death and are capable of liver engraftment. (A) Infection of R26R mice with the AAV-TTRcre virus results in widespread hepatic expression of beta-galactosidase (left panel). Negative controls are shown; the X-gal negative toe from the virus injected mouse (left panel-inset) and liver from a cadaveric R26R mouse that did not receive virus (right panel). (B) Beta-galactosidase positive binucleated hepatocytes were successfully isolated 24 hours post-mortem from R26R mice that received virus, white arrowheads mark the two nuclei of the binucleated hepatocyte (B-left panel). Successful engraftment by mature hepatocytes was confirmed by the presence of x-gal positive nodules in the recipient liver, visualized with X-gal stain in whole liver (Bright panel) and tissue sections (C-left panel), as well as by immunohistochemistry for Fah protein (C-right panel).

Figure 6.

Viable hepatocytes are present in genomic DNA non-human primate and human liver. Non-human primate liver (A and B): Both cadaveric cells (left column) and fresh liver cells (right column) stain positively for hepatocyte markers Fah (A) and Albumin (B). The white arrow in the left panel (A, far left) denotes a Fah positive, binucleated, cell from cadaveric primate liver. Human liver (C and D): Both cadaveric and fresh hepatocytes from human liver were positive for G6PD (C) and HNF4a (D). Negative controls were run for each antibody, no primary antibody.