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Successful Transplantation of Primary Hepatocytes from DsRed Mice into Fah$^{-/-}$;Scid/Scid Mice; A Future In Vivo Model System for Receiving Human Hepatocytes

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Abstract
We have developed a system for studying hepatocellular growth potential in which liver cells are introduced into the diseased livers of Fah$^{-/-}$; Scid/Scid double knockout mice. To use this system to study cell transplantation, DsRed liver cells were introduced into severe immunodeficient Fah$^{-/-}$; Scid/Scid double knockout mice. In regenerated recipient livers, up to 20% of the mouse liver is repopulated by DsRed mouse hepatocytes demonstrating the creation of a functional mouse liver in which parenchyma is derived from DsRed mouse hepatocytes. The severe immunodeficient Fah$^{-/-}$;Scid/Scid double knockout mice provide a tool for studying hepatocellular biology.
Keywords: Human Hepatocytes, Transplantation, DsRed and Fah$^{-/-}$;Scid/Scid Mice.

1. Introduction

In vitro cultures of primary cells often do not reflect their biological functions in the organism. For instance, cultured human hepatocytes display a different gene expression profile than hepatocytes in the liver. Therefore, substantial efforts have been made to repopulate murine livers with human hepatocytes. Yet the ability to propagate specialized human cells with intact biological functions in experimental animal models remains technically challenging. Until recently, only the urokinase type plasminogen activator (uPA) mouse model was permissive for repopulation of human hepatocytes. This mouse model, developed to study bleeding disorders, contains an additional copy of uPAexpressed from an albumin promoter. As anticipated, the mouse had increased fibrinolysis, but hepatotoxicity was also observed, which usually led to the death of pups because of intestinal bleeding or liver failure[1]. A few years later, congenic and xenogenic (rat and woodchuck) hepatocyte transplantation was reported[2–4.] Jonathana A. Rhim *et al.* have developed a transgenic mouse system to assess the regenerative capacity of hepatocytes. In this system, albuminurokinase (Alb-uPA) transgenic mice are recipients of donor mouse hepatocytes. The transplanted hepatocytes grow within the Alb-uPA liver[4], replacing transgene-expressing hepatocytes that are functionally compromised by transgene expression[1]. Using this approach, they demonstrated that hepatocytes from adult liver have extensive replicative potential[4]. The growth of the transplanted cells was occurred over several weeks. The resulting fully regenerated livers were chimeric, composed both of donor-derived cells and of host-derived cells that had deleted transgene DNA and therefore no longer expressed the transgene[4]. Like transplanted hepatocytes, these host-derived cells also had a growth advantage relative to transgene-expressing hepatocytes and expanded. In hemizygous transgenic animals, transgene inactivation is a relatively frequent event[1]; complete replacement of the transgenic liver occurs by 8 weeks of age. By comparison, in homozygous mice, transgene inactivation is a less common event, consistent with the fact that two transgene arrays must be inactivated. Thus, hepatocyte transfer into homozygous Alb-uPA mice would be ideal for assessing liver cell growth. Even though Alb-uPA transgenic mouse liver supported the growth of transplanted mouse hepatocytes[4] but this mouse model was immunotolerant. So, we hypothesized that immunodeficient mice with lacking the enzyme fumarylacetoacetate hydrolase (Fah$^{-/-}$) would be better model to assess the growth of transplanted hepatocytes from other types of mouse or species. That's why we generated Fah$^{-/-}$; Scid/Scid double knockout mice and successfully transplanted hepatocytes from DsRed mouse. We have shown that upto 20% of diseased mouse liver hepatocytes were replaced by hepatocytes from donor mice DsRed. This study supports the previous study of Karl-Dimiter B. *et al.*[5] Finally, if our current methods were feasible, Fah$^{-/-}$; Scid/Scid double knockout mice livers repopulated with hepatocytes from dsRed mice would be valuable

tools for studying liver biology and in near future these mice would be *in vivo* model systems for receiving human hepatocytes.

2. Materials and Methods

Mice: The Fah$^{-/-}$ mouse[6] was crossed with the mice homozygous for the severe combined immune deficiency spontaneous mutation *Prkdc*^{scid}, commonly referred to as *scid*, are characterized by an absence of functional T cells and B cells. These mice were obtained from The Jackson Laboratory. Homozygous mice were identified by genotyping of tail DNA. Genotyping was done with the same primers and conditions as described elsewhere[6] or according to the protocols provided by vendors. The severe immunodeficient Fah$^{-/-}$; Scid/Scid double knockout mice were kept on 7.5 mg/ml NTBC (100%) in the drinking water. All mice were bred in a pathogen-free facility and tested routinely for mouse diseases. Mice were also kept in temperature-and humidity-controlled animal quarters with a 12-h light-dark cycle.

Animal ethics: Research involving animals complied with protocols approved by the Hamamatsu University School of Medicine Committee of Laboratory Animal Experimentation, Japan.

Liver Cell Isolation and Transplantation: Mouse liver cells were isolated from 2- to 4-month-old male DsRed mice (The Jackson Laboratory) by two-step EDTA/collagenase perfusion using a protocol modified from Klaunig *et al.*.[7] In the isolation procedure, liver cell suspensions were centrifuged twice at 50X g to enrich for hepatocytes. Cell viability was determined by trypan blue exclusion and cells were selected for transplantation which were approximate 90% viable. After isolation, liver cells were kept on ice and transferred within 2 hr. Liver cells (1-2x10^5) were transplanted into recipient mice of around 3 months of age by intrasplenic injection as described[4].

Western blotting for detection of homozygosity of Scid mice: About 200µl blood was collected from ~ 24 gm of adult Fah$^{-/-}$; Scid/Scid double knockout mice by using retro-orbital bleeding method as described earlier[8]. Blood was kept at room temperature for few minutes to hours for clotting and then centrifuged in order to collect sera. These sera were then subjected to western blotting as described earlier[9]. We described the western blotting procedure a little bit details that the sera were separated by electrophoresis on 10% SDS-polyacrylamide gel and transferred to a PVDF membrane (Cat. No. RPN2020F, GE Healthcare, Buckinghamshire, UK). The membranes were then incubated with the HRP-conjugated anti-mouse IgG (Cat. No. P0447, Dako, Glostrup, Denmark) antibodies. Finally, the signals of specific proteins were detected using the chemiluminescent ECL kit (Cat. No. NEL 104, NEL 105, Perkin-Elmer Life Sciences, MA, USA).

Immunohistochemistry: Liver tissues were excised from ~4 month-old of Fah$^{-/-}$; Scid/Scid double knockout mice after exogenous hepatocyte transplantation and frozen in OCT compound immediately (Tissue Tech, Torrance, CA, USA) for liver sectioning. The 4µm thickness of liver sections were prepared. The sections were then rinsed with PBS and counter-stained with DAPI, and observed with a confocal microscope FV-1000 (Olympus).

3. Results

Generation of Fah$^{-/-}$; Scid/Scid double knockout mice: In order to generate the Fah$^{-/-}$; Scid/Scid double knockout mice, we crossed the Fah$^{-/-}$ mice with the mice homozygous for the severe combined immune deficiency spontaneous mutation *Prkdc*^{scid}, commonly referred to as *scid* characterized by an absence of functional T cells and B cells. Finally, we got eight desired mice by crossing with maintaining the following figure-1 and these mice were used for receiving the primary hepatocytes from DsRed mice.

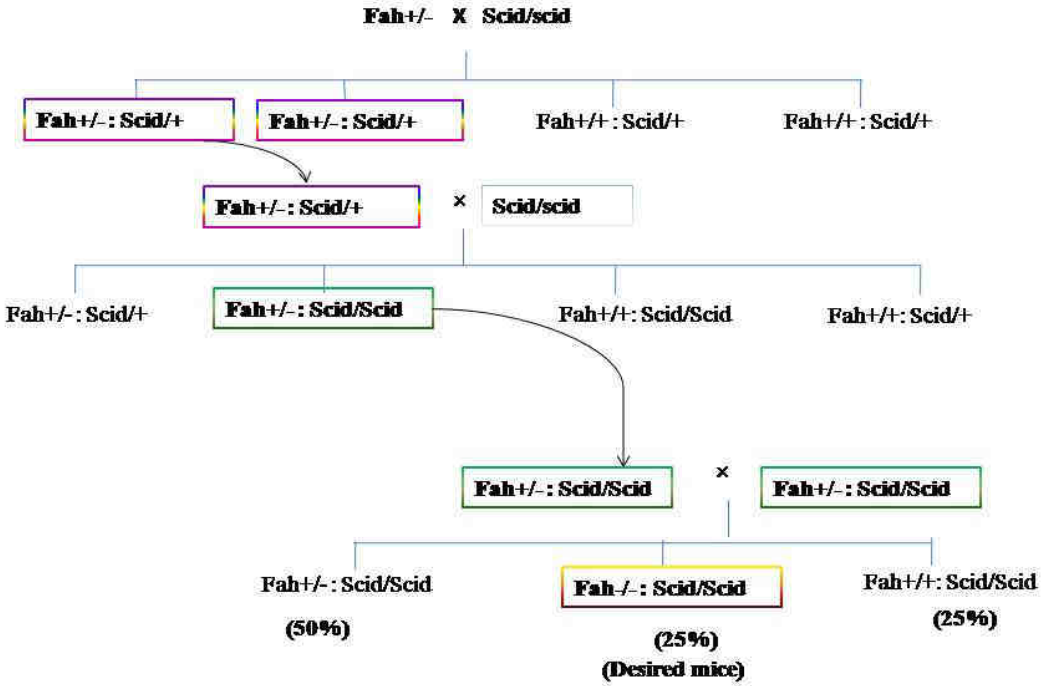

Figure-1. Generation Fah-/-; Scid/Scid mice

Detection of Fah genotype and homozygosity of *scid* in Fah$^{-/-}$;Scid/Scid mice:
Heterozygous and homozygous mice of *fah* genotype were identified by genotyping of tail DNA (Figure 2). Genotyping was done with the same primers and conditions as described elsewhere[6] or according to the protocols provided by vendors.

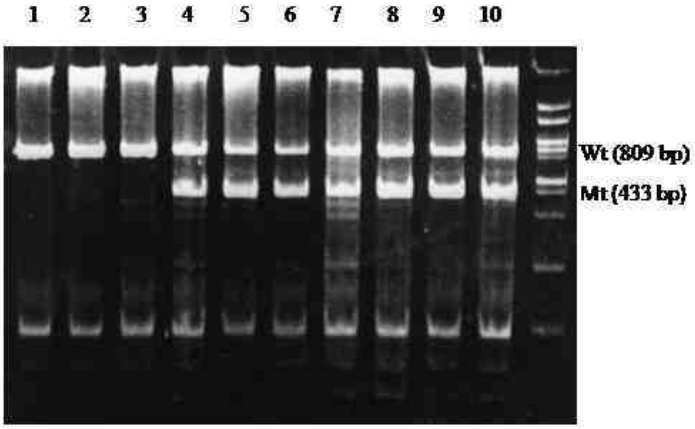

Figure-2. PCR for *fah* genotype with tail DNA isolated fromdifferent mice. Band size 809 bp and band size 433 bp indicated for wild type and *fah* gene mutant mice respectively. Lanes1 through 3 for wild type mice, lanes 4 through 10 for heterozygous mice for *fah* gene; lane11,1-kb Plus DNA ladder (Invitrogen) used as a molecular size marker.

We next determined the homozygosity of Scid (Figure-3) in recipient Fah$^{-/-}$;Scid/Scid mice by Western blotting as described in materials and methods section.

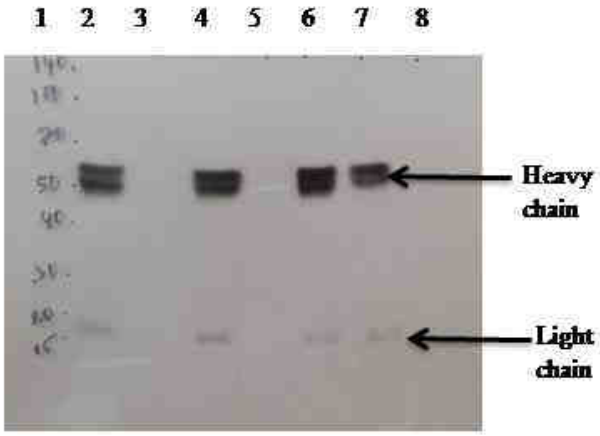

Figure-3. Western blotting for detection of IgG heavy and light chains for Scid homozygosity in recipient Fah$^{-/-}$;Scid/Scid mice. Lanes 3, 5 and 8 indicated the homozygosity of Scid mice; lanes 2, 4, 6 and 7 indicated either wild type and heterozygous mice. Lane 1 indicated the prestained protein ladder (Wako, Japan).

Detection of transplanted DsRed mice hepatocytes into Fah$^{-/-}$;Scid/Scid recipient mice:

For transplantation of DsRed mouse hepatocytes, we first generated a suitable immunodeficient mouse by crossing the Fah$^{-/-}$ mouse with the Scid mouse, which is depleted of mature B and T lymphocytes. These double knock-out mice allow more efficient engraftment of forign cells[10]. Hepatocytes from the DsRed mice were transplanted by intrasplenic injection[11] into Fah$^{-/-}$;Scid/Scid recipient mice. After transplantation, NTBC was withdrawn, and mice were monitored everyday for changes in body weight. A constant or increasing body weight reflects a balanced hydration status and is an indirect indicator of successfull engraftment. Fah$^{-/-}$;Scid/Scid mice that did not receive hepatocyte transplantation and are withdrawn from NTBC progressively lose weight and die within 30-35 days. DsRed positive hepatocytes could be detected from periportal to centrilobular over the whole liver acinus in all liver lobes (Fig. 4A and 4B). DsRed cells were found in small clusters, reflecting the selective expansion after successful engraftment. 1.5 months after transplantation, some areas of the Fah$^{-/-}$;Scid/Scid murine liver had 20% DsRed hepatocytes (Fig. 4A and 4B).

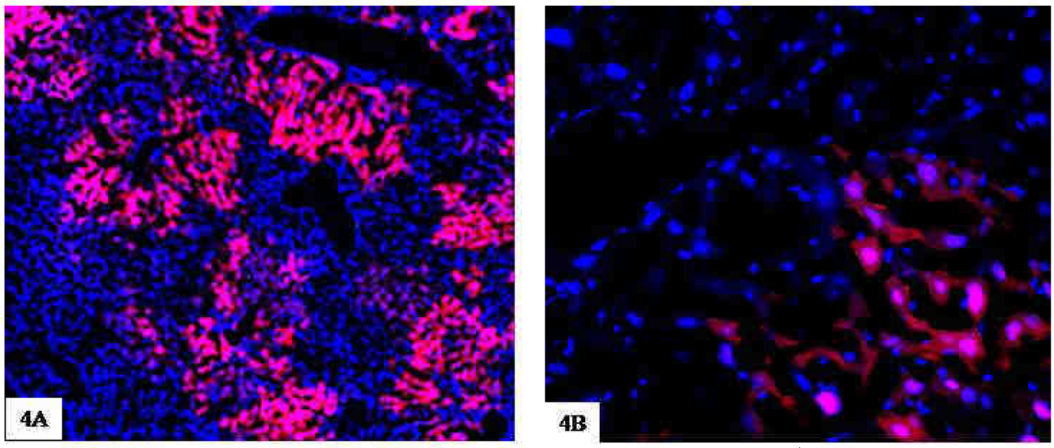

Figure-4. DsRed mouse hepatocyte transplantation into recipient Fah$^{-/-}$;Scid/Scid mice. (A) & (B) DsRed mouse hepatocyte clusters (Red fluorescent) are found over the entire left lower lobe of recipient mice liver. The liver sections were counter-stained with DAPI (blue). Photographs were obtained at 100X and 200X magnifications for 4A and 4B respectively.

Survival rate and weight gain in Fah$^{-/-}$;Scid/Scid recipient mice post exogenous hepatocyte transplantation and withdrawing NTBC water:

In hereditary tyrosinaemia type I (HT1) (McKusick 276700), fatal liver disease results either because of liver failure during infancy or early childhood or because of development of hepatocellular carcinoma during childhood or adolescence. This is caused by toxic metabolites which accumulate because of deficiency of fumarylacetoacetase (Fah), the last enzyme in the tyrosine catabolic pathway. 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) is a potent inhibitor of 4-hydroxyphenylpyruvate dioxygenase and has been shown to efficiently prevent tyrosine degradation, and production of succinylacetone,

in patients with tyrosinaemia[12].The triketones such as NTBC, mesotrione acts as a herbicides which has been proposed for the treatment of Type 1 hereditary tyrosinaemia. Inhibition of p-hypodroxylphenylpyruvate dioxygenase (HPPD) prevents production of homohentistic acid and the resulting accumulation of toxic products in patients with a Fah deficiency. Thus, NTBC has replaced the liver transplantation as the first line treatment for HT1[13].

After transplantation, NTBC was withdrawn, and mice were monitored every second day for changes in body weight. A constant or increasing body weight reflects a balanced hydration status and is an indirect indicator of successful engraftment. Fah$^{-/-}$;Scid/Scid mice thatdid not receive hepatocyte transplantation and are withdrawn from NTBC progressively lose weight and die within 30-35 days. We performed transplantation of DsRed mouse hepatocytes into 15 of Fah$^{-/-}$:Scid/Scid mice. In all cases, mice become very weak gradually after transplantation. Their weight decreased day by day. Ten of them died just after 30-35 days after withdrawing NTBC water. Finally, we observed that five of the transplanted mice were looking healthy and also their body weight was increased and sometimes constant (Table 1). So, we have found that the survival rate is 33.33% after transplantation of DsRed hepatocytes. Even after 45 days, the mice were very good looking without NTBC water. Finally, we isolated the hepatocytes on 45th day after transplantation and performed fluorescence microscopy (Figure A and 4B).

Table 1. Mouse weight observation after exogenouse hepatocyte transplantation

No. of mice	**Sex**	**Age on NTBC withdrawing and hepatocyte transplantation date**	**Properties of mice**	**Mouse weight (gm) on NTBC withdrawing and hepatocyte transplantation date**	**Mouse weight (gm) on the 45th days after hepatocyte transplantation**
05	♂	~ 3 months	Fah$^{-/-}$;Scid/Scid	24.01±0.64	23.8±0.55

4. Discussion:

We report the development of a method for successful transplantation of hepatocytes from DsRed mice into Fah$^{-/-}$:Scid/Scid mice. The recipient mice lacked to produce fumarylacetoacetase enzyme. This is caused by toxic metabolites which accumulate because of deficiency of fumarylacetoacetase (Fah), the last enzyme in the tyrosine catabolic pathway. NTBC is a potent inhibitor of 4-hydroxyphenylpyruvate dioxygenase and has been shown to efficiently prevent tyrosine degradation, and production of succinylacetone, in patients with tyrosinaemia.[12] The recipient mice also lacked to produce B cells, T cells, and any residual NK cell activity. This property enhances the transplantation rate in recipient mice.Our transplantation success rate was 33.33%.

To extend the usefulness of the Fah$^{-/-}$ knockout mouse model for studying liver cell growth, we crossed the Fah$^{-/-}$ mice with the immunodeficient Scid mice to generate Fah$^{-/-}$;Scid/Scid double knockout mice. Transplantation of liver cells from DsRed mice into these mice resulted in 20% reconstitution of mouse liver with DsRed hepatocytes and even these knockout mice can be survived after withdrawing NTBC water. This is a good sign for the treatment of hereditary tyrosinaemia type I. This is also a remarkable demonstration that a functional liver can be formed from transplanted hepatocytes and suggests that Fah$^{-/-}$ knockout mouse livers can be reconstituted with hepatocytes from a range of species.

5. Conclusion

In conclusion, we demonstrate that the Fah-/-; Scid double knockout mouse is a suitable recipient for hepatocyte transplantation. If we are able to transplant human hepatocytes in these knockout mice, then these mice reconstituted with human hepatocytes can offer the opportunity to study drug metabolism and pursue gene therapy strategies.

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6. References

1. Sandgren EP, Palmiter RD, Heckel JL, Daugherty CC, Brinster RL, Degen JL. 1991. Complete hepatic regeneration after somatic deletion of an albumin-plasminogen activator transgene. *Cell* 66, 245–256.
2. Petersen J, Dandri M, Gupta S, Rogler CE. 1998. Metabolic Labeling of Woodchuck Hepatitis B Virus X Protein in Naturally Infected Hepatocytes Reveals a Bimodal Half-Life and Association with the Nuclear Framework. *Proc Natl Acad Sci USA* 95, 310–315.
3. Rhim JA, Sandgren EP, Palmiter RD, Brinster RL. 1995. Complete reconstitution of mouse liver with

xenogeneic hepatocytes. *Proc Natl Acad Sci USA* 92, 4942–4946.
4. Rhim JA, Sandgren EP, Degen JL, Palmiter RD, Brinster RL. 1994. Replacement of diseased mouse liver by hepatic cell transplantation. *Science* 263, 1149– 1152.
5. Karl-Dimiter Bissig, Tam T. Le, Niels-Bjarne Woods, and Inder M. Verma. 2007. Repopulation of adult and neonatal mice with human hepatocytes: A chimeric animal model. *Proc Natl Acad Sci USA104,* 20507–20511.
6. Grompe M, Al-Dhalimy M, Finegold M, Ou CN, Burlingame T, Kennaway NG, Soriano P. 1993. Loss of fumarylacetoacetate hydrolase is responsible for the neonatal hepatic dysfunction phenotype of lethal albino mice. *Genes Dev* 7, 2298–2307.
7. Klaunig, J. E., Goldblatt, P. J., Hinton, D. E., Lipsky, M. M., Chacko, J. & Trump, B. F. 1981. Mouse liver cell culture. I. Hepatocyte isolation. *In Vitro* 17, 913-925.
8. Sharma A, Fish BL, Moulder JE, Medhora M, Baker JE, Mader M, Cohen EP. 2014. Safety and blood sample volume and quality of a refined retro-orbital bleeding technique in rats using a lateral approach. *Lab animal 43*(2): 63.
9. Islam, M. J., Amin, M. B., Uddin, M. K. M., Hikosaka, K., Noritake, H., Wu, Y-X., Aoto, K., Miura, N. 2016. Mouse homologues of hepatitis C virus human entry factors inhibit the entry of HCV pseudo- particles (HCVpp) into human hepatoma cells. *Biores Comm.* 2(1), 128-133.
10. Shultz LD, Ishikawa F, Greiner DL. 2007. Humanized mice in translational biomedical research. *Nature Rev* 7:118–130.
11. PonderKP, GuptaS,Leland F, Darlington G, Finegold M, DeMayo J, Ledley FD, Chowdhury JR, Woo SL, *et al*. 1991. *In vivo* transfer and expression of a human epidermal growth factor gene accelerates wound repair. *Proc Natl Acad Sci USA* 88:1217–1221.
12. Holme E , Lindstedt S. 1998. Tyrosinaemia type I and NTBC (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione). *J Inherit Metab Dis.* 21(5):507-17.
13. Sumaira M, Sinead N, Christina J, Lina E, John W, Sandrine R, John W. Treatment Adherence in Type 1 Hereditary Tyrosinaemia (HT1): A Mixed-Method Investigation into the Beliefs, Attitudes and Behaviour of Adolescent Patients, Their Families and Their Health-Care Team. DOI 10.1007/8904_2014_337. 2014.