Biochemical and Biophysical Research Communications 381 (2009) 276–282

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A novel method of mouse *ex utero* transplantation of hepatic progenitor cells into the fetal liver

Mima Shikanai [a], Kinji Asahina [a,1], Sachiko Iseki [b,*], Kenichi Teramoto [c], Tomohiro Nishida [a], Keiko Shimizu-Saito [a], Masato Ota [b], Kazuhiro Eto [b], Hirobumi Teraoka [a,*]

[a] Department of Pathological Biochemistry, Medical Research Institute, Tokyo Medical and Dental University, 2-3-10 Kandasurugadai, Chiyoda-ku, Tokyo 101-0062, Japan
[b] Department of Molecular Craniofacial Embryology, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan
[c] Department of Hepato-Biliary-Pancreatic Surgery, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan

ARTICLE INFO

Article history:
Received 15 January 2009
Available online 13 February 2009

Keywords:
Ex utero surgery
Cell transplantation
Hepatic progenitor cell
Hepatoblast
Hepatocyte
Fetal liver
Liver development
Biliary epithelial cell
Embryonic stem cell

ABSTRACT

Avoiding the limitations of the adult liver niche, transplantation of hepatic stem/progenitor cells into fetal liver is desirable to analyze immature cells in a hepatic developmental environment. Here, we established a new monitor tool for cell fate of hepatic progenitor cells transplanted into the mouse fetal liver by using *ex utero* surgery. When embryonic day (ED) 14.5 hepatoblasts were injected into the ED14.5 fetal liver, the transplanted cells expressed albumin abundantly or α-fetoprotein weakly, and contained glycogen in the neonatal liver, indicating that transplanted hepatoblasts can proliferate and differentiate in concord with surrounding recipient parenchymal cells. The transplanted cells became mature in the liver of 6-week-old mice. Furthermore, this method was applicable to transplantation of hepatoblast-like cells derived from mouse embryonic stem cells. These data indicate that this unique technique will provide a new *in vivo* experimental system for studying cell fate of hepatic stem/progenitor cells and liver organogenesis.

The developing hepatic parenchyma consists of hepatoblasts, which are defined as a bi-potent progenitor cell differentiating into both hepatocytes and biliary epithelial cells [1]. Hepatoblasts are characterized by expression of E-cadherin, a transmembrane glycoprotein which promotes and maintains cell adhesion, and Delta-like 1/Pref-1 (Dlk), an EGF-like transmembrane protein [2,3]. The isolated Dlk^+ cells are capable of differentiating into both hepatocytes and biliary epithelial cells *in vitro* [3]. It has been reported that 62.7% of E-cadherin$^+$ cells in ED12.5 fetal liver are positive for Dlk by the double-immunostaining [4].

Adult injured liver models have been used to study experimental transplantation of hepatic progenitor cells, including hepatoblasts [3–7], as well as of hepatocytes [8]. Transplantation of these cells into the liver is generally performed through portal vein or via spleen of adult mice under the experimental conditions inhibiting proliferation of recipient parenchymal cells and potentiating proliferation of donor cells [8]. In hepatic injury models, donor hepatoblasts migrate to the injured liver and differentiate into hepatocytes, but not into biliary epithelial cells [3–6]. In contrast, it has been observed that transplanted hepatoblasts differentiate into biliary epithelial cells in the bile duct injury model [4,7]. Since the environment of these adult injured livers is not necessarily suitable for natural differentiation of hepatoblasts, an *in vivo* monitoring assay system using fetal liver would be required to characterize hepatoblasts or hepatic stem/progenitor cells. However, there has long been no available way to analyze those hepatic immature cells along with embryology. Therefore, we tried to establish a method of *ex utero* transplantation of hepatic stem/progenitor cells into mouse embryonic liver.

An *ex utero* surgical technique itself was originally developed on limb development research, including limb amputation and engraftment [9], and it has subsequently been applied to the transplantation of 3T3 cells [10] and AtT-20 cells [11]. Iseki's group also reported implantation of FGF2-soaked beads [12] and injection of DiI [13] into fetal mouse heads by *ex utero* surgery. However, no report has appeared on successful cell transplantation into internal organs such as liver by using *ex utero* surgery. Thus, such a mouse *ex utero* manipulation might provide a unique *in vivo* experimental system for studying liver development if the fetuses could survive

* Corresponding authors. Fax: +81 3 5803 5579 (S. Iseki), +81 3 5280 8075 (H. Teraoka).
E-mail addresses: s.iseki.emb@tmd.ac.jp (S. Iseki), hteraoka.pbc@mri.tmd.ac.jp (H. Teraoka).
[1] Present address: Department of Pathology, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA.

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.02.037

and develop normally after the invasive surgical procedures, including injection of hepatic stem/progenitor cells into the fetal liver.

In this report, we demonstrated a novel *ex utero* transplantation model using the mouse fetal liver in order to monitor the behavior of transplanted hepatic stem/progenitor cells not only in the short-term fetal stage but also during the long-term postnatal period.

Materials and methods

Mice. Mice that were purchased from Sankyo Labo Service Corporation (Tokyo, Japan) and transgenic mice that express green fluorescent protein (GFP) [14] were maintained in the animal house of Tokyo Medical and Dental University. All animal experiments were performed according to the Guide for Care and Use of Laboratory Animals by Tokyo Medical and Dental University. All efforts were made to minimize the number of animals used and their suffering.

Preparation of hepatoblasts from ED14.5 fetal liver. ED14.5 fetal livers from GFP-expressing mice obtained by mating C57BL/6N mice with GFP-expressing mice were digested with 1 U/ml dispase (Gibco, Grand Island, NY, USA). Hepatoblasts were isolated by immuno-magnetic beads-assisted cell sorting (CELLection Biotin Binder Dynabeads; Dynal A. S, Oslo, Norway) as in [2] with slight modification. Briefly, digested liver cells were treated with rat monoclonal antibodies against mouse Dlk (1:100; D187-3, MBL, Nagoya, Japan) at 4 °C for 30 min, and then incubated with goat anti-rat IgG antibody-coated beads at a ratio of at least four beads per cell. The hepatoblasts decorated with the beads were collected with a magnetic particle concentrator, and were liberated with DNase I treatment according to the manufacturer's instruction (Dynal). E-cadherin$^+$ hepatoblasts were prepared with anti-E-cadherin antibodies (1:100; ECCD-1, Takara, Otsu, Japan).

Ex utero transplantation. ED14.5 C57BL/6N fetuses were used as recipients. Under a surgical microscope at low-power magnification, uteri were exposed from anaesthetized mothers as follows: a ventral midline was made through the skin and abdominal wall of mother; the uterus was taken out of the peritoneal cavity with sterile instruments; the uterus was incised along anti-placental side; the yolk sac, amnion, and skin of fetus were opened to enable a glass needle to have a direct access to the injection site (see Fig. 1A and B). ED14.5 hepatoblasts were counted microscopically, and located into pulled glass capillary injection pipettes with an inside diameter of approximately 100 μm. To insert approximately 1 mm length of the pulled pipette tip, the level of 1 mm from the tip was marked. These donor cells (20–30 cells) were directly injected into the exposed fetal liver. Usually, operated 2 or 3 fetuses were maintained in uterus and unoperated fetuses were removed. After manipulation, the operated fetuses in uterus were repositioned in the abdominal cavity, followed by closing the wall and skin of mother with sutures. Since it was impossible to obtain neonates by natural parturition owing to dissection of uterine wall, the neonates (ED19.5) 5 days after the operation were recovered by caesarean section. When the neonates were fostered by appropriate ICR female mice, some of them were able to grow, and 6-week-old mice were obtained.

Immunohistochemical and histological analyses. Neonatal livers obtained from 8 and 10 ED14.5 mice transplanted with Dlk$^+$ and E-cadherin$^+$ hepatoblasts, respectively, were subjected to immunostaining and periodic acid-Schiff (PAS) staining (Supplementary methods). For each neonate, one section every seven serial liver sections was collected, and resulting 12–15 liver sections per neonate were immunohistochemically analyzed. Each lobe of the livers from transplanted 6-week-old mice was also examined with immunostaining and PAS staining.

Differentiation of embryonic stem cells (ESCs). ESCs were established from the GFP-expressing mice (Supplementary methods and Supplementary Fig. 1A). To induce ESC differentiation toward hepatoblasts, ESCs were cultured in a serum-free culture medium composed of DMEM/F12, B27 Supplement (Gibco) [15], ITS-X (Gibco), 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, and 10 mM Hepes in the absence and presence of activin A (2.5 ng/ml); 4 days later, FGF2 (20 ng/ml), HGF (10 ng/ml), and EGF (20 ng/ml) were added without activin A in an additional 4-day culture. Into ED14.5 fetal livers, 200–300 differentiated cells containing approximately 40–60 hepatoblast-like cells were transplanted, the ratio of which was estimated by FACS analysis with antibodies against Dlk and E-cadherin.

Results and discussion

Ex utero surgery and transplantation of hepatoblasts into the fetal liver

A procedure of the *ex utero* surgery is shown in Fig. 1A and B. Under anesthesia, the uterus of a pregnant mouse 14.5 days of gestation was exposed by a ventral midline incision, and the fetuses in the uterus were exposed by an incision of the uterine wall at a position opposite that of the placenta (Fig. 1B). Then, the yolk sac surrounding the embryo and fetal skin were incised at the right side of the embryo, and cells were directly injected into the fetal liver with a glass capillary pipette (Fig. 1A).

For the *ex utero* cell transplantation, hepatoblasts were prepared from ED14.5 fetal liver of the GFP-expressing mouse. The isolated hepatoblasts expressed α-fetoprotein (Afp), albumin (Alb), Dlk, and E-cadherin, whereas cytokeratin19 (CK19), a biliary epithelial marker, was slightly detected, and mature hepatocyte markers including glucose 6-phosphatase (G6Pase), tyrosine aminotransferase (Tat), and tryptophan dioxygenase (To) were not observed in the isolated hepatoblasts (Fig. 1C). Next, we determined the injection volume limited to 1 μl, because the fetal liver was split open owing to a larger amount of injection medium than 1 μl. The isolated hepatoblasts were suspended in the culture medium and were injected into the ED14.5 fetal liver by using the *ex utero* surgery. After transplantation, the yolk sac, the abdominal wall, and skin of the mother were sutured (Fig. 1A), while leaving the uterine wall open. We expected that the manipulated fetuses would survive in the abdominal cavity of the mother until the 19.5 days of gestation, the time of birth (5 days after transplantation), because the umbilical blood vessels and placenta are intact.

Regulation of littermates with and without cell transplantation

C57BL/6N pregnant mice have average 10 fetuses distributed in right and left horns of the uterus. When we transplanted isolated hepatoblasts into 1–3 fetuses and left the same number of unoperated fetuses in a mother, many of operated fetuses died during 5 days after transplantation and only 17–25% of the operated fetuses survived (Fig. 2A, open bars). We assumed that impairment of the survival rate of the fetuses is due to a growth disadvantage of the operated fetuses compared to the unoperated fetuses in a mother. To test our assumption, we transplanted hepatoblasts into 1–3 fetuses by removing the unoperated littermates from the uterus, resulting in survival of almost all operated fetuses after the transplantation (Fig. 2A, closed bars). Leaving only 2 or 3 operated fetuses without unoperated ones in a mother, the average body weight of these pups was comparable to that of control pups (99.3%; manipulated 1181 mg vs. control 1189 mg) (Fig. 2B). When 4–6 operated fetuses were left, the body weight was lower than that of the control fetuses (73.4%) with subsequent death of many