Enhancing Survival of Human Hepatocytes by Neonatal Thymectomy and Partial Hepatectomy in Micro-miniature Pigs

H.C. Hsu[a,b], S. Enosawa[a,b], T. Yamazaki[b,c], S. Tohyama[d], J. Fujita[d], K. Fukuda[d], and E. Kobayashi[a,*]

[a]Department of Organ Fabrication, Keio University School of Medicine, Tokyo, Japan; [b]Division of Advanced Medical Sciences, National Center for Child Health and Development, Tokyo, Japan; [c]Kohno Clinical Medicine Research Institute, Tokyo, Japan; and [d]Department of Cardiology, Keio University School of Medicine, Tokyo, Japan

ABSTRACT

Background. With the goal of in vivo cultivation of human hepatocytes that have not been sufficient in full differentiation in vitro, the advantage of neonatal thymectomy was verified on expansion of xenogeneic human hepatocyte in the micro-miniature pig (MMP).

Methods. The thymus was excised immediately after the birth of the MMPs via cesarean section. Newborns were fed by artificial feeding under specific pathogen-free conditions. The thymectomized and nonthymectomized littermates were transplanted with human hepatocytes via a portal vein with or without partial hepatectomy at the MMP adult stage.

Results. The growth of thymectomized MMPs and the sham operated littermates was not significantly different; the former weighed 1.98 ± 0.30 kg (average ± standard deviation, n = 4) and the latter weighed 2.28 ± 0.39 kg (n = 4) at 1 month of age, and 17.48 ± 1.92 kg and 16.75 ± 2.68 kg at 12 months of age. Blood thymosin α_1 concentrations in the thymectomy group were significantly lower than in the control group (0.22 ± 0.05 ng/mL vs 0.46 ± 0.16 ng/mL; n = 4, 12 months old, $P = .029$). After human hepatocyte transplantation, human albumin levels were detectable on day 28 in the peripheral blood of the thymectomy plus hepatectomy group (14.3 ± 4.9 ng/mL [± range, n = 2]) but were not detectable even on day 21 in the control group.

Conclusions. Neonatal thymectomy was successfully achieved in infantile MMPs born via cesarean section. These pigs were considered to be an ideal in vivo bioreactor for human hepatocytes.

LIVER TRANSPLANTATION IS THE ULTIMATE treatment for the end stage of hepatic failure, although the donor shortage is the critical issue. It is hopeful that hepatocyte transplantation can be a substitution for liver transplantation, and the therapeutic advantages of this procedure have been proved [1,2]. Unlike liver transplantation, isolated hepatocytes can be transplanted to numerous patients multiple times in a less invasive manner. In addition, long-term storage and long-distance transport are available. In recent years, cell therapy and regenerative medicine have been highlighted, which has brought more attention to hepatocyte transplantation [2,3].

The pig has been recognized as a suitable preclinical experimental animal. Because rearing environment, medication amount, and experiment duration depend on body size, small-size experiment-oriented pigs are advantageous [4]. The world's smallest experimental pig has been developed in Japan, labeled the micro-miniature pig (MMP) [5]. MMPs enable us to more easily perform long-term experiments and to reduce the amount of medication needed.

Although it is known that neonatal thymectomy induces immunodeficiency in rodents [6,7], it is still unclear whether the procedure is effective in pigs [8]. In the present study,

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*Address correspondence to Eiji Kobayashi, Department of Organ Fabrication, Keio University School of Medicine, Room 7S4 of Research Park Building, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: organfabri@keio.jp

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we conducted a neonatal thymectomy with infantile MMPs born via cesarean section and estimated the immunosuppressive effect by human hepatocyte transplantation.

MATERIALS AND METHODS

Animals

Pregnant MMPs were purchased from Fuji Micra Inc (Shizuoka, Japan). After cesarean section and thymectomy, neonates were fed with artificial milk until 1 month old; they were then switched to MMP pellets (Fuji Micra Inc) according to a regular protocol under specific pathogen-free (SPF) conditions.

Cesarean Section and Thymectomy

The pregnant MMPs were anesthesized with isoflurane at a maintenance dose of 2.0% to 2.5% just before full term. The 8 piglets obtained were randomized into 2 groups; 4 newborns underwent thymectomy under isoflurane anesthesia as described previously [9]. Briefly, an incision was made along the median line of the ventral cervical regions, and the cervical thymus was excised. The chest was then opened along the median line of the ventral thoracic regions with continuous bagging to retain breath. The thoracic part of the thymus was dissected (Fig 1), and the incision was immediately closed with a 3-0 suture. The other 4 newborns were not operated on and were maintained under SPF conditions. The weight of the piglets was measured monthly, and the blood thymosin α_1 concentration was evaluated at the end of the study (at 12 months old).

Thymosin Determination and Characterization of Peripheral Lymphocytes

Serum thymosin α_1 levels were determined by using an enzyme-linked immunoassay (porcine thymosin α_1 enzyme-linked immunosorbent assay [ELISA] kit; MyBioSource, Inc, San Diego, Calif, United States). Flow cytometry of peripheral blood lymphocytes was performed with FITC mouse anti-pig CD3ε, PE-Cy 7 mouse anti-pig CD4a, and Alexa Fluor 647 mouse anti-pig CD8a monoclonal antibodies (Becton, Dickinson and Company, San Jose, Calif, United States). Mitogen response of the lymphocytes was tested by the incubation of concanavalin A (10 μg/mL) and 50,000 cells in 100 μl of 10% fetal bovine serum containing RPMI1640 using a 96-well plate. After 72-hour culture in a carbon dioxide incubator, 20 μl of Cell Counting Kit-8 (347-07621; Dojindo Molecular Technologies, Inc, Kumamoto, Japan) was added to each well, and the colorimetric change was determined at 450 nm 2 hours later.

Human Hepatocyte Preparation

The human hepatocytes were isolated from an excess liver specimen from a living liver transplantation. The isolation was basically performed according to the method of Seglen [10] with modifications specific for human liver tissue [11]. Isolated hepatocytes were frozen by using a programmable freezer and stored in liquid nitrogen until use. For transplantation, the hepatocytes were thawed with 37°C water bath and washed 3 times using saline, and then re-suspended in 20 mL of saline containing 5 U/mL of heparin.

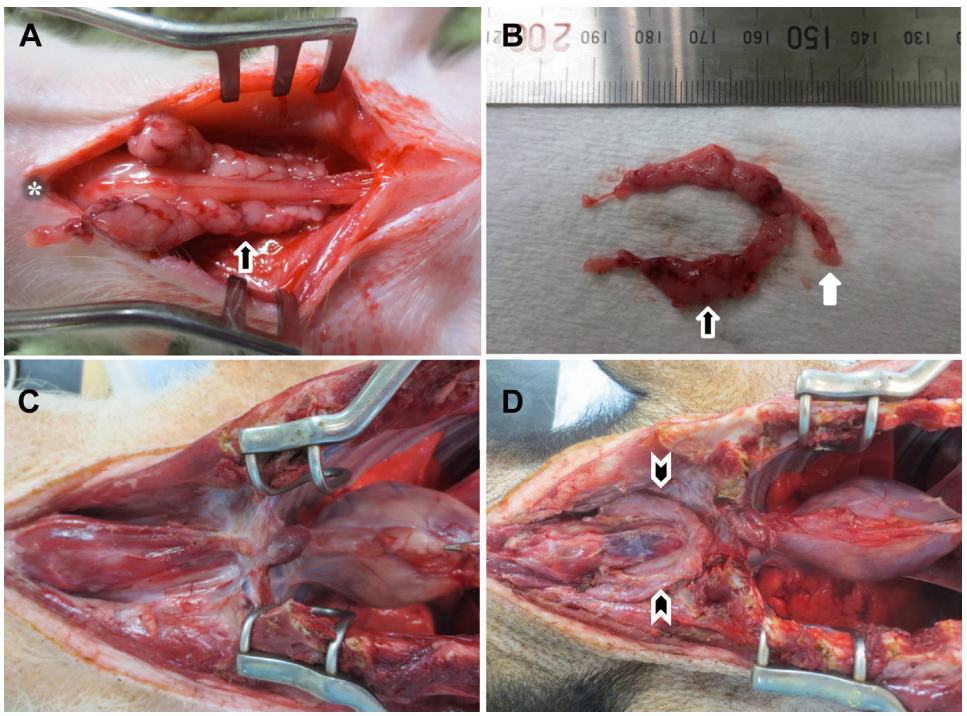

Fig 1. Thymectomy of a micro-miniature pig. (**A**) The midline incision was made on the ventral region of the neck, and the cervical lobe (black arrow) was excised. The asterisk indicates head side. (**B**) Total image of the resected thymus. The black arrow indicates the cervical lobe, and the white arrow indicates the thoracic lobe. (**C**) The image of the ventral cervical region of the neck after thymectomy. (**D**) The image of the ventral cervical region of the neck before thymectomy. The black arrowhead indicates the cervical lobe.

Hepatocyte Transplantation

Under isoflurane anesthesia, the midline incision was made on the abdomen of the MMPs, and the liver and the portal vein were exposed. Before hepatocyte transplantation, the left medial lobe of the liver was excised (Fig 2). Hepatocyte transplantation was performed as described previously [12,13]. Briefly, a 14-G cannula (1714-27-P; Covidien, Dublin, Ireland) was inserted from the portal vein into the right medial lobe portal branch of the liver. To confirm that the apex of the cannula was in the right position, 10 mL of indocyanine green was injected, and the human hepatocytes were slowly transplanted into their liver lobes by injection for 10 minutes (Fig 3).

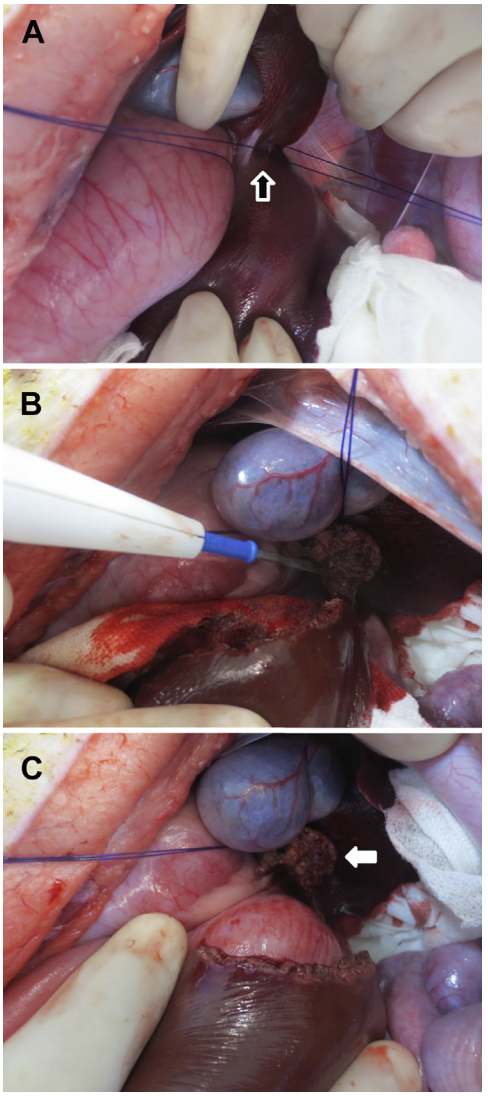

Fig 2. Partial hepatectomy of the lateral left lobe. (**A**) Intrahepatic portal branch flowing in the lateral left lobe was ligated with 2-0 sutures (black arrow). (**B**) The lobe was resected by using an electric scalpel. The lobe was ~15% of the total liver in weight. (**C**) The resection was completed. The white arrow indicates the cut surface.

The MMP blood human albumin level on postoperative day (POD) 7, POD 14, POD 21, and POD 28 were investigated by using an human albumin ELISA quantitation kit (Bethyl Laboratories, Inc, Montgomery, Tex, United States). The blood lymphocyte specimens were analyzed according to fluorescence-activated cell sorting.

Statistical Analysis

Statistical significance was determined by 1-way analysis of variance followed by an unpaired Student t test. P values $<.05$ were considered statistically significant.

Ethical Considerations

All procedures in the animal experiments were approved by the institutional animal ethics committee (reference number 2000-001A). The use of human hepatocytes was approved by the institutional review board (reference numbers 385 and 395).

RESULTS

Effect of Neonatal Thymectomy on Growth and Thymosin Concentrations

The weight of the piglets that underwent thymectomy was slightly less than the sham operated piglets at 1 month of age (average ± standard deviation, 1.98 ± 0.30 kg [n = 4] vs 2.28 ± 0.39 kg [n = 4]). However, their weights at 12 months of age were almost equal (17.48 ± 1.92 vs 16.75 ± 2.68 kg). Blood thymosin α_1 concentrations of the thymectomized pigs were significantly lower than those of the sham operated pigs at 12 months old (0.22 ± 0.05n g/mL vs 0.46 ± 0.16 ng/mL [n = 4]; $P = .029$) (Fig 4).

Profile of Peripheral Leukocytes

The numbers of neutrophils and lymphocytes were 6056 ± 794 cell/mL and 2831 ± 62 cell/mL in the thymectomized group and 4731 ± 222 cell/mL and 1737 ± 160 cell/mL in the sham operated group (average ± range, n = 2), respectively (Fig 5A). The percentages of CD4-positive and CD8-positive lymphocytes were 68.9% and 43.9% in the thymectomized group and 57.0% and 52.9% in the sham operated group (Fig 5B). Mitogen response of the lymphocytes was lower in the thymectomized group than in the sham operated group (Fig 5C).

Human Albumin Concentration After Hepatocyte Transplantation

In terms of the effectiveness of the partial hepatectomy on the survival of transplanted hepatocytes, the human albumin concentrations 7 days after hepatocyte transplantation were 59.8 ± 0.57 ng/mL in the nonhepatectomized pigs (average ± range, n = 2) and 120.4 ± 120.4 ng/mL in the partially hepatectomized pigs (average ± standard deviation, n = 6) (Fig 6A). We then performed a partial hepatectomy. Time course changes in the concentrations are shown in Fig 6B. The human albumin concentration at 7 days after hepatocyte transplantation was almost equal in the thymectomized and sham operated groups (122.3 ± 99.5 ng/mL [n = 3] and 146.1 ± 129.7 ng/mL [n = 3]). However, the concentration

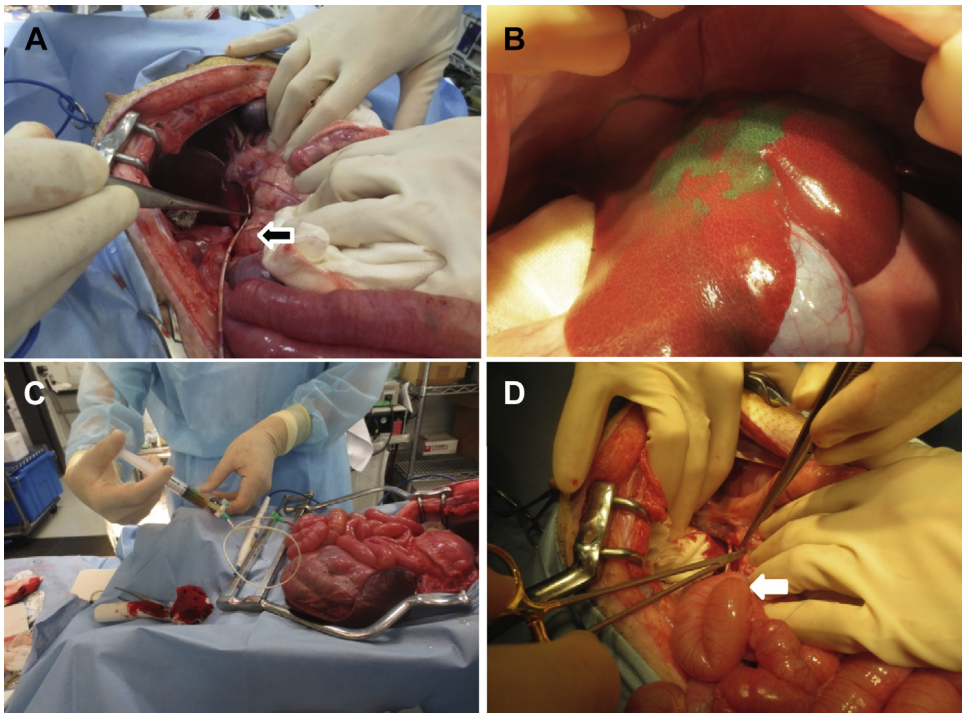

Fig 3. Hepatocyte transplantation in a micro-miniature pig. (**A**) A 14-G cannula was inserted into the portal vein (black arrow), and the apex was located at the portal branch in the left medial liver lobe. (**B**) Indocyanine green was infused into the cannula to confirm the apex was at the right position. (**C**) Thawed human hepatocytes were slowly infused through the cannula for 10 minutes. (**D**) After the transplantation, the needle puncture was closed with 5-0 (white arrow).

of the thymectomized group was 1.5-fold higher than that of the control group at 14 days after hepatocyte transplantation (56.7 ± 35.7 ng/mL [n = 3] and 38.2 ± 16.1 ng/mL [n = 3]). Noticeably, human albumin levels remained at detectable levels at days 21 and 28 in the thymectomized group but not in the sham operated group.

Discussion

We showed that human hepatocytes were successfully engrafted and functionally recovered in partial liver–resected MMPs, from which the thymus gland was totally resected.

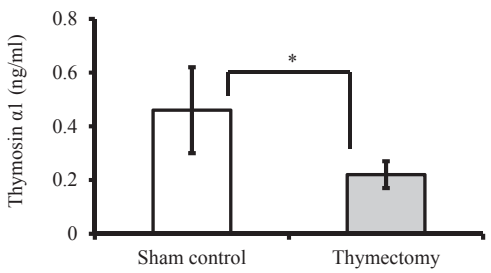

Fig 4. Blood thymosin α_1 concentrations at 12 months old. The white and gray columns indicate the levels of sham operated and thymectomized pigs, respectively. The bars indicate standard deviations, and the asterisk indicates statistical significance ($P = .029$).

MMP is the smallest experimental pig available; it weighs ~20 kg as an adult. Therefore, it is not only easy to handle for surgical procedures but also suitable for translational research [5]. Compared with farmer pigs and other miniature pigs, MMPs have enabled us to research disease models in adult stages and provide long-term observations. For clinical hepatocyte transplantation, a large amount of cells is required. It is hard to prepare a large amount of cells for preclinical studies in farmer pigs. MMPs have smaller livers, and thus a relatively small amount of hepatocyte is sufficient for experiments. For example, it will be very helpful to research stem cell–derived hepatocyte transplantation because it is difficult to acquire a large amount of hepatocytes in vitro.

Researches with immunodeficient mice boosted the progress cell transplantation research, although the lack of an appropriate large animal model hampered the preclinical studies of cell transplantation. A study of severe combined immunodeficiency pigs has been reported [14]. However, 45% of the fetuses were stillborn, and most of the survivors died before postnatal day 70; only 13% of the fetuses survived for >1 year. Farmer pigs grow to >300 kg, and it is therefore difficult to build isolated cages for them. Moreover, propagation of severe combined immunodeficiency pigs requires huge cost and time. To overcome these

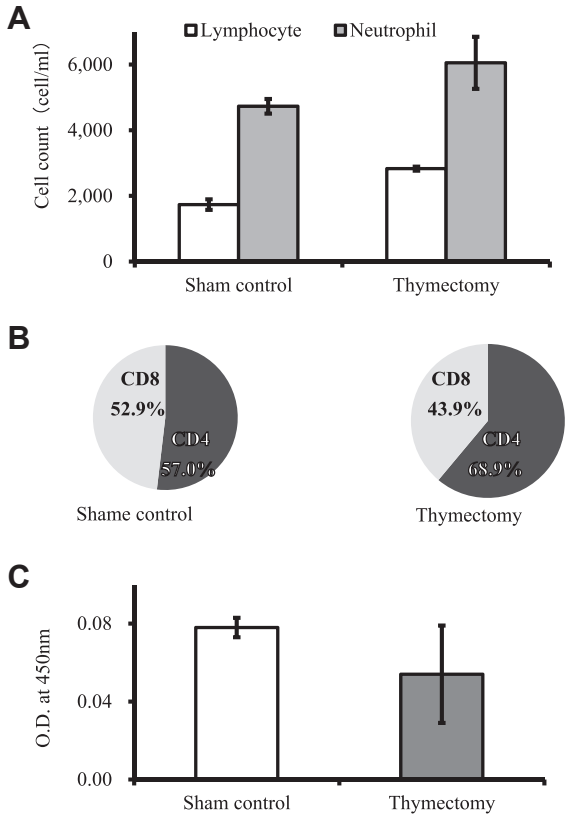

Fig 5. Characterization of peripheral leukocytes of the thymectomized and sham operated pigs. (**A**) Lymphocyte and neutrophil count of the micro-miniature pigs. The open column represents lymphocytes, and the gray column represents neutrophils. The bars indicate range (n = 2). (**B**) Percentage of CD4-positive (dark gray) and CD8-positive (light gray) lymphocytes. All lymphocytes were first gated by using anti-CD3 antibodies. (**C**) Relative mitogen response of peripheral blood lymphocytes of sham operated (open column, n = 2) and thymectomized (gray column, n = 2) groups are shown. The bars indicate range. Experimental details are described in the text.

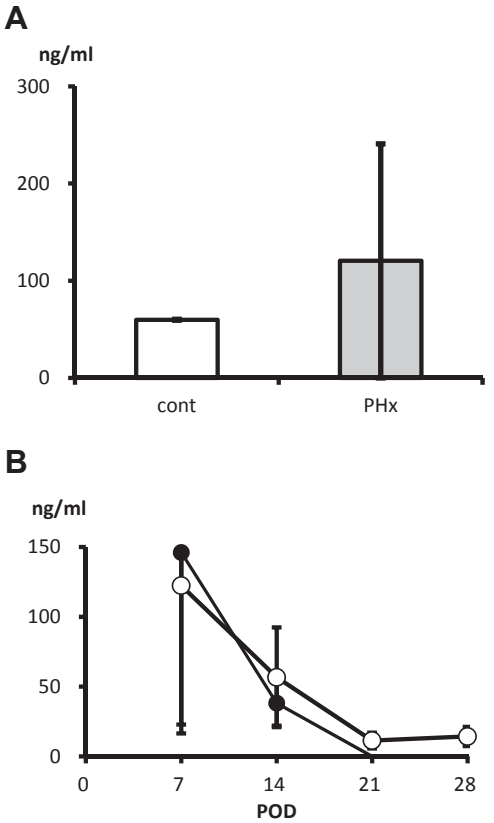

Fig 6. Human albumin concentrations after hepatocyte transplantation. (**A**) The open and gray columns represent the level of sham operated (n = 2) and partially hepatectomized (n = 6) pigs, respectively. (**B**) Time course changes of albumin levels in the sham-operated (closed circle) and thymectomized (open circle) pigs. Abbreviation: cont, sham control; PHx, partial hepatectomy; POD, postoperative day.

limitations, another type of immunodeficiency pig was needed. It is preferable to have pigs of smaller body size, especially to keep them healthy in the adult stage.

We conducted a total thymectomy in newborn piglets at cesarean section because the immunosuppression effect was insufficient with the young piglet [15]. We hypothesized that the immunity of the piglet would be well developed in a few weeks before birth. After the operations, newborn MMPs were raised in an SPF environment to avoid complications. All thymectomized MMPs were raised to the adult stage with no growth inhibition.

Because a partial hepatectomy performed in experimental animals showed cell population proliferation in the liver, we performed a partial hepatectomy, followed by hepatocyte transplantation, in the MMPs. Judging by the blood human albumin concentration on POD 28, the engraftment of the hepatocyte exhibited a longer retention tendency in the thymectomized MMP. To the best of our knowledge, this study was the first premature birth pig model in hepatocyte transplantation.

CONCLUSIONS

The present study findings support our hypothesis and prove that neonatal thymectomy extended the lifespan of the transplanted human hepatocytes. Neonatally thymectomized MMPs pretreated with partial hepatectomy are an ideal in vivo bioreactor for human hepatocyte expansion.

REFERENCES

[1] Enosawa S, Horikawa R, Yamamoto A, Sakamoto S, Shigeta T, Nosaka S, et al. Hepatocyte transplantation using a living donor reduced graft in a baby with ornithine transcarbamylase deficiency: a novel source of hepatocytes. Liver Transpl 2014;20:391–3.

[2] Forbes SJ, Gupta S, Dhawan A. Cell therapy for liver disease: from liver transplantation to cell factory. J Hepatol 2015;62:S157–69.

[3] Fox IJ, Chowdhury JR. Hepatocyte transplantation. Am J Transplant 2004;4:7–13.

[4] Cooper DK, Satyananda V, Ekser B, van der Windt DJ, Hara H, Ezzelarab MB, et al. Progress in pig-to-non-human primate transplantation models (1998-2013): a comprehensive review of the literature. Xenotransplantation 2014;21:397–419.

[5] Kobayashi E, Hishikawa S, Teratani T, Lefor AT. The pig as a model for translational research: overview of porcine animal models at Jichi Medical University. Transplant Res 2012;1:8.

[6] Resnitzky P, Zipori D, Trainin N. Effect of neonatal thymectomy on hemopoietic tissue in mice. Blood 1971;37:634–46.

[7] Hard GC. Thymectomy in the neonatal rat. Lab Anim 1975;9:105–10.

[8] Binns RM, McFarlin DE, Sugar JR. Lymphoid depletion and immunosuppression after thymectomy in the young pig. Nat New Biol 1972;238:181–3.

[9] Kobayashi E, Kamada N, Enosawa S, Toyama N, Delriviere L, Goto S, et al. A technique for complete thymectomy in adult rats. J Immunol Methods 1994;171:33–6.

[10] Seglen PO. Preparation of isolated rat liver cells. Methods Cell Biol 1976;13:29–83.

[11] Enosawa S. Isolation of GMP grade human hepatocytes from remnant liver tissue of living donor liver transplantation. Methods Mol Biol 2017;1506:231–45.

[12] Enosawa S, Yuan W, Douzen M, Nakazawa A, Omasa T, Fukuda A, et al. Consideration of a safe protocol for hepatocyte transplantation using infantile pigs. Cell Med 2012;3:13–8.

[13] Kobayashi E, Enosawa S, Nagashima H. In: Stock P, Christ B, editors. Hepatocyte Transplantation. Methods Mol Biol 2017;1506:149-60.

[14] Suzuki S, Iwamoto M, Saito Y, Fuchimoto D, Sembon S, Suzuki M, et al. Il2rg gene-targeted severe combined immunodeficiency pigs. Cell Stem Cell 2012;10:753–8.

[15] Sahara H, Weiss MJ, Ng CY, Houser SL, Pujara AC, Sayre JK, et al. Thymectomy does not abrogate long-term acceptance of MHC class I-disparate lung allografts in miniature Swine. Transplant Proc 2006;38:3253–5.