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肝臓の再生・線維化・発癌に関与する自己骨髄由来細胞についての基礎的研究

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はしがき

骨髄由来幹細胞 (haematopoietic stem cells)から hepatocyte-like cells への分化・誘導が報告され、肝障害・再生時の肝細胞供給源として自己の骨髄由来幹細胞が注目されている。これらの報告は、各種トランスジェニックマウス・ラットを用いた実験系が多く、導入したマーカー遺伝子発現が安定していない場合があり他の移植モデルでの検討が必要であるとされる。我々は、先天的にアルブミン合成能を欠く無アルブミンラット(F344-alb)を用いて同系アルブミン産生ラット(F344)分離肝細胞を F344-alb の脾臓内や肝臓内に移植し、これらの臓器内で移植肝細胞が生着し肝固有機能を長期間維持することを報告してきた (K. Ogawa, M. Inagaki et al. Transplantation 1993, H. Ikebukuro, M. Inagaki, et al. Eur Surg Res 1999)。分離肝細胞移植は各種の先天性肝代謝異常疾患モデル動物実験において優れた効果を認め、すでに遺伝子・細胞治療法の１つとして米国において臨床応用が試みられているが、1)移植 Donar 不足、2) 移植肝細胞増殖には、時間がかかる、3)外来遺伝子の導入効率が低く、長期間の安定した発現が困難などさまざまな問題が指摘されている。従って、より分離・回収しやすい細胞供給源として自己骨髄細胞の利用が試みられている。

我々は、正常ラット(F344) 骨髄由来細胞を無アルブミンラット(F344-alb)肝臓内に移植する実験動物モデルを確立し報告てきた。(J. Arikura, M. Inagaki, et al. J Hepatol 2004;41;215-221, X. Huiling, M. Inagaki, et al. J Surg Res 2004;122,75-82) これらの実験結果から、骨髄由来細胞には hepatocyte-like cells に分化可能な幹細胞が存在することを確認したが、その数はきわめて少ないことが判明した。今後、自己骨髄細胞群から肝細胞へ分化可能な幹細胞を効率よく分離・回収し、さらに in vitro において細胞培養することにより多くの幹細胞を得ることが可能となれば細胞供給源としての価値が高まる。そのために、無アルブミンラットを用いて１）自己骨髄細胞の凍結保存法の開発、２）新たな自己細胞供給源の探索、３）ラット疾患動物モデルを用いた骨髄細胞移植の効果判定を計画した。これらの結果より、肝臓の再生・線維化・発癌に関与する自己骨髄由来細胞についての基礎的知見を得る事を目的とした。

研究組織

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Albumin-Producing Hepatocytes Derived from Cryopreserved F344 Rat Bone Marrow Cells Transplanted in the Livers of Congenic Nagase's Analbuminemic Rats

Many studies have shown that bone marrow cells (BMCs) have the potential to differentiate into various kinds of somatic cells, including hepatocytes. Furthermore, livers with severe hepatic damage have been shown to contain hepatocytes derived from BMCs. It is speculated that these hepatocytes are formed either by the fusion of pre-exiting differentiated hepatocytes with BMCs, or by the trans-differentiation of BMCs into hepatocytes without cell fusion. Thus, BMCs may be a facilitating source for cell transplantation in hepatic disease. If cryopreserved BMCs from the patient's own bone marrow could be used as a source for cell transplantation, it would be a major breakthrough in transplantation therapy for severe hepatic failure. Although previous studies have described optimizing conditions for cryopreservation of hematopoietic stem cells (SCs) and cord blood SCs, neither, the capacity of transdifferentiation of cryopreserved BMCs into hepatocytes, nor the

protocols for this procedure, have been reported.

Nagase' s analbuminemic rats (NAR) lack the ability to produce albumin because of intronic 7 bp deletion near the splicing donor site in the albumin exon H, which leads to the skipping of exon H, exon G+H, and exon H+I during mRNA processing. The transplantation model in which normal F344 hepatocytes are transplanted into the livers of F344alb, being congenic F344 rats (F344) with the NAR genetic defect, has proved useful for investigating the fate and function of transplanted cells without immunosuppressants. This is because the action of transplanted cells is demonstrated by the albumin production and the presence of the normal albumin gene. We previously reported that transplanted BMCs from F344 can raise clusters of albumin-producing hepatocytes in the livers of F344alb.

We conducted this study to investigate whether cryopreserved BMCs from F344 rats can induce the growth of albumin-producing hepatocytes in the livers of F344alb. Because University of Wisconsin (UW) solution is now currently used for the cryopreservation of various tissues and cells, F344 BMCs were cryopreserved in UW solution containing 10% fetal bovine serum (FBS) and 12% dimethylsulfoxide (DMSO). Thawed cells were infused into

the portal vein of F344alb immediately after 70% hepatectomy (PH), so we could evaluate whether they increased the numbers of clusters of albumin positive (alb+) hepatocytes within the recipient livers.

Materials and Methods

Animals

Male F344 rats (Charles River Japan, Tokyo, Japan) and male F344alb (bred in the Asahikawa Medical College animal laboratory) were used in this study. All care and procedures were approved by the institutional animal care and use committee. We divided 6-week-old F344alb into four groups: Group 1 were untreated; Group 2 were subjectedb to PH alone; Group 3 were transplanted with freshly-isolated BMCs immediately after PH; and Group 4 were transplanted with cryopreserved BMCs immediately after PH. All the rats were killed humanely 4 weeks after the treatment.

Cryopreservation of BMCs and Cell Transplantation

BMCs were isolated from the femurs of 6-week-old male F344 and suspended in Dulbbeccco' s modified Eagle medium (DMEM) (Gibco BRL, Grand-Island, NY). The cells were mixed with Histopaque-1077 (Sigma, St. Louis, MO), centrifuged at 1,800 rpm for 30 min and suspended in the UW solution

containing 10% FBS with 12% DMSO. Each tube containing twenty million cells was cooled on ice and then stored at - 80℃ for 7 days. After thawing the cells at 37℃ in a water bath, BMCs were suspended in DMEM. Cell viability was determined by the trypan blue exclusion test using a hemocytemeter under a phase-contrast microscope. We injected twenty million cryopreserved or freshly-isolated BMCs into the portal vein of each recipient immediately after PH.

Immunostaining

The livers were perfusion-fixed with periodate-lysine-paraformaldehyde (PLP) solution via the portal vein, then cut into slices, further fixed in the PLP solution overnight at 4℃, embedded in paraffin, and cut into 3・m-thick sections. After deparaffinization, the slides were reacted with 1:500 diluted rabbit anti-rat albumin antibody (Ig Fab fraction, Cappel, Malver, PA), followed by incubation with biotin-conjugated goat anti-rabbit IgG (Dako, Carpinteria, CA), and then with the diaminobenzidine/H_2O_2 solution. Single and double alb+ hepatocytes and clusters consisting of more than 3 alb+ cells were counted microscopically, and their numbers/cm^2 liver sections were determined for each animal.

RT-PCR and Southern Blot Analysis

Total RNA was isolated from the livers of F344alb in each group at 4 weeks after the treatment, and reverse transcriptase PCR (RT-PCR) was done to amplify albumin mRNA. The primer sequences for rat albumin cDNA were 5' -TTGCCAAGTACATGTGTGAG-3' (exon G, forward) and 5' -GGTTCTTCTACAAGAGGCTG-3' (exon I, reverse), respectively, which were designed to amplify the mRNA containing exon H in F344, and the mRNA lacking it in F344alb. PCR was done using the RNA with 35 cycles at 94℃ for 30 sec, at 58℃ for 30 sec, and at 72℃ for 1 min. We electrophoresed 10 ・l of the aliquots on 1% agarose gels and stained then with ethidium bromide. The PCR products were then electrophoresed on 1% agarose gels and transferred to nylon membranes. After prehybridization in the hybridization buffer for 1 h at 60℃, the membranes were hybridized with the oligonucleotide probe (5' -ACACTGGAGAAGTGCTGTGCTGAAGGCGAT-3'), including part of the albumin exon H. After washing the membranes with 5x SSC/0. 1% sodium dodecyl sulfate, the hybridization signals were detected by using a CDP star kit (Amersham, Buckinghamshire, UK).

Western Blot Analysis

Blood samples were collected from the animals before they were killed, and the serum was isolated and stored at -80℃ until assay. Aliquots of the serum were electrophoresed on 13% polyacrylamide gels, and then transferred to a nitrocellulose membrane, followed by incubation with 1:8000 diluted peroxidase-conjugated rabbit anti-rat albumin antibody. The hybridization signals were then detected by using ECL Western blotting detection reagents (Amersham).

Statistical Analysis

Statistical differences in the numbers of single and double alb+ hepatocytes and alb+ clusters between the groups were analyzed by one-way ANOVA and the Bonferroni test. P values < 0.05 were considered significant.

Results

Emergence of Alb+ Hepatocyte Clusters after Transplantation with the Cryopreserved BMCs

After quick thawing of the cryopreserved BMCs, cell viability was 76% to 80%. The viability did not decrease for at least 4 weeks with this protocol. The F344alb livers contained a few alb+ hepatocytes, as previously

described, but these always existed as single or double cells and never formed clusters of more than three cells. By 4 weeks after PH alone (Group 2), the number of alb+ cells was unchanged from that in Group 1, remaining as single or double cells. In the F344alb that received transplantation with freshly-isolated BMCs immediately after PH (Group 3), the size and numbers of alb+ hepatocyte clusters were increased, with large clusters of more than 60 cells. In the F344alb that received transplantation of cryopreserved BMCs immediately after PH (Group 4), the size and number of alb+ hepatocyte clusters were also increased. When the clusters were divided into four size classes (single, double, 3-10 and >11 cells), although the numbers of single and double cells were almost the same in all the groups, clusters of more than three cells were detected only in Groups 3 and 4.

Donor Origin of Alb+ Hepatocyte Clusters

RT-PCR using total RNA could amplify a 373 bp fragment including albumin exon H from F344, and a 240 bp fragment lacking exon H from F344alb. When the membranes blotted with the PCR products were hybridized with the exon H probe, a very weak 373 bp band was detected for F344alb after PH,

consistent with previous reports that the analbuminemic hepatocytes could produce a very small amount of normal albumin mRNA. In the F344alb that received transplantation with the freshly-isolated (Group 3) or cryopreserved BMCs transplantation after PH (Group 4), the 373 bp bands were much more intense than those in Groups 1 and 2. On the other hand, no albumin mRNA was detected in the BMCs of F344, indicating that donor-derived cells can produce albumin only after differentiating into hepatocytes.

By using Western blot analysis, we detected a very small amount of albumin in the serum of untreated F344alb (Group 1) and those subjected to PH alone (Group 2). The serum albumin levels increased remarkably in the F344alb transplanted with freshly-isolated BMCs (Group 3) or cryopreserved BMCs after PH (Group 4), and their livers contained clusters of alb+ hepatocytes.

Discussion

Cryopreservation of the patient' s own BMCs is essential in an autologous transplantation; however, the capacity of cryopreserved BMCs to differentiate into hepatocytes and the optimal protocols have never been

reported. We previously reported that when F344 BMCs were infused into the portal vein of F344alb immediately after PH or when the bone marrow of F344alb was substituted with F344 BMCs, the number of alb+ hepatocyte clusters in the livers of recipient F344alb increased. The donor origin of these alb+ hepatocytes was demonstrated by the increase in normal albumin mRNA in the recipient livers, detection of the normal albumin gene sequence in the alb+ hepatocyte clusters, the presence of the Y-chromosome marker in alb+ hepatocytes when male donor and female recipients were used, and the increase in serum albumin levels in the recipient F344alb.

In the present study, when cryopreserved F344 BMCs were transplanted into the portal vein of F344alb after PH, clusters of alb+ hepatocytes emerged, and the donor origin of these cells was demonstrated by the presence of normal albumin mRNA in the recipient livers and increased serum albumin levels. Therefore, cryopreserved BMCs also have the capacity to form albumin-positive clusters, and they might be useful for cell transplantation therapy in liver diseases.

In our previous study, when freshly-isolated BMCs of F344 were transplanted into the portal vein without prior PH, there were fewer alb+

hepatocyte clusters. This is consistent with many reports stating that trans-differentiation from BMCs to hepatocytes was evident under regenerating conditions. Therefore, the proliferating tissue environment created by PH may be important for transplanted F344 BMCs to colonize within the liver. Considering that the smallest clusters consisted of only three cells, whereas the large ones consisted of more than 60 cells on cross-section, we think that the cell number of clusters may be made of about 10-500 cell masses. Assuming that each cluster was derived from a single cell, the most engrafted BMCs might have a much greater proliferating capacity than the recipient hepatocytes and divide three to nine times to form the colonies. This indicates that transplanted F344 BMCs may have greater proliferating capacity than recipient hepatocytes because the latter divide only once or twice times during liver regeneration after PH.

The UW solution was originally developed for hypothermic preservation of organs and is now widely used in cardiac, hepatic, and renal transplantation. In a previous study, hepatocytes cryopreserved with UW solution were significantly functional in terms of plating efficiency, ammonia metabolism, hepatic enzyme leakage, and *in vivo* transplantation. The

present study also demonstrated that the UW solution is also useful for cryopreservation of bone marrow cells.

In conclusion, cryopreserved BMCs isolated from normal rats can induce growth of albumin-producing hepatocytes in the analbuminemic rat liver. Although the frequency of these hepatocytes is relatively low, they might maintain a high capacity to form colonies, as they do after the transplantation of freshly-isolated BMCs.

Albumin (+) Hepatocytes Derived from the Bone Marrow Reconstituted by the F344 Rat Liver Nonparenchymal Cells in the Livers of Analbuminemic Rats

It was demonstrated that there exist hematopoietic stem cells in the liver, and after liver transplantation, those hematopoietic stem cells migrate to the recipient's bone marrow, proliferate and maintain a hematologically chimeric state. The chimerism generated after liver transplantation is thought to be responsible for donor-specific tolerance not only for the liver graft but also for grafts of other organ such as heart, kidney, skin and small intestine. Transplantation of liver nonparenchymal cells (LNPCs) into the lethally-irradiated rats and mice was shown to reconstitute the bone marrow of the recipients, generating all lineages of blood cells including T and B lymphocytes, granulocytes, macrophages, megakaryocytes and erythrocytes.[6] Taniguchi et al. demonstrated that dendritic cells expressing c-kit (stem cell factor receptor tyrosine kinase), sca-1 antigen (a marker of primitive hematopoietic cells) and $Lin^{lo/-}$ (lack of bone marrow cell lineage markers) in the normal mouse liver are capable to reconstitute the bone marrow of the lethally-irradiated mice. Recently, Kotton et al. further showed that CD45+ cells in the adult mouse liver had potent hematopoietic reconstitution activity.

Numbers of recent studies demonstrated that transplantation of bone marrow cells (BMCs) or hematopoietic stem cells from the peripheral blood raise hepatocytes in the host livers in man and rodents. Some studies reported that the mechanism of generation of such donor-derived hepatocytes was

fusion of BMCs with the host hepatocytes, while other studies suggested that BMCs are capable to trans-differentiate into hepatocytes without fusion.

Fischer 344 congenic rats (F344alb) carry the genetic defect of Nagase' s analbuminemic rats with the genetic background of Fischer 344 rats (F344). F344alb are otherwise normal except for a genetic 7 base pair deletion downstream of the exon H splice site within the 9th intron of albumin gene, which results in an inability of hepatocytes to produce albumin. When hepatocytes of F344 were transplanted into the livers of F344alb, they resided within the host liver. In this model, the transplanted F344 hepatocytes could be demonstrated by immunohistochemistry for albumin, RT-PCR for albumin mRNA and PCR for the normal albumin gene sequences. This model also has an advantage that there is no need to use immunosuppressants. We previously showed that, when F344 BMCs were transplanted into the portal vein of F344alb immediately after 70% hepatectomy or into the penile vein after whole body irradiation, colonies of albumin positive (alb+) hepatocytes of the F344 origin were formed within the liver of F344alb.

In the present study, we investigated whether the BMCs derived from the F344 LNPCs can raise alb+ normal hepatocytes within the liver of F344alb in which the bone marrow was reconstituted by the transplantation with F344 LNPCs after whole body irradiation.

Materials and Methods

Treatment of Animals

Male F344 were purchased from Charles River Japan (Yokohama, Japan), and

F344alb were bred in the Asahikawa Medical College animal laboratory. Male F344 and F344alb, 6 to 7 weeks old, were used as the donors and recipients, respectively. F344alb were divided into 5 groups: Group I (n=5), untreated; Group II (n=8), whole body irradiation; Group III (n=5), LNPC transplantation without irradiation; Group IV (n=15), LNPC transplantation immediately after irradiation; Group V (n=5), BMC transplantation immediately after irradiation. All the animals were sacrificed 8 weeks after the treatment. All procedures performed on the animals were approved by the institutional committee according to the guidelines for humane care of laboratory animals.

Preparation and Transplantation of LNPCs and BMCs

LNPCs which mainly consist of endothelial cells together with other nonparechymal cells such as fibroblasts and Kupffer, hepatic stellate and hematopoietic cells were isolated from F344 livers according to the method of Shimaoka et al.[26] Briefly, the liver was flowed through via the portal vein with calcium-free Hanks' solution at the rate of 10 ml/min for 5 min at 39 °C, followed by perfusion with a 0.05% collagenase (Sigma, type V) solution at the rate of 10 ml/min for 10 min at 39 °C. The liver was transferred to a Petri dish, gently brushed and minced in Hanks' solution at 4 °C. The isolated cells were suspended in the Hanks' solution, filtered through a steel mesh with 70-120 mm pore size and centrifugated at 50 g for 1 min three times to precipitate hepatocytes. LNPCs were collected from the supernatants by centrifugation at 400 g for 5 min and suspended in the Hanks' solution. BMCs were prepared as described elsewhere.[25] The

LNPCs or BMCs (10^7 cells/animal, viability of >85) were infused into the penile vein of the recipients immediately after whole body irradiation (7.5 Gy/rat).

Albumin Immunoassaying

The livers were perfusion-fixed with periodate-lysine-paraformaldehyde (PLP) solution via the portal vein. Four slices were cut out from each hepatic lobe, further fixed in the PLP solution at 4℃ overnight, dehydrated through gradient series of ethanol and embedded in paraffin. The tissues were cut into 3 mm-thick sections. After deparaffinization and immersion with 3% H_2O_2 for 5 min, the slides were reacted with 1:500 diluted rabbit anti-rat albumin antibody (Ig Fab fraction, Cappel, Malver, PA). Then the slides were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG polymer (Dako, Carpinteria, CA). After washing in phosphate-buffered saline (PBS), the antibody binding was visualized by the diaminobenzidine substrate-chromogen system (DAKO), followed by counter-staining with hematoxylin. Single and double alb+ hepatocytes and clusters consisting of more than 3 alb+ hepatocytes were counted microscopically, and their numbers/cm^2 sections were determined for each animal by the Scion Image software.

Microdissection

Alb+ hepatocytes were isolated from the albumin-immunostained sections by the laser capture microdissection system (Olympus, Tokyo, Japan).

PCR Detection of the Albumin Gene Sequences

The BMCs of the F344alb treated as above and the microdissected tissues

were incubated in a proteinase K solution for 16 hr at 56 °C, and DNA was purified by QIAamp DNA Micro Kit (50) (QIAGEN GmbH, Hilden, Germany). The primer pairs, 5' -GAAGGCGATCCTCCTGCCTGC-3' (forward) and 5' -CCACCGTGAGTG TTCCTAGC-3' (reverse), were designed to amplify a region including the intronic 7 base pairs lacking in the F344alb albumin gene.[21] PCR was performed using the aliquots of DNA solutions with 38 cycles of 94 °C for 30 sec, 58 °C for 30 sec and 72 °C for 1 min. The PCR products were electrophorased on 10% polyacrylamide gels, stained with ethidium bromide and visualized under UV illumination.

Statistical Analysis

The values were analyzed by one-way ANOVA analysis of variance or the Bonferroni test. *P* values less than 0.05 were considered significant.

Results

Prolonged Survival by LNPC Transplantation after Irradiation

Although only 1 of 8 (12.5%) F344alb that had received irradiation alone (Group II) survived until 8 weeks, 11/15 (73.3%) F344alb that had received LNPC transplantation after whole body irradiation (Group IV) were alive. All the animals untreated (Group I, n=5), transplanted with F344 LNPCs without irradiation (Group III, n=5) or received F344 BMC transplantation after irradiation (Group V, n=5) were alive until 8 weeks.

Alb+ Hepatocytes in the Livers of F344alb

The livers of untreated F344alb (Group I), those with irradiation alone (Group II) and F344alb with LNPC transplantation without irradiation (Group III) contained a very few alb+ hepatocytes. These alb+ hepatocytes were

always present as single or pairs of cells. In contrast, clusters consisting of more than 3 alb+ hepatocytes were observed in the livers of F344alb that received F344 LNPC transplantation with irradiation (Group IV) or BMC transplantation with irradiation (Group V). There was no significant difference according to the numbers of single and pairs of alb+ hepatocytes as compared between each group (Table 1) bers of alb+ hepatocyte clusters (>3 cells) between Group IV and V.

Normal Albumin Gene Sequences in the BMCs and Alb+ Hepatocyte Clusters

A 67 bp band was generated by PCR against the albumin gene sequences spanning the 7 bp, that are deleted in F344alb, from the F344 BMC DNA, while a 60 bp band was generated from the F344alb BMC DNA. PCR for DNA isolated from BMCs of F344alb that received transplantation with the F344 LNPCs (Group IV) or BMCs (Group V) after irradiation generated both 60 and 67 bp bands, indicating that their bone marrow was chimeric due to the presence of both F344 and F344alb cells. The 67 bp band was not detected in the F344alb that received F344 LNPC transplantation without irradiation (Group III). PCR for the DNA purified from the Alb+ hepatocytes detected a single 67 bp band for 2 clusters and double 60/67 bp bands in 1 cluster dissected from albumin-immunostained sections of F344alb that received F344 LNPC transplantation after irradiation (Group IV).

Discussion

Because the liver is a hematopoietic organ during the fetal period, it is thought that the liver may retain a low hematopoietic activity throughout the life.[28] In consistent to the previous reports,[4-6] we could successfully

reconstitute the bone marrow of the whole body irradiated F344alb by transplantation with the F344 LNPCs. This was evidenced by the fact that the normal albumin gene sequences lacking in the F344alb were demonstrated by PCR in the BMCs of F344alb transplanted with F344 LNPCs after irradiation. However, because both normal 67 bp and aberrant 60 bp bands were detected in the BMCs of F344alb transplanted with F344 LNPCs after irratiation, the bone marrow was chimeric due to the presence of both F344 and F344alb cells. In consistence to the bone marrow reconstitution, survival rate of irradiated F344alb was much improved by transplantation with F344 LNPCs. The normal albumin gene sequences, on the other hand, could not be detected in the BMCs from the F344alb transplanted with the F344 LNPCs without prior irradiation, indicating that the repopulation of the F344 LNPCs does not or much less efficiently occur without irradiation.

The livers of untreated F344alb contained a few alb+ hepatocytes as described previously. These alb+ hepatocytes were thought due to intracellular accumulation of aberrant albumin which is generated from the exon H, I-deleted albumin mRNA. Such cells were usually present as single or double cells and increased in number in association with aging or treatment with hepatic carcinogens. The numbers of single and double alb+ cells were not increased by transplantation with the F344 LNPCs or BMCs, indicating that most, if not all, of such single and double cells in the recipient livers were of host origin.

The livers of F344alb transplanted with F344 LNPCs or BMCs after irradiation, on the other hand, contained larger clusters of alb+ hepatocytes consisting

of >3 cells. We previously demonstrated that the alb+ hepatocyte clusters of the donor origin were generated by transplantation with F344 BMCs in F344alb after 70% hepatectomy or after whole body irradiation. In the present study, the fact that normal albumin gene sequences were detected by PCR from the alb+ hepatocyte clusters dissected from the albumin-stained tissue sections clearly indicated that they were derived from the BMCs reconstituted by the F344 LNPCs. It was also noteworthy that the large clusters were not detected in Group III in which the bone marrow was not reconstituted by the F344 LNPCs, indicating that the alb+ hepatocyte clusters were generated from the BMCs reconstituted by the F344 LNPCs, but not directly from the transplanted F344 LNPCs.

Since the frequency of alb+ hepatocyte clusters (>3 cells) was not different between Group IV and V, there may be no remarkable difference between the BMCs derived from LNPCs and those from BMCs in regard to the ability to raise the alb+ hepatocytes. However, as the transplantation of allogenic LNPCs may provide the immuno-tolerance to the host (Starzl et al. Lancet), the LNPC transplantation may have advantage against the BMC transplantation in regard to prevention of the graft versus host disease (GVHD) that is occasionally caused by transplantation with allogenic BMCs.

The alb+ hepatocyte clusters after the LPNC transplantation was small in number like in the case with the BMC transplantation. However, in the chronic liver damage model of Fah-/- mice in which host hepatocytes are continuously lost due to the metabolic damage, about 30% of liver mass was reconstituted by the hepatocytes derived from the BMCs of Fah+/+ mice after

the BMC transplantation. It is then possible that, although hepatocytes derived from BMCs may be small in number, they may repopulate to reconstitute the liver under the condition of chronic hepatic damage. On the other hand, as BMCs were suggested to provide hepatotrophic growth factors and cytokines directly or indirectly after converting to LPNCs, it remains to investigate the BMCs derived from LNPCs in these aspects.