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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

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Abstract

Purpose. Hematopoietic stem cells (SC) are though to have the potential to differentiate into hepatocytes; however, this potential has not been reported for cryopreserved SCs. We investigated if cryopreserved bone marrow cells (BMCs) from F344 rats (F344) can induce the growth of albumin-producing hepatocytes in the livers of congenic Nagase's analbuminemic rats (F344alb).

Methods. F344 BMCs were cryopreserved in University of Wisconsin (UW) solution containing 10% fetal bovine serum and 12% dimethylsulfoxide, at –80℃. After thawing, 20 x 10^7 cells were infused via the portal vein into the livers of F344alb immediately after 70% hepatectomy (PH). We examined the recipient livers for albumin positive (alb+) hepatocytes and albumin mRNA and measured the serum albumin levels 4 weeks later.

Results. Single and double alb+ hepatocytes were occasionally seen in the F344alb livers without the BMC transplantation. However, clusters consisting of more than three alb+ hepatocytes were seen in the livers of recipients transplanted with the cryopreserved BMCs after PH, the same as in the livers transplanted with freshly-isolated BMCs. Normal albumin mRNA was detected in the recipient livers, and the serum albumin levels were increased.

Conclusion. Cryopreserved F344 BMCs can induce the growth of alb+ hepatocytes after transplantion in the F344alb liver after PH.

Introduction

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 [1-12]. It is speculated that these hepatocytes are formed either by the fusion of pre-exiting differentiated hepatocytes with BMCs [13, 14], or by the trans-differentiation of BMCs into hepatocytes without cell fusion [15, 16]. 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) [17] and cord blood SCs [18], 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 [19, 20]. 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 [21, 22]. We previously reported that transplanted BMCs from F344 can raise clusters of albumin-producing hepatocytes in the livers of F344alb [23, 24].

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 [25, 26], 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 3μm-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 μl 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 [17, 18], but these always existed as single or double cells and never formed clusters of more than three cells (Table 1). 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 (Fig. 1A, Table 1). 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 (Fig. 1B, Table1). 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 (Fig. 1C, Table 1).

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 (Table 1).

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 (Fig. 2A). 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 (Fig. 2B), consistent with previous reports that the analbuminemic hepatocytes could produce a very small amount of normal albumin mRNA [13, 14]. 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 (data not shown).

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) (Fig. 3). 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 [23], 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 [7, 10-12]. 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 [25, 26]. 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 [26]. 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.

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Figure Legends

Fig. 1. (A)-(C) Albumin-immunostaining of the livers from) congenic F344 analbuminemic rats (F344alb). (A The liver form 10-week-old 70% hepatectomized F344alb contained a few single albumin-positive (alb+) hepatocytes (Group 1). (B) A large alb+ hepatocyte cluster consisting of more than 60 cells was seen 4 weeks after fresh bone marrow cell (BMC) transplantation and partial hepatectomy (PH) (Group 2). (C) A large alb+ hepatocyte cluster consisting of more than 60 cells was seen 4 weeks after cryopreserved BMC transplantation and PH (Group 3). (A, B; X 200、C; X 100).

Fig. 2. Albumin mRNA and the normal albumin gene in the livers of congenic F344 analbuminemic rats (F344alb). (A) Reverse-transcriptase PCR (RT-PCR) using the exon G and I primers amplified the 373 bp band from the total hepatic RNA (F344). Although the RT-PCR amplified the 240 bp band lacking exon H from F344alb hepatic RNA, no normal 373 bp band was apparent in the F344alb subjected to either partial hepatectomy (PH) and fresh bone marrow cell (BMC) or PH and cryopreserved BMC transplantation after 4 weeks. (Stained with ethidium bromide). (B) The RT-PCR products in (A) were blotted on a nylon membrane and hybridized with the exon H probe. A weak signal was detected at the position of the normal 373 bp band in the untreated livers and after PH alone. The intensity of the 373 bp bands was remarkably

increased in the livers after PH (F344albPH, Group 1) and fresh BMC (Fresh BMCs, Group 2) or cryopreserved BMC (Cryo-BMCs, Group3) transplantation.

Fig. 3. Western blotting analysis of serum albumin. Although an intense 67 Kd band was evident in 1:100 diluted F344 serum (F344), only a very weak band was detected in the serum of congenic F344 analbuminemic rats (F344alb) with partial hepatectomy (PH) (F344albPH). However, the band intensity increased remarkably after PH and fresh BMC (Fresh BMCs, Group 2) or cryopreserved BMC (Cryo-BMCs, Group 3) transplantation.

Table 1 Numbers of albumin-positive hepatocytes and cell clusters

Group[a]	weeks after BMCTx[b]	no. of rats[c]	no. of each size class of alb+ clusters /cm^2			
			single	double	3-10 cells	>11 cells
1	−	5	6.1±0.1	2.7±0.3	0	0
2	−	5	6.7±2.2	3.5±1.2	0	0
3	4	5	5.8±2.5	2.5±0.9	4.0±1.5*[d]	1.2±0.6*[d]
4	4	5	6.4±2.3	2.5±0.5	1.9±0.7*	0.3±0.3*

[a]Group 1, untreated; group 2, PH alone; group 3, fresh BMC transplantation plus PH; group 4, cryopreserved BMC transplantation plus PH

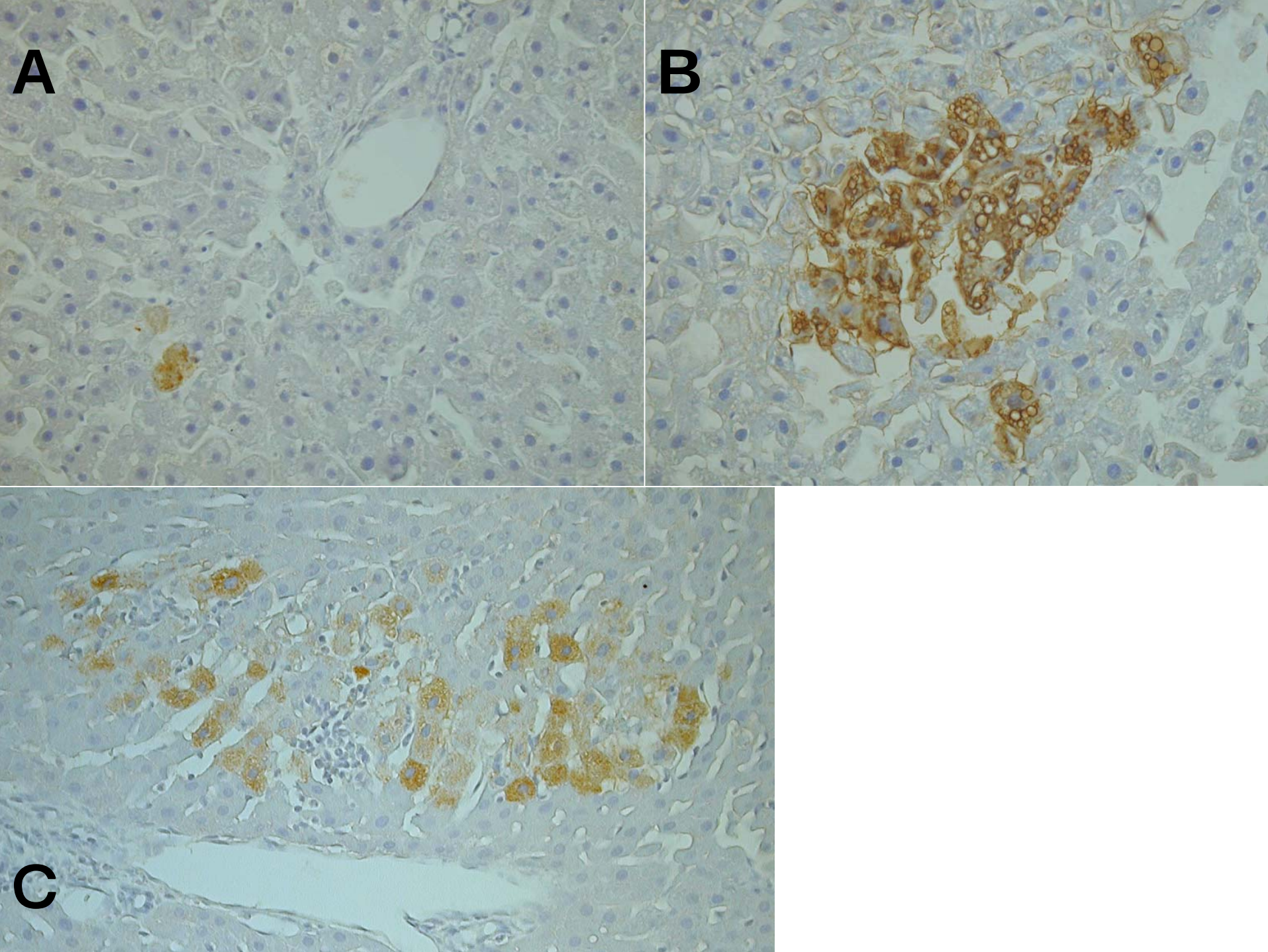
[b]weeks after BMC transplantation

[c]number of rats examined

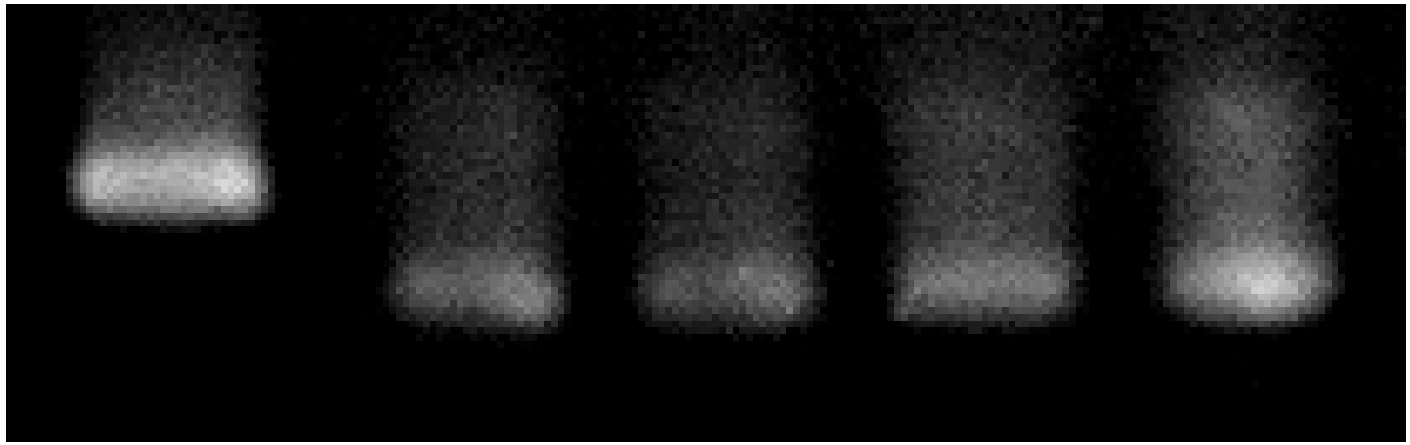
[d]The difference between Group 4 and Group 3 was not significant.

* P < 0.05 in comparison with Group 2.

PH, partial hepatectomy; BMC, bone marrow cell


A
B
C

A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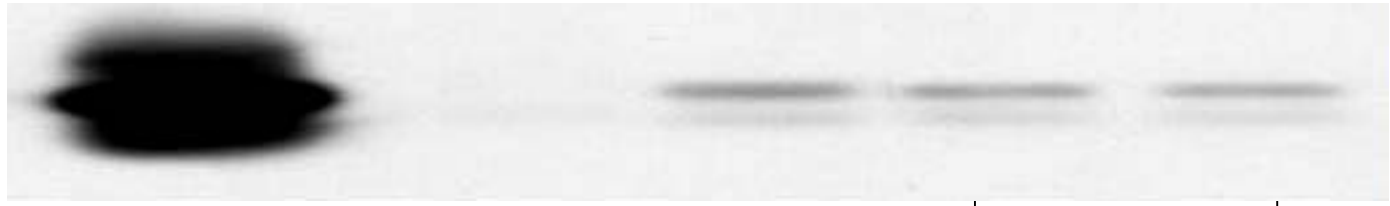
373
240
F344
F344alb PH
Fresh BMCs
Cryo-BMCs

B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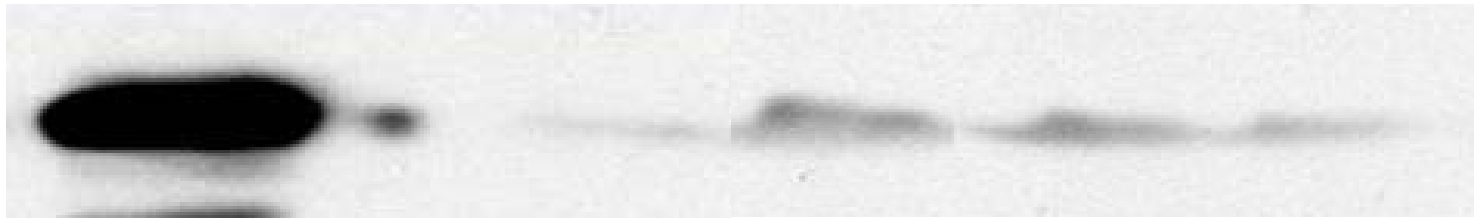

F344

F344alb PH

Fresh BMCs

Cryo-BMCs