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Production of Mouse Embryoid Bodies with Hepatic Differentiation Potential by Stirred Tank Bioreactor

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Embryonic stem (ES) cells can differentiate into functional hepatic lineage cells, which can potentially be used in biomedicine. To obtain hepatic lineage cells from ES cells, embryoid bodies (EBs) must be formed. In this study, we developed an EB formation system using a spinner flask for mass production of EBs. ES cells were inoculated into the spinner flask, where they formed EBs within 4 d. The EBs were then transferred into an attached culture for hepatic differentiation. To verify the hepatic lineage cells, albumin secretion and hepatic-specific gene expression were examined. We found that EBs formed by either the spinner flask or hanging drops exhibited similar albumin secretion potential and hepatic-specific gene expression. We conclude that the spinner flask method can be used to produce mouse EBs that can be used to mass produce hepatic lineage cells for use in biomedicine.

Key words: ESCs; embryoid bodies; hepatic lineage cells; bioreactor

Although liver transplantation appears to be the best cure to date for end-stage liver failure, because there is a shortage of donor livers, most patients with liver diseases such as acute fulminant hepatic failure or liver cirrhosis die waiting for a liver transplantation. To give such patients more time, bioartificial livers have been developed as temporary liver support for these patients until they recover from their liver disease or receive a liver transplant.[1,2] One possible alternative to transplanting the liver itself is to transplant hepatocytes, epithelial parenchymatous cells of the liver.[3,4] However, due to a shortage of functional human hepatocytes, this method is undeveloped and impractical.

Embryonic stem cells are pluripotent and self-renewing cells, and can be used to develop a variety of tissue-specific cells. Recently, hepatic lineage cells have been derived from ES cells *in vitro*.[5] These lineage cells might be used as a potential source of hepatocytes which can in turn be used in transplantations or used in the development of bioartificial livers. In addition, they might possibly be used in drug screening to examine the metabolism of xenobiotics *in vitro*.

During *in vitro* differentiation, ES cell aggregation is commonly induced to form embryoid bodies (EBs).[6] EBs are embryo-like structures that consist of three germ layers: endoderm, mesoderm, and ectoderm.[7] All three germ layer cells can further differentiate into tissue specific cells *in vitro*. EB formation methods are based on preventing ES cells from attaching to the surfaces of culture vessels, allowing the suspended ES cells to aggregate and form EBs.[6] The most common way of achieving this is to use the hanging drop method and static suspension culture to allow small scale formation of EB. In this way, cell differentiation can be observed. The hanging drop method enables the ES cells to

aggregate in a drop of medium that is suspended from the lid of a petri dish. Because the drops contain almost the same number of ES cells, this method offers uniform-sized EBs. However, because the hanging drop method requires more culture space and longer operating time, it is not very well suited to the mass production of EBs. When mass producing EBs, it is easier to use the static suspension culture method, in which ES cells are suspended in a static petri dish in which EBs are formed through cell division from a single ES cell or through aggregation from groups of cells. One drawback of this method, however, is that the EBs often fuse together to become large cell aggregates. This reduces the efficiency of EB formation[8] and inhibits cell growth and differentiation.[9]

Several approaches to increasing the production of EBs have been proposed. They include using a spinner flask for antiangiogenesis drug screening,[10] encapsulating ES cells for cardiac[11] and hematopoietic differentiation[9] potential, and recently, using a new type of rotating bioreactor for the production of primate EBs.[12] To date, however, no new EB formation system for mass producing hepatic lineage cells has been developed.

In this study, we developed a cultivation system using a spinner flask for the production of mouse EBs with hepatic differentiation potential. EBs produced by hanging drops were used as a study control. To examine the performance of the EB formation system, we measured the kinetics of albumin production of EBs, and measured the hepatic-specific gene expression of the differentiated EBs by RT-PCR. We found similar kinetics of albumin production and hepatic-specific gene expression in both EBs formed using the spinner flask and the hanging drop method. Therefore, EBs formed using the spinner flask can be a proper method to produce EBs with hepatic differentiation potential in a large-scale system.

Materials and Methods

ES cell cultivation. The mouse ES-D3 cells used in this study were obtained from Bioresources Collection and Research Center (Hsinchu, Taiwan). To maintain the ES cells in an undifferentiated state, they were co-cultured with feeder cells in mouse ES cell culture medium (MES medium) in 5% CO_2 at 37 °C. The MES medium contained 85% DMEM (Gibco Invitrogen, Grand Island, NY), 15% fetal bovine serum (FBS, ES cell grade, Gibco Invitrogen), 12.5 U/ml penicillin, 12.5 µg/ml streptomycin (Gibco Invitrogen), 0.1 mM non-essential amino acid (Gibco Invitrogen), and 0.1 mM β-mercaptoethanol (Gibco Invitrogen). Feeder cells were mouse primary embryonic fibroblasts cultivated in mouse embryonic fibroblast culture medium (MEF medium). MEF medium consisted of 90% DMEM, 10% FBS, 12.5 U/ml penicillin, and 12.5 µg/ml streptomycin. The feeder cells were inactivated by 10 µg/ml

mitomycin C (Sigma-Aldrich, St. Louis, MO) for 2.5 to 3 h before being co-cultured with ES cells.

EB formation. When using the spinner flask method, ES cells were first harvested and trypsinized. Then 1×10^7 cells were inoculated into 100 ml of MES medium in a 125 ml spinner flask (Corning, NY) and cultivated in 5% CO_2 at 37 °C at 60 rpm. Half of the medium was exchanged with fresh medium every 2 d. At day 4, the EBs were harvested.

When using the hanging drop method, the trypsinized ES cells were cultured in a drop of MES medium suspended from the lid of a petri dish. Each drop was 30 µl and had 900 cells. After incubation at 37 °C in 5% CO_2 for 2 d, the EBs were transferred into petri dishes containing MES medium for suspension culture for an additional 2 d, and then harvested.

Induction of hepatic differentiation of EBs. After the EBs had formed for 4 d, they were collected, washed once with MES medium, and inoculated into a collagen type I (rat tail, BD Falcon, Bedford, MA) coated 48-well plate containing MES medium, and incubated at 37 °C in 5% CO_2 for attached culture. One EB per well was cultivated. After 3 d in attached culture, using a method described by Hamazaki *et al.*[13] and Chinzei *et al.*,[14] we induced EB differentiation into hepatic lineage cells. For early-stage induction, 10 ng/ml bFGF (R&D Systems, Minneapolis, MN) was added at day 7; for mid-stage induction, 20 ng/ml HGF (R&D Systems) was added at day 9; and for late-stage induction, 20 ng/ml HGF, 10 ng/ml OSM (R&D Systems), 100 nM dexamethasone (Dex, Sigma-Aldrich), and 5 µg/ml ITS (based on transferrin, Gibco Invitrogen) were added at day 11.

EB size measurement. The size of EBs was measured by image analysis. During the EB formation period, EBs were sampled and images of the EBs were captured by a microscope attached to a digital camera. The projected area of the EB was computed by image analysis software (Scion Image, Frederick, MD), and converted to a diameter assuming a circular cross-sectional area.

Albumin secretion assay. Albumin secreted by the differentiated cells was measured with a mouse albumin ELISA quantitation kit (Bethyl, Montgomery, TX) according to the manufacturer's instructions. The specificity of the antibody used in the mouse albumin ELISA kit was tested and found to have no cross reactivity with bovine albumin (data not shown).

RT-PCR analysis of hepatic-specific gene expression. mRNA of ES cells, differentiated EBs, mouse fetal (E13.5), and adult (two month old) liver cells were extracted with an RNeasy Mini Kit (Qiagen, Hilden, Germany); RT-PCR was performed utilizing the OneStep RT-PCR kit (Qiagen) in a GeneAmp PCR

System 9700 (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

To ensure ES cell differentiation into hepatic lineage cells, the expression of hepatic-specific genes, including α1-anti-trypsin (AAT), α-fetoprotein (AFP), albumin, carbamoyl-phosphate synthetase 1 (CPS1), cytochrome P450 7A1 (CYP7A1), glucose-6-phosphatase (G6P), tyrosine aminotransferase (TAT), and transthyretin (TTR), were analyzed. β-actin was used as the endogenous control. The genes, sequence of the primers, annealing temperature, reaction cycles, and RT-PCR product size were as follows: AAT, AATGGAAGA-AGCCATTCGAT, AAGACTGTAGCTGCTGCAGC, 55 °C, 30 cycles, 484 bp; AFP, TCGTATTCCAACAG-GAGG, AGGCTTTTGCTTCACCAG, 55 °C, 25 cycles, 173 bp; albumin, GCTACGGCACAGTGCTTG, CAG-GATTGCAGACAGATAGTC, 55 °C, 25 cycles, 260 bp; CPS1, ATGACGAGGATTTTGACAGC, CTTCACA-GAAAGGAGCCTGA, 60 °C, 35 cycles, 126 bp; CY-P7A1, TACGCATGTTTCTCAACGATAC, TCTTGG-ACAGCAAATAGTCTTC, 55 °C, 35 cycles, 583 bp; G6P, CTACCTGCTACTAAAAGGGCTAGG, GCTA-GGCAGTATGGGATAAGACTG, 60 °C, 30 cycles, 369 bp; TAT, TATCCTGAGGGTACCAGTTTACC, TCT-TCGACTTCTCTCTGGTGTAG, 58 °C, 30 cycles, 211 bp; TTR, CTCACCACAGATGAGAAG, GGCTGAG-TCTCTCAATTC, 55 °C, 25 cycles, 225 bp; and β-actin, TTCCTTCTTGGGTATGGAAT, GAGCAATGATCT-TGATCTTC, 55 °C, 20 cycles, 200 bp.

Results

EB morphology and size distribution between days 1 and 4

Stirred tank bioreactors are widely used to bioprocess culture cells. Here we developed a cultivation system using a spinner flask to produce mouse EBs with hepatic differentiation potential. About 1×10^7 ES cells were cultured in a 125 ml spinner flask; they formed EBs within 4 d. Figure 1 shows the shape of the EBs after they had formed for 4 d, after being cultured using the spinner flask and hanging drop methods. Both methods produced simple EBs, though those produced by the spinner flask method were smaller than those formed by the hanging drop method. The hanging drop method employs almost the same cell number in each drop at the initial cell aggregation stage, and no further aggregation occurs during the EB formation period. Consequently, EBs from hanging drops showed size uniformity. By contrast, a stirred spinner flask provides a hydrodynamic environment that results in random cell aggregation. Moreover, cell aggregation is sustained through the EB formation period, and EB size decreases due to shear stress. Hence, EBs from the spinner flask showed smaller size and greater variation. We measured the sizes of the EBs while they were being formed using the two methods, and studied the size distribution (Fig. 2). Because the spinner flask produced some single cells
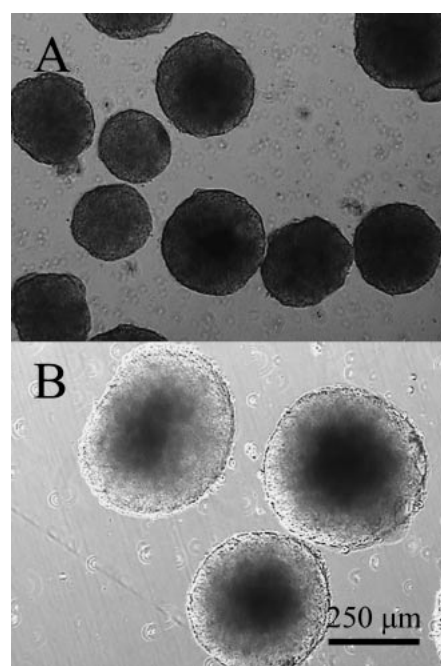

Fig. 1. Morphology of EBs with Cultivation at Day 4.
EBs were produced by spinner flask (A) or hanging drops (B). These day-4 EBs were harvested for further hepatic differentiation studies.
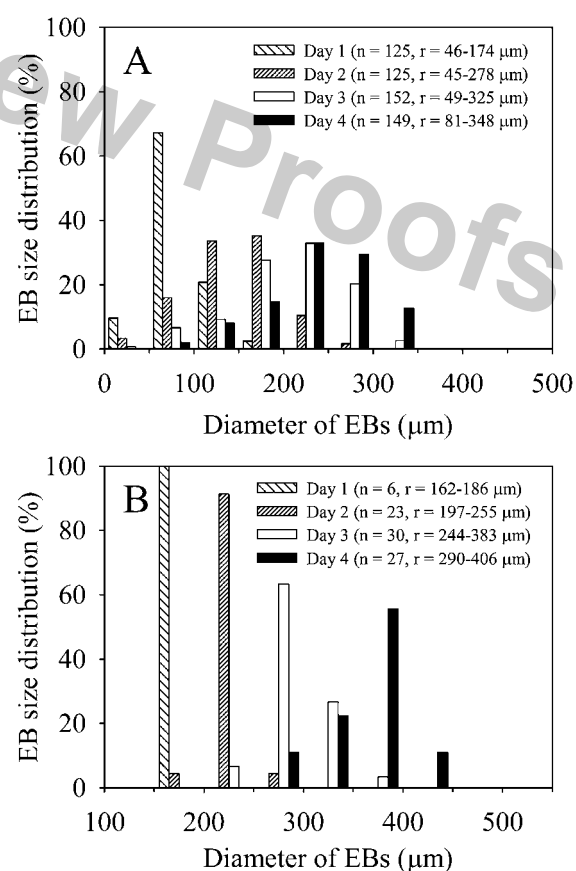

Fig. 2. Size Distribution of EBs during the Cultivation Period.
EBs from spinner flask (A) or hanging drops (B) at each time point were harvested for size measurement. In the legends, "n" denotes sample numbers, and "r" denotes EB diameter ranges.
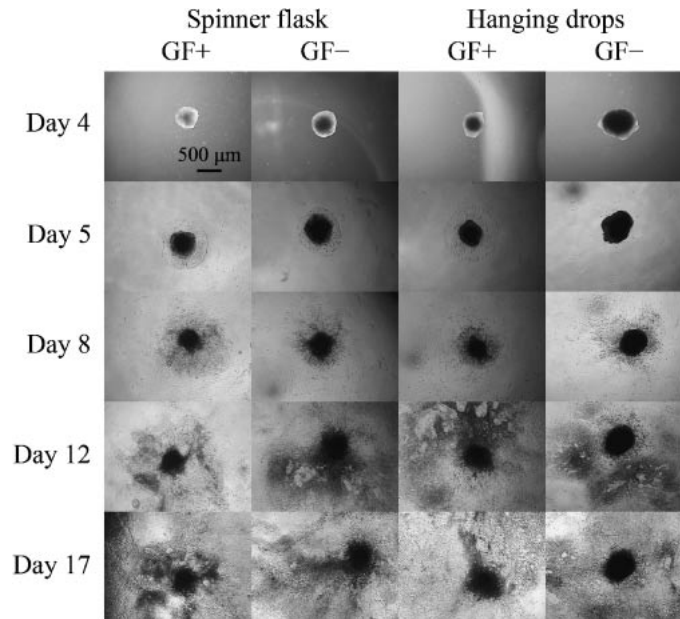
Fig. 3. Morphology of EBs in an Attached Culture during the Cultivation Period.

　　The day 4, EBs from spinner flask or hanging drops were transferred to collagen coated a 48-well plate at one EB per well with (GF+) or without (GF−) growth factor induction for hepatic differentiation. Photos in each column show the morphology of the same EB during the cultivation period.

and small cell-aggregates consisting of only a few cells, we took only aggregates of cells whose diameters were greater than 35 μm into consideration. At day 1, EBs formed by the spinner flask showed a size distribution pattern ranging from 46 to 174 μm, 67% ranging between 50 and 100 μm (Fig. 2A). The EBs increased in size over cultivation time. The size distribution broadened to a range of 81 to 348 μm by day 4. Compared to the spinner flask method, the hanging drop method produced larger, more uniform EBs, with sizes ranging between 162 and 186 μm at day 1 and between 290 and 406 μm by day 4 (Fig. 2B).

EB morphology and EB albumin production during the cultivation period

EBs were formed in the spinner flask or hanging drops for 4 d, and then transferred to an attached culture to induce differentiation of hepatic lineage cells. In the present study, we cultured EBs into collagen type I-coated 48-well plates as one EB per well so that we could trace the morphology of each EB during the cultivation period. To improve hepatic differentiation, growth factors, including bFGF, HGF, and OSM, were added step by step; hepatotrophic factors including insulin, transferrin, selenium, and Dex were also added at late-stage induction. Figure 3 shows the morphology of the EBs during the cultivation period. At day 4, EBs were inoculated into each well of the cell culture plates. At day 5, all the EBs attached to the collagen coated wells, and cells started to spread from the EBs to form a monolayer at the periphery. At day 8, some EBs started to show beating cardiomyocytes. During the culture

period, the cell numbers of all EBs increased continuously. At day 17, the colonies of EBs almost covered the entire area of the wells of a 48-well plate. In addition, all EBs showed similar morphology, whether they were produced by the spinner flask or by hanging drops cultured with or without growth factors.

To monitor the hepatic differentiation potential of the same EBs during the cultivation period, we measured the specific albumin productivity of each EB, because albumin is the major hepatocyte-secreted protein. Albumin secreted by the differentiated EBs was measured every 2 d from day 11 to day 17 (Fig. 4). They did not secrete a noticeable amount of albumin at day 11, the specific albumin level measuring lower than 0.01 ng/h·EB. The EBs still showed low specific albumin production at day 13. These data for day 11 and 13 were not further statistically analyzed by t-test because the deviation of measurement at low albumin levels can be interfered as statistical results. However, EB secretion of albumin gradually increased after day 13. When growth factors was used to induce production, the spinner flask produced EBs with significantly higher specific albumin productivity from day 15 to day 17 than when growth factors were not used in the same method (p = 0.002 and 0.0004 at day 15 and 17 respectively), highest specific albumin productivity reaching 1.80 ± 0.265 ng/h·EB at day 17. When growth factors were used with the hanging drop method, the EBs were found to have higher specific albumin productivity than with the same method without growth factor induction only at day 17 (p = 0.038), highest specific albumin productivity reaching 1.34 ± 0.193 ng/h·EB at day 17. Analyzed
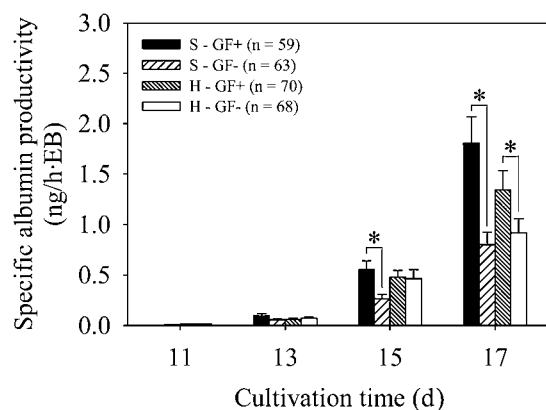
Fig. 4. Specific Albumin Productivity of EBs during the Cultivation Period.

The day 4, EBs obtained from spinner flask (S) and hanging drops (H) in an attached culture with (GF+) or without (GF−) growth factor were monitored for their specific albumin productivity during the cultivation period. All data are shown as mean with standard error, and "n" denotes sample numbers. The data were also statistically analyzed by t-test, where "*" indicates $p < 0.05$.

statistically, there was no significant difference in the specific albumin productivity of EBs formed by the two methods regardless of whether growth factors were used from day 15 to day 17 ($p > 0.05$), indicating that the choice of EB formation method did not affect the ability of the EBs to secrete albumin.

EB albumin secretion potential

There was variation in the amount of specific albumin produced by EBs formed by the spinner flask and the hanging drop method (Fig. 4). To compare the ability of each EB to secrete albumin, we measured the albumin yield (total albumin produced from day 11 to day 17) (Fig. 5). More than 50% of the EBs produced by either method secreted between 0 and 100 ng/EB of albumin. Using growth factor to induce differentiation of hepatic lineage cells yielded higher albumin yields. With growth factors, 42% of the spinner flask-produced EBs and 26% of the hanging drop-produced EBs generated more than 100 ng/EB of albumin from day 11 to day 17. Furthermore, with growth factor, 2% of the spinner flask-produced EBs secreted more than 500 ng/EB of albumin during the cultivation period. Without growth factor, the EBs formed by either method secreted less albumin, as confirmed by histogram estimations, which found that 89% of spinner flask-produced EBs and 84% of those produced by the hanging drop method secreted between 0 to 100 ng/EB of albumin.

Effect of EB size on albumin production

There were variations in the size of EBs formed by the spinner flask and hanging drop methods. We compared different sizes of EBs with their ability to secrete albumin (Fig. 6). With growth factors, spinner flask-formed EBs ranging in size from 250 and 300 μm secreted the most albumin (163 ± 36.6 ng/EB), and
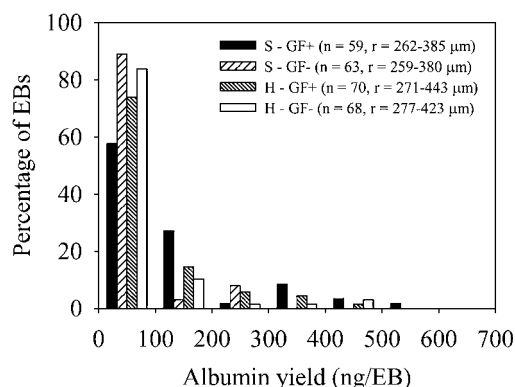

Fig. 5. Distribution of Albumin Yield of EBs.

EBs were sorted by albumin yield to determine their distribution by albumin yield. The albumin yield of each EB is the sum of albumin production from day 11 to day 17. The EBs were produced by spinner flask (S) or hanging drops (H), and cultured with (GF+) or without (GF−) growth factor induction for hepatic differentiation. In the legends, "n" denotes sample numbers, and "r" denotes EB diameter ranges.
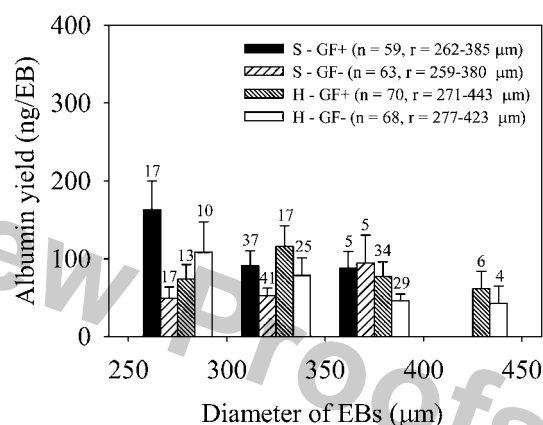

Fig. 6. Albumin Yield of EBs of Different EB Sizes.

EBs were sorted by size to evaluate the size effect on albumin yield. The EBs were formed by spinner flask (S) or hanging drops (H), and cultured with (GF+) or without (GF−) growth factor induction for hepatic differentiation. Albumin yield is the sum of albumin production of EBs from day 11 to day 17. All data are shown as mean with standard error, and the numbers above the error bar denote sample numbers. In the legends, "n" denotes total sample numbers, and "r" denotes EB diameter ranges.

those ranging in size from 350 to 400 μm secreted the least (88.1 ± 21.4 ng/EB). Without growth factors, the spinner flask-formed EBs, ranging in size from 350 to 400 μm, secreted the most albumin (92.3 ± 36.2 ng/EB), and those ranging in size from 250 to 300 μm secreted the least (48.9 ± 14.5 ng/EB). With growth factors, the hanging drop-formed EBs, ranging in size from 300 to 350 μm, secreted the most albumin (116 ± 26.6 ng/EB), and those ranging in size from 400 to 450 μm secreted the least (61.5 ± 22.5 ng/EB). Without grow factors, hanging drop-formed EBs, ranging in size from 250 to 300 μm, secreted the most albumin (108 ± 39.3 ng/EB), and those from 400 to 450 μm secreted the
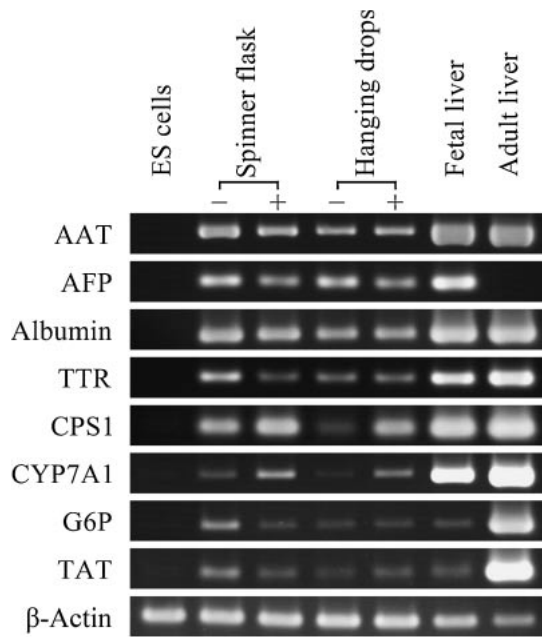
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Fig. 7. Hepatic-Specific Gene Expression in Differentiated EBs.
Hepatic-specific gene expression was examined by RT-PCR for differentiated EBs with (+) or without (−) growth factor induction at day 17. AAT, AFP, albumin, and TTR showed endoderm gene expression. These genes were also expressed in mature hepatocytes. CPS1, CYP7A1, G6P, and TAT showed mature hepatic-specific gene expression. Fetal liver (E13.5) and adult liver (two months old) were used as a positive control.

least (42.2 ± 22.5 ng/EB). The data were further analyzed by ANOVA, and the EB size effect was evaluated with to the confidence coefficient set at 0.05. No significant differences were found between the amount secreted by growth factor added spinner flask-formed EBs ($p = 0.13$) and those in which growth factor was not used ($p = 0.35$). Nor were any significant differences found between the amount secreted by growth factor added hanging drop-formed EBs ($p = 0.50$) and those in which growth factor was not used ($p = 0.22$). We found no significant differences in the amounts between the two methods. Taken together, these results indicate that the size of an EB did not affect its ability to secrete albumin.

Hepatic-specific gene expression of differentiated EBs
RT-PCR was used to analyze hepatic-specific gene expression of differentiated EBs (Fig. 7). EBs formed by the spinner flask and hanging drop methods expressed endoderm markers (AAT, AFP, albumin, and TTR) and mature hepatic markers (CPS1, CYP7A1, G6P, and TAT). It has been reported that the gene CYP7A1 is expressed in hepatocytes only, not in the yolk sac.[15] Hence, hepatic lineage cells in this study were differentiated from EBs formed by either method. However, the use of growth factors to induce hepatic differentiation showed the differentiated EBs to have suppressed hepatic-specific gene expression. Only CPS1 and CYP7A1 were improved by growth factor induction.

Discussion

Because the liver, the largest organ in the human body, is composed of 1.5×10^{11} hepatocytes, a large number of hepatocytes are needed to treat a patient with liver failure. Preparing a bioartificial liver, for example, is estimated to require about 1×10^{10} hepatocytes,[2] or 150 to 450 g of hepatocytes.[1] To find a more abundant source of hepatocytes, we developed a system using a spinner flask to culture ES cells to produce mouse EBs that can be used later to induce hepatic differentiation.

To evaluate the potential of this method to produce EBs capable of hepatic differention, we measured the ability of EBs to secrete albumin as an index to evaluate the potential of EBs formed by the spinner flask method and the hanging drop method of hepatic differentiation. We also used RT-PCR to evaluate semi-quantitatively the ability of the differentiated EBs to express hepatic-specific genes. The two methods were found to have similar albumin secretion abilities and similar hepatic-specific gene expression. These results indicate that hepatic differentiation of EBs was not affected by the method by which they were formed, a finding similar to that of another recent study, in which the hematopoietic differentiation potential of EBs was not affected by different EB formation methods.[8] Although shear stress in a stirred tank bioreactor is still a problem for cell cultures, we did not observe any damage to EB formation or hepatic differentiation of EBs caused by shear stress in a spinner flask.

Because spinner flask shear stress caused a wide distribution in EB size, we examined the effect of size on hepatic differentiation. EBs are considered embryo-like structures,[7] and embryo size is well controlled during embryogenesis.[16] Although we found EB size to be proportional to cell numbers, we did not find a significant difference in EB size or albumin yield, an indicator of hepatic differentiation potential.

The use of growth factors and other hepatotrophic factors might improve the hepatic differentiation of ES cells. One previous study reported the use of aFGF, HGF, OSM, ITS, and Dex to induce hepatic differentiation of ES cells and improved mature hepatic-specific gene expression of differentiated EBs.[13] In the present study, however, spinner flask-formed EB production of albumin protein and expression of albumin genes was not consistent by the use of growth factors. In addition, hepatic-specific gene expression of differentiated EBs was either suppressed or not improved by growth factor induction, a finding similar to other studies.[14,17] Moreover, another study, proposing that the use of human insulin and Dex might improve hepatic differentiation of ES cells, reported that HGF, an essential growth factor to induce hepatic differentiation during embryogenesis, did not improve hepatic differentiation of ES cells *in vitro*.[18] Together, these results suggest that a protocol for hepatic differentiation of ES cells should be designed specifically for each ES cell line.

In conclusion, the advantage of using a spinner flask to produce EBs is that it can be used to inoculate a large quantity of ES cells at one time, which cannot be done using the hanging drop method. Although the spinner flask produced a wide variety of EB sizes in this study, size was not found to affect albumin secretion potential. Furthermore, the albumin secretion potential and hepatic-specific gene expression of the EBs that were produced by the spinner flask method were similar to those produced by the hanging drop method, a widely accepted method. We conclude that the spinner flask can be used in the production of EBs for large-scale production of hepatic lineage cells for use in biomedicine.

Acknowledgment

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