Effects of extracellular matrixes and growth factors on the hepatic differentiation of human embryonic stem cells

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Effects of extracellular matrixes and growth factors on the hepatic differentiation of human embryonic stem cells. Am J Physiol Gastrointest Liver Physiol 295: 313–321, 2008. First published June 5, 2008; doi:10.1152/ajpgi.00072.2008.—Hepatocytes derived from human embryonic stem cells (hESCs) are a potential cell source for regenerative medicine. However, the definitive factors that are responsible for hepatic differentiation of hESCs remain unclear. We aimed to evaluate the effects of various extracellular matrixes and growth factors on endodermal differentiation and to optimize the culture conditions to induce hepatic differentiation of hESCs. The transgene vector that contained enhanced green fluorescent protein (EGFP) under the control of human α-fetoprotein (AFP) enhancer/promoter was transfected into hESC lines. The transgenic hESCs were cultured on extracellular matrixes (collagen type I, laminin, and Matrigel) in the presence or absence of growth factors including hepatocyte growth factor (HGF), bone morphogenetic protein 4, fibroblast growth factor 4, all-trans-retinoic acid, and activin A. The expression of AFP-EGFP was measured by flow cytometry. The culture on Matrigel-coated dishes with 100 ng/ml activin A showed 19.5% of GFP-positive proportions. Moreover, the sequential addition of 100 ng/ml activin A and 20 ng/ml HGF resulted in 0.7% and produced a higher yield of EGFP-positive cells than the group stimulated by activin A alone. RT-PCR and immunocytochemical staining produced a higher yield of EGFP-positive cells than the group stimulated by activin A alone. RT-PCR and immunocytochemical staining revealed these EGFP-positive cells to differentiate into mesendoderm-like cells by use of activin A and then into hepatic endoderm cells by use of HGF. Two other hESC lines also differentiated into endoderm on the hepatic lineage by our method. In conclusion, we therefore found this protocol to effectively differentiate multiple hESC lines to early hepatocytes using activin A and HGF on Matrigel.

Materials and Methods

Construction of transgene vector. The human AFP promoter region (−328 to −16, the adenine of the ATG start codon was numbered as nucleotide 1) and enhancer region (−465 to −3272) were obtained by long-range polymerase chain reaction (PCR) using a LA Taq polymerase (Takara Bio, Otsu, Japan). The genomic DNA extracted from hESCs was used as a PCR template. The specific primers for the AFP promoter region were as follows: 5′-TCTGCAACTTGGGACAAGTCA (sense), 5′-TGTTATTGGCAATGGTTGAA (antisense). The AFP promoter region was ligated with Apa-I (Takara Bio) digested pEGFP-1 (BD Biosciences, Franklin Lakes, NJ) (pAfpPcEGFP). The specific primers for the AFP enhancer region were as follows: 5′-GAAGATCTTTGGG-GATAGATCTTTTTCA (sense), 5′-AAAACTGAGGATAGA- CTAATGGAAGGAC (antisense). The BglII and PstI recognition sites were inserted into the sense and antisense primer, respectively, as underlined. The AFP enhancer region was ligated with BglII-PstI (Takara Bio) digested pAfpPcEGFP, thus resulting in a construct in which EGFP was expressed under the control of the AFP enhancer/promoter (Fig. 1A).

Generation of transgenic hESCs. The transgenic vector was transfected into three hESC lines (Khes1, Khes2, and Khes3) by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol (9). Stable transfected cells were selected in the presence of 100 μg/ml G418 (Sigma-Aldrich, St. Louis, MO) on a G418-resistant mouse embryonic fibroblast feeder layer. The proper transgene insertion was confirmed by PCR. The stable transgenic hESCs were cultured in an undifferentiated state as described previously (25).

Preliminary Results

Hepatocytes Derived from Human Embryonic Stem Cells (hESCs) are anticipated to become a potentially useful cell source for various cell therapies, bioartificial livers, and drug discovery support systems. Although several articles have reported the differentiation of hESCs into hepatocyte-like cells in vitro by various methods (2, 5, 6, 16), it remains unknown which extracellular matrixes (ECMs) or growth factors are responsible for the induction of hepatic differentiation of hESCs (3, 23). Moreover, it has also been difficult to obtain fully functional hepatocytes derived from hESCs (8).

This laboratory previously reported that mouse embryonic stem cells (mESCs) differentiated and matured into hepatocytes using the transgenic mESCs that expressed enhanced green fluorescent protein (EGFP) under the control of mouse α-fetoprotein (AFP) promoter (12, 13). AFP is one of the common markers for hepatic differentiation, because the hepatoblasts in developing livers abundantly produce AFP (24).

In this study, we generated hESCs that express EGFP under the control of human AFP enhancer/promoter. These hESCs were cultured on several ECMS in the presence or absence of some growth factors, and the expressions of AFP-EGFP were measured. Using the transgenic hESCs, we aimed to evaluate and compare the effects of several ECMS and growth factors on hESC-endodermal differentiation and to optimize the culture conditions for the hepatic differentiation of hESCs.

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on gelatin-coated dishes for 7 days. In method 2, the hESCs were differentiated without forming EBs. To induce differentiation into endoderm in flat cultures, undifferentiated hESCs were transferred directly to some ECM-coated dishes under low-serum conditions. Collagen type I-coated dishes (BD Biosciences), laminin-coated dishes (BD Biosciences), and Matrigel thin-coated dishes were used. To make Matrigel thin-coated dishes, the plastic culture dishes were coated with growth factor-reduced Matrigel (1:80 dilution, BD Biosciences) for 16 h at 37°C. Following enzymatic dissociation, the hESCs were selected by gravity sedimentation and then were replated on each ECM-coated dish in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 (Sigma-Aldrich) supplemented with 20% knockout SR (GIBCO, Grand Island, NY), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), and MEM nonessential amino acids (GIBCO) for the first day (day 0). From the next day (day 1), the hESCs were cultured in RPMI 1640 (GIBCO) with 0.5% fetal bovine serum (FBS) (HyClone, Logan, UT), 1 mM sodium pyruvate (Sigma-Aldrich), 10 mM nicotinamide (Sigma-Aldrich), 2 mM l-ascorbic acid phosphate (Wako Pure Chemical, Osaka, Japan), insulin-transferrin-selenium supplement (GIBCO), and 1 × 10^{-7} M dexamethasone (Sigma-Aldrich). From day 1, 20 ng/ml hepatocyte growth factor (HGF, R&D Systems, Minneapolis, MN), bone morphogenetic protein 4 (BMP4, R&D Systems), fibroblast growth factor 4 (FGF4, R&D Systems), 10 μM all-trans-retinoic acid (ATRA, Sigma-Aldrich), or 100 ng/ml activin A (R&D Systems), were also added.

Flow cytometry. The differentiated hESCs were dissociated with 0.05% trypsin (GIBCO)-EDTA (Dojindo Laboratories, Kumamoto, Japan) solution and then resuspended in 3% FBS-PBS. The cells were analyzed and isolated by use of a FACSVantage SE (BD Biosciences).

Cell proliferation assay. The cell numbers were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Cell Titer 96 Aqueous One Solution Reagent, Promega, Madison, WI), according to the manufacturer’s protocol. After 1 h of incubation, the absorbance value was measured with a plate reader at 490 nm.

Immunocytochemistry. The cultured cells were fixed and stained as previously described (12, 13). Alkaline phosphatase staining and immunocytochemistry for Oct-3/4 and SSEA4 were performed as described previously (9, 25). An anti-EGFP rabbit antibody (Invitrogen), anti-AFP mouse antibody (Sigma-Aldrich), anti-albumin goat antibody (Bethyl, Montgomery, TX), anti-E-cadherin mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-Sox17 mouse antibody (R&D Systems) were used as the first antibodies. Alexa 488-conjugated donkey anti-rabbit IgG (Molecular Probes, Eugene, OR) for EGFP staining, Alexa 546-conjugated donkey anti-goat IgG

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**Fig. 1.** Human embryonic stem cells (hESCs) that express enhanced green fluorescent protein (EGFP) under the control of α-fetoprotein (AFP) enhancer/promoter. A: a graphical display of the transgenic vector. Alkaline phosphatase staining (B) and immunostaining for Oct-3/4 (C) and SSEA4 (D) demonstrated the hESCs to be cultured in an undifferentiated state. E: EGFP expression localized at the AFP-producing cells in the embryoid bodies (EBs) differentiated by method 1. Green fluorescence indicates EGFP, red fluorescence indicates AFP, and blue fluorescence represents 4,6-diamidino-2-phenylindole (DAPI). The yellow color, which is obtained by merging the green and red fluorescence, indicates the coexpression of EGFP and AFP in the same cells. Original magnifications: 100× (B–D); 200× (E). F: an RT-PCR analysis was performed using the total RNA extracted from the undifferentiated hESCs (left lane) and the EBs (right lane).
(Molecular Probes) for albumin staining, and Alexa 555-conjugated goat anti-mouse IgG (Molecular Probes) for AFP, E-cadherin, and Sox17 staining were used as the secondary antibodies. All first antibodies were diluted at 1:200, and the secondary antibodies were diluted at 1:500. The stained cells were covered with Vectashield mounting medium with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA).

RT-PCR. The total RNA was extracted from cultured cells 1 day, 5 days, and 10 days after the initiation of differentiation and from isolated EGFP-AFP-positive cells just after cell sorting, by use of an RNeasy Mini Kit (Qiagen, Chatsworth, CA), and treated with RNase-free DNase (Qiagen). The total RNA of the cultured cells (2 μg) was reverse-transcribed into cDNA as described previously (12). The total RNA extracted from a normal human liver was purchased from Cell Applications, Inc. (San Diego, CA). One microgram of the total RNAs extracted from the human liver, the isolated EGFP-positive cells, and the EGFP-negative cells were reverse-transcribed into cDNA. The primers were generated for the EGFP gene and the following human genes: Oct-3/4, Nanog, placental lactogen 1 (PI-1), cystic fibrosis transmembrane conductance regulator (CFTR), TGFβ receptor II (TβRII), matrix metalloproteinase 3 (MMP3), brachyury (T), Meox1, Meox2, GATA4, GATA6, mebox 2 (Cdx2), musashi homolog 1 (musashi), nestin, goosecoid (Gsc), brachyury (T), α-smooth muscle actin (α-SMA), AFP, albumin, GATA4, tyrosine aminotransferase (TAT), treptophan 2,5-deoxyxogengase (TO), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Their sequences and PCR conditions are summarized in Table 1.

Table 1. PCR primer sequences

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Annealing Temperature</th>
<th>PCR Cycles</th>
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<tr>
<td>Oct-3/4</td>
<td>5'-GAGAACAATTGAGAAGACCTTCAAGAGA</td>
<td>5'-TTCTGCGGCGGTTAAGGAGA</td>
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<tr>
<td>Nanog</td>
<td>5'-AAAGACAGGCTCGGCTAACAGG</td>
<td>5'-CTCTGCGTCTTCATATTTTACAG</td>
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<tr>
<td>PI-1</td>
<td>5'-ACCTTCAAGCTTAGGGAATGGA</td>
<td>5'-CTCTGCGTCTTCATATTTTACAG</td>
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<tr>
<td>Cdx2</td>
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<td>5'-CTCTGCGGCGGTTAAGGAGA</td>
<td>60°C</td>
<td>30</td>
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<tr>
<td>Musashi</td>
<td>5'-CGAGTCTGACGCTCAGAAAACAAATGGACC</td>
<td>5'-TCTCACGAGAATTTCTCTGCGAATTCGTAGTT</td>
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<td>30</td>
</tr>
<tr>
<td>Nestin</td>
<td>5'-CTCTTGCAAGAATCTCCAGGGACCTTG</td>
<td>5'-CTCTGCGTCTTCATATTTTACAG</td>
<td>60°C</td>
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</tr>
<tr>
<td>Gsc</td>
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<td>5'-CTCTGCGGCGGTTAAGGAGA</td>
<td>60°C</td>
<td>30</td>
</tr>
<tr>
<td>T</td>
<td>5'-CTCTTGCAAGAATCTCCAGGGACCTTG</td>
<td>5'-CTCTGCGGCGGTTAAGGAGA</td>
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<td>30</td>
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<tr>
<td>eMHIC</td>
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<td>5'-CTCTGCGGCGGTTAAGGAGA</td>
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<td>AFP</td>
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<td>5'-TCTCTTGCAAGAATCTCCAGGGACCTTG</td>
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<td>30</td>
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<tr>
<td>Albumin</td>
<td>5'-TCGAGGTGAGAATCTTGGGAGGCTGTT</td>
<td>5'-CTCTGCGGCGGTTAAGGAGA</td>
<td>60°C</td>
<td>30</td>
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<tr>
<td>GATA4</td>
<td>5'-ATGAGGCTGACTGATCTAGTG</td>
<td>5'-AGAGGAGAATCTTGGGAGGCTGTT</td>
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<td>30</td>
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<tr>
<td>TAT</td>
<td>5'-CTCTTGCAAGAATCTCCAGGGACCTTG</td>
<td>5'-CTCTGCGGCGGTTAAGGAGA</td>
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<td>30</td>
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<tr>
<td>TO</td>
<td>5'-AGAGGAGAATCTTGGGAGGCTGTT</td>
<td>5'-AGAGGAGAATCTTGGGAGGCTGTT</td>
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<td>30</td>
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<tr>
<td>EGFP</td>
<td>5'-AGAGGAGAATCTTGGGAGGCTGTT</td>
<td>5'-AGAGGAGAATCTTGGGAGGCTGTT</td>
<td>60°C</td>
<td>30</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GGAGGAGGCTGTCATCTTATTAA</td>
<td>5'-TCTCTTGCAAGAATCTCCAGGGACCTTG</td>
<td>60°C</td>
<td>30</td>
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qPCR. For quantitative PCR (qPCR), the total RNA was extracted from the cultured cells of the undifferentiated state (day 0), 5 days, and 10 days after differentiation. Real-time PCR was performed as described previously (26). The quantified values of each gene were normalized against GAPDH. The annealing reactions were performed at 60°C. The gene-specific primers are listed in Table 2.

Statistical analysis. All experiments were performed in three replications. The results are given as means ± SD. The statistical analyses were performed by either Dunnett’s test or Student’s t-test. P < 0.05 was considered to be statistically significant.

RESULTS

Differentiation of hESCs into AFP-producing endoderm cells. To examine the specific expression of EGFP for AFP-producing cells, the transgenic hESCs were differentiated by method 1. In one clone of KhES3, both the EGFP and AFP expression were observed in the same cells by method 1 (Fig. 1E). An RT-PCR analysis of EBs revealed that all three germ layers and trophoderm markers were observed, thus suggesting their pluripotency, and that EGFP expressed in the AFP-expressing EBs (Fig. 1F). This transgenic hESC clone grew normally in the presence of the mouse embryonic feeder cell layer compared with its parental hESC line, and it also possessed a normal human karyotype (data not shown). Therefore, this clone was used for the subsequent experiments. The other G418-resistant clones did not express EGFP despite their expression of AFP, probably owing to silencing of the transgene.

Effects of ECMs and growth factors on endodermal differentiation. The proportion of EGFP-positive cells was extremely low (less than 5%) by method 1. We have therefore tried to elucidate the most effective culture conditions for differentiating hESCs into hepatocytes by method 2. The transgenic hESCs were cultured on collagen type I-, laminin-, and Matrigel thin-coated dishes in the presence of either HGF, BMP4, FGF4, ATRA, or activin A or in the absence of these factors. The proportion of EGFP-positive cells was determined by use of a flow cytometer. Laminin is a principal component of Matrigel. However, not only the transgenic hESCs but also the other hESC line cells, including KhES1, KhES2, and the parental KhES3 cells, did not attach to the laminin-coated dishes in any cases. HGF, BMP4, and FGF4 had little effect on the endodermal differentiation compared with the cases in the absence of growth factor on both Matrigel-coated dishes (Fig. 2A) and collagen type I-coated dishes (Fig. 2D). ATRA had no effects on endodermal differentiation whereas, in contrast, it was found to possibly inhibit endodermal differentiation (Fig. 2B). Activin A had the best effect of these growth factors on the Matrigel-coated dishes at the time point of day.
and the proportion of EGFP-positive cells was 19.5 ± 5.3% (Fig. 2C).

To find more effective protocols for hepatic differentiation, various methods using sequential or combined additions of growth factors were evaluated. Activin A or ATRA were added for the first 4 days, and then HGF, BMP4, or FGF4 were added for the next 5 days to the culture medium on Matrigel-coated dishes (Fig. 2E). The expression of EGFP was hardly observed in the

Table 2. Quantitative PCR primer sequences

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<tr>
<td>Oct-3/4</td>
<td>5'-TCTCCGCCGCTCCAGGT</td>
<td>5'-GCCCCACTCCAGCTGG</td>
</tr>
<tr>
<td>Nanog</td>
<td>5'-CTGGCTAGATGCCACAGG</td>
<td>5'-TGCTTTGGAGCTGGGAAG</td>
</tr>
<tr>
<td>T</td>
<td>5'-TGCTGGCTGGAGCCGAGTT</td>
<td>5'-GATGACTCCTTCTGTCCTG</td>
</tr>
<tr>
<td>Gsc</td>
<td>5'-GAGAGAAGTTGAGGTCTGTT</td>
<td>5'-CTCTGATAGGACCCCTTCCTG</td>
</tr>
<tr>
<td>AFP</td>
<td>5'-CACGCTCCAGTTGCTCTTCT</td>
<td>5'-AGCTTGGCTGGGAAGTTGA</td>
</tr>
<tr>
<td>Albumin</td>
<td>5'-GCCAGAAAACTGGATTGGAAGACGG</td>
<td>5'-ATGGAAGGTTGATAAGTTGACG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GAAGTGGTGGGAGGATGGTCACG</td>
<td>5'-GAAGATGGTGGGAGGATGGTCACG</td>
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Fig. 2. Expressions of EGFP-AFP after the induction of the endoderm differentiation by method 2. A: transgenic hESCs were cultured on Matrigel-coated dishes. Expressions of EGFP were measured 7 days and 14 days after the initiation of differentiation. A statistical analysis was performed by Dunnett’s test. The group with no added growth factors was used as the control group (n=3). Flow cytometric analyses revealed that the EGFP-positive proportion was 2.7 ± 2.7% with use of all-trans-retinoic acid (ATRA; B) and 19.5 ± 5.3% with use of activin A (C) on Matrigel-coated dishes at day 7. D: EGFP expression of the differentiated hESCs on the collagen type I-coated dishes. The statistical analysis was performed by Dunnett’s test (n=3). E: expression of EGFP by the sequential additional method. Flow cytometric analyses revealed that the EGFP-positive proportion was 29 ± 0.1% when ATRA was added for the first 4 days and then HGF was added for the next 5 days (F), and 21.7 ± 1.8% when activin A was added for the first 4 days and then HGF was added for the next 5 days (G) on the Matrigel-coated dishes. H: total cell numbers were compared between the group using activin A alone (dashed line) and the group using activin A and HGF (solid line). The statistical analysis was performed by Student’s t-test (n=3). *P < 0.01.
groups that were stimulated by ATRA initially, even if other growth factors were added later (Fig. 2F). The highest proportion of EGFP-positive cells (21.7 ± 1.8%) was obtained in the group that was differentiated first by activin A and then by HGF (Fig. 2G). The presence of BMP4 and/or FGF4, however, reduced the effects of activin A and HGF on endodermal differentiation. The converse order of activin A and HGF addition resulted in 6.4 ± 0.3% EGFP-positive cells. Although there was no statistical difference in the EGFP-positive proportion between the group that was stimulated by activin A alone and the group that was induced by activin A-HGF, an MTT assay revealed that a larger number of total differentiated cells was obtained in the activin A-HGF group, to a statistically significant degree (Fig. 2H). Taken together with these results, 10 undifferentiated hESCs were estimated to generate ~7.0 AFP-producing cells by using the protocol of the sequential addition of activin A and HGF on Matrigel-coated dishes, whereas 10 hESCs were estimated to generate ~2.3 AFP-producing cells when stimulated by activin A alone. Therefore, the protocol of the sequential addition of activin A and HGF on Matrigel-coated dishes produced higher yields of EGFP-positive AFP-producing cells than that of using activin A alone.

Characterization of the differentiated hESCs. To examine the characteristics of the hESCs differentiated by the protocol of the sequential addition of activin A and HGF on Matrigel-coated dishes, RT-PCR and immunocytochemical analyses were performed. RT-PCR showed that the expression of undifferentiated marker genes (Nanog and Oct-3/4) decreased in a time-dependent manner (Fig. 3A). The expression of goosecoid (a mesoderm maker) increased from day 5. Goosecoid is one of the markers for early gastrula organizer mesoderm, from which the definitive endoderm originates (22, 27). This mesoderm is often referred to as “mesendoderm,” and it is considered to contain a precursor of definitive endoderm (10, 17). AFP expressed highly at day 10. However, the mature hepatocyte markers (TAT, TO) either were weakly expressed or were not expressed at all. Immunostaining revealed EGFP and AFP to be expressed in the same cells (Fig. 3B). Sox17 was expressed in the nuclei of some EGFP-positive cells strongly at both day 5 and day 10 (Fig. 3C). Although both EGFP-positive
and EGFP-negative cells expressed E-cadherin at day 5, the expression of E-cadherin localized at the EGFP-positive cells at day 10 (Fig. 3D). Only a small portion of the EGFP-AFP-positive cells expressed albumin at day 5 and day 10 (Fig. 3E).

Characterization of the isolated EGFP-positive and EGFP-negative cells. To examine the characteristics of both the isolated EGFP-positive and EGFP-negative cells that were differentiated by activin A and HGF, both EGFP-positive and EGFP-negative cells were harvested at day 10 by use of a flow cytometer, and an RT-PCR analysis was performed on their RNA (Fig. 4). In the EGFP-positive cell fraction, the self-renewal undifferentiated markers (Nanog and Oct-3/4) were downregulated, and the expression of the early hepatic markers including GATA4 and AFP increased. The hepatocyte markers, including albumin and TO, showed a weak expression compared with the normal adult livers. Although the expression of goosecoid was weakly detected, brachyury, which displays a similar expression pattern to goosecoid, was not detected at all.

On the other hand, the expression of Nanog and Oct-3/4 were still detected in the EGFP-negative cell fraction. Although AFP was detected in the EGFP-negative fraction, its expression was extremely weaker than that of the EGFP-positive fraction. The hepatocyte-related genes including albumin, TAT, and TO were expressed at lower levels. Furthermore, the EGFP-negative cells expressed the mesodermal markers. It was therefore suggested that the EGFP-negative cell fraction included various cell types, such as undifferentiated ESCs.

Hepatic differentiation of other hESC lines. To investigate whether our endodermal differentiation method was effective for other hESC lines, both KhES1 and KhES2 were differentiated and their whole cultures were analyzed by qPCR (Fig. 5). The expression level of Oct-3/4 and Nanog decreased gradually. At day 5 when the addition of activin A was finished, the expression of mesodermal markers (goosecoid and brachyury) increased transiently. However, by day 10, their expressions disappeared in the presence of HGF. The hepatic markers of AFP and albumin were upregulated in a time-dependent manner. At day 10, the AFP expression showed a 22-fold increase in KhES1 and a 300-fold increase in KhES2. Approximately a 11-fold increase in KhES1 and a 30-fold increase in KhES2 in the albumin expression were observed. Therefore, these hESC lines were differentiated into hepatic endoderm cells via the mesendoderm-like cells, just as in the KhES3 line.

DISCUSSION

Human hepatocytes are useful not only for cell transplantation therapies but also for pharmacological studies. Human ESCs are one of the most promising cell sources, and some studies have reported that hESCs differentiated into hepatocyte-like cells after undergoing various procedures (2, 5, 6, 8, 16). In the present study, we aimed to compare the relative merits of ECMs and growth factors on the hepatic differentiation of hESCs.

Differentiated hESCs are heterogeneous and contain various cell types (4). To clarify the differentiated cells in the hepatic lineage, some cell markers are needed. We focused on AFP as an endodermal marker, because AFP is one of the most abundant serum proteins and it is produced by fetal hepatocytes (24). Human AFP promoter locates 0.3 kb 5′ upstream, and the AFP enhancer region is located ~4 kb 5′ upstream of the AFP gene (21, 33). The hybrid promoter of AFP enhancer/promoter that lacks any silencers in the 5′ flanking region of the AFP gene is reported to have a higher promoter activity than the AFP promoter region (0.3 kb) alone (30). Therefore, we generated the hESCs that expressed EGFP under the control of the AFP enhancer/promoter to increase their sensitivity to AFP-producing cells.

Activin has been shown to act as a mesoderm inducer in Xenopus (7). Activin A has also been demonstrated to play an important role in the definitive endoderm differentiation of both hESCs and mESCs (5, 15, 19, 35). Activin A alone induced ~20% AFP-producing cells in the study. However, the total number of differentiated cells did not increase in the presence of activin A alone. On the other hand, the sequential additions of activin A and HGF showed ~22% of the propor-
tion of AFP-producing cells, thus achieving the same level of the group that was differentiated by activin A alone. Moreover, a higher yield of AFP-producing cells was obtained. Neither the addition of HGF alone nor the reverse order of activin A and HGF had better effects on endodermal differentiation. Taken together with these findings, activin A might thus induce the production of early endodermal cells from undifferentiated hESCs. On the other hand, HGF may induce the proliferation of AFP-producing cells, while not specifying any endodermal fate for the hESCs. A recent article demonstrated that hESC's differentiated into hepatocyte-like cells with the proportion of 11.2% α1-antitrypsin-producing cells by forming EBs, using the hESCs that expressed EGFP under the control of α1-antitrypsin promoter (6). Another report showed that 80% cells expressed Sox17 by immunostaining (5). However, it would not be easy to compare the differentiation efficiency with previous studies because of different experimental systems. In previous studies, the mESCs were differentiated into AFP-producing cells in the presence of ATRA (12, 13). Unlike mESCs, hESCs did not commit to the hepatic lineage by the addition of ATRA. Therefore, there might be some differences between mice and humans regarding the differentiation mechanism of hepatic endoderm.

Because AFP is expressed not only in the fetal liver, which is a derivative of the definitive endoderm, but also in the extraembryonic endoderm, such as the yolk sac endoderm (10), it was therefore impossible to distinguish between the definitive and extraembryonic endoderm by using AFP. During gastrulation, the definitive endoderm is derived from the early gastrula organizer (node) of mesoderm cells (10, 17, 32). Goosecoid and brachyury are expressed in these mesoderm cells (1, 31). The definitive endoderm at the early stage is contiguous with the mesoderm-derived notochord and is, therefore, often termed “mesendoderm” (10). Mesendoderm cells are assumed to have bipotency to differentiate into a mesoderm and definitive endoderm (22, 27). The EGFP-positive cells differentiated in the protocols using activin A and HGF showed a transient mesodermal marker expression followed by graded increased expressions of the early endoderm markers. Moreover, E-cadherin expresses in the undifferentiated ESCs and then its expression specifies in the mesendoderm and endoderm (34), which were compatible with our results. Taken together with these findings, our findings suggest that the differentiated cells induced by activin A might be mesendoderm-like cells that possess an intermediate character, namely between mesoderm and endoderm, and that HGF might commit them to an early endoderm lineage, thereby causing them to proliferate. It was therefore also suggested that the hESC differentiation using both activin A and HGF on Matrigel-coated dishes might trace the normal developmental processes of definitive differentiation of hESCs.
endoderm (10, 27). Chemokine (C-X-C motif) receptor 4 (CXCR4) has been proposed as a marker for definitive endoderm (5, 35). However, the EGFP-AFP-positive cells did not express CXCR4 at any stage in the present study (data not shown). Because CXCR4 is expressed in not only the developing definitive endoderm but also in various tissues such as the bone marrow, thymus (20), and developing mesoderm (18), the expression pattern of CXCR4 is therefore expected to be different between the ESC lines.

Only a small number of the AFP-producing cells were positive for albumin, and they did not express any hepatic maturation indicators. Furthermore, they did not exhibit the ammonia removal activity (data not shown). Therefore, the exogenous stimuli by ECMs and growth factors might not induce the AFP-producing cells to mature into hepatocyte-like cells. We previously reported a coculture system to maturate murine hepatic progenitors and mESC-derived AFP-producing cells, using hepatic mesenchymal cells as the feeder layer (11, 12, 14). Therefore, we attempted to purify the AFP-EGFP-positive cells using a flow cytometer. However, it was difficult to isolate and culture them because of their decreased viability after the cell sorting. Further technical improvements will therefore be needed to purify the endoderm cells and promote their maturation into hepatocytes.

In conclusion, we successfully achieved a high proportion and high yield of AFP-producing cells on multiple hESC lines using our protocol of the sequential additions of activin A and HGF on Matrigel-coated dishes. The AFP-producing cells were supposed to be early definitive endoderm cells. Our findings may apply to the differentiation of induced pluripotent stem cells into hepatocytes (28, 29, 36) and will additionally contribute to the availability of hESC-derived hepatocytes with sufficient capabilities to be applied for use in both cell therapy and drug discovery research.

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