New primary culture systems to study the differentiation and proliferation of mouse fetal hepatoblasts

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Hepatoblasts are endodermal precursors in the liver that have the potential to differentiate into both hepatocytes and biliary epithelial cells. Signals from the cardiac mesoderm and septum transversum mesenchyme induce the ventral foregut endoderm to differentiate into hepatoblasts at embryonic day (E) 8 (4, 6, 10, 13, 26, 43). Hepatoblasts express α-fetoprotein (AFP) and albumin (Alb) and penetrate into the septum transversum mesenchyme (2, 28, 29). At midgestation, hepatoblasts surrounding the portal vein differentiate into biliary epithelial cells expressing cytokeratin 19 (CK19) (30-33). During late gestation and perinatal stages, hepatoblasts differentiate into hepatocytes, which produce a number of metabolic enzymes, including carbamoylphosphate synthetase I, glucose-6-phosphatase (G6Pase), tyrosine aminotransferase (TAT) and glycoconjugate synthase (Gys) (5, 7, 8, 11, 24, 25).

Some cytokines are known to regulate the proliferation and differentiation of hepatoblasts (17). Hepatocyte growth factor (HGF) produced by nonparenchymal cells promotes the proliferation of hepatoblasts and hepatocytes (12). The HGF receptor, c-met, is expressed in hepatoblasts and hepatocytes (35). Oncostatin M (OSM) is a member of the interleukin-6 (IL-6) family of cytokine, which also includes IL-11 and leukemia inhibitory factor. Mouse OSM signals through the OSM receptor (OSMR) and gp130 (14). It is known that OSM promotes the maturation of hepatoblasts (15, 18). However, whereas OSMR−/− mice display a normal liver phenotype (20), gp130−/− mice exhibit maturation of only a fraction of hepatocytes in vivo, although hepatocytes from gp130−/− mice do differentiate when cultured at high cell density (19). These findings suggest that OSMR/gp130 signaling is not sufficient for hepatocyte maturation and that this process thus requires another unidentified factor(s).

As shown recently, hepatoblasts have been isolated by using various cell-surface markers. The isolated cells have been shown to be able to proliferate and differentiate in vivo and in vitro. Suzuki et al. (39, 40) purified CD45+TER119−c-kit+CD49f+lowCD29− cells from E13.5 fetal mice liver using fluorescence-activated cell sorting (FACS). These cells included hepatic progenitor cells and lineage-limited cells and differentiated into hepatocytes and biliary epithelial cells in vitro and in vivo. Tamimizu et al. (34) showed that Dlk, a type I membrane protein, is a specific marker for hepatoblasts. Dlk+ cells from E14.5 mouse fetal liver were able to differentiate into both hepatocytes and biliary epithelial cells in vitro (42). Recently, E-cadherin has been shown to be a specific cell surface marker for hepatoblasts (22, 41). Nitou et al. (23) indicated that E-cadherin+ cells isolated from E12.5 mouse liver require coculture with cells of the E-cadherin-negative fraction, which includes endothelial and mesenchymal cells, for their survival and differentiation in vitro (23). Nierhoff et al. (21) showed that E12.5 E-cadherin+ cells transplanted into the spleen, differentiated into mature hepatocytes, and formed hepatic lobes. All of the culture conditions require the addition of both HGF and epithelial growth factor (EGF) for maintenance of hepatoblasts, suggesting that the previously reported culture conditions are not ideal for identifying factor(s) involved in the control of the proliferation and differentiation of hepatoblasts.

In this report, we aimed to establish a culture system for identifying cytokine(s) that are involved in the differentiation and proliferation of hepatoblasts. We purified hepatoblasts
from E12.5 fetal mouse liver by autoMACS by using the E-cadherin cell surface marker. The isolated E-cadherin+ cells were able to differentiate into hepatocytes or biliary epithelial cells in the CCl₄ and 4–4′-diaminodiphenylmethane (DAPM) models, respectively. We established new culture systems that permit the study of the mechanisms underlying the differentiation and proliferation of hepatoblasts. A low-cell-density culture system is suitable to identify factors that affect the proliferation of hepatoblasts and differentiation of hepatoblasts into biliary epithelial cells. A high-cell-density culture system is appropriate to identify factors involved in the differentiation of hepatoblasts into hepatocytes.

**MATERIALS AND METHODS**

**Mice.** Institute of Cancer Research strain (ICR) mice (Charles River, Kanagawa, Japan) were used for all experiments. Animals were maintained at a constant temperature of 20°C in 12:12-h light-dark cycle. All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and received approval from the Kumamoto University Animal Care and Use Committee.

**Isolation of E-cadherin+ cells from fetal liver.** E-cadherin+ cells were isolated by using an autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). E12.5 or E14.5 fetal mouse livers were digested with Dispase I (5 mg/ml; Roche, Basel, Switzerland) in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA) for 1 h at 37°C. The cell aggregates were dissociated into single cells by pipetting and filtered with a nylon mesh filter (pore size: 132 µm; Nihon Rikagaku Kikai, Tokyo, Japan). After filtration, the cell preparation was centrifuged at 800 g for 10 min. The pellet was washed with cell culture medium three times. After being centrifuged and washed in autoMACS running buffer [Tris-buffered saline solution (TBS) pH 7.6, containing 10 mM CaCl₂ and 0.5% BSA], the cell suspension was incubated with rat anti-mouse E-cadherin (clone ECCD-1) (Takara Biomedicals, Shiga, Japan) for 30 min at room temperature. Following incubation with ECCD-1 antibody, the cell suspension was washed and centrifuged with the running buffer. Liver cells were re-suspended in running buffer and incubated with goat anti-rat IgG microbeads (Miltenyi Biotec) for 15 min at 4°C. After being washed with the running buffer, the cells were centrifuged and suspended in autoMACS running buffer. The cell suspension was applied to the autoMACS, and E-cadherin+ cells were separated according to the manufacturer’s protocols. The proportions of E-cadherin+ cells in the E12.5 and E14.5 liver cell populations were 4.5 ± 0.3 and 5.3 ± 0.3%, respectively. In both cases, the Trypan blue dye exclusion test indicated that more than 85% of the E-cadherin+ cells were viable. Isolation of the E12.5 and E14.5 E-cadherin+ cells was performed 41 and 23 times, respectively, including preliminary experiments. Freshly isolated cells were used in each of the culture experiments.

**Transplantation of E-cadherin+ cells.** E-cadherin+ cells were isolated from E12.5 mouse by autoMACS (Miltenyi Biotec). The cells were labeled with PKH26 fluorescent dye (Sigma, St. Louis, MO), according to the manufacturer’s protocol. The PKH26-labeled E12.5 E-cadherin+ cells were re-suspended to a density of 1 × 10⁶ cells/ml in culture medium. The recipient mice were 4-wk-old female ICR mice (Charles River). Acute liver injury was induced in the recipient mice by subcutaneous injection of CCl₄ (2 ml/kg body wt; Wako Pure Chemicals, Osaka, Japan) dissolved in olive oil. Bile duct injury was induced in recipient mice by administration of DAPM (80–120 mg/kg body wt, Wako) by gavage (16). After 48 h of the administration of CCl₄ or DAPM, the PKH26-labeled E12.5 E-cadherin+ cells (1 × 10⁶ cells) were transplanted into the spleens of recipient mice under anesthesia. Recipient mice were euthanized 5 days after the transplantation. Three mice were used in each treatment group, and three independent replicate experiments were performed.

Negative control (nontransplanted) mice were euthanized 7 days after treatment with CCl₄ or DAPM. Three mice were used in each treatment group, and two independent replicate experiments were performed.

**Immunohistochemistry.** E-cadherin+ cells were collected on glass slides by cytocentrifugation. Cytocentrifugation was performed by using the Cytospin 3 (Shandon Scientific, Cheshire, UK) for 3 min at 400 rpm followed by air drying. Transplanted livers were fixed with 4% paraformaldehyde (PFA) (Sigma) in phosphate-buffered saline (PBS) at 4°C overnight and embedded in optimal cutting temperature compound (Sakura Finechemical, Tokyo, Japan). Frozen sections were prepared by using a cryostat. Frozen sections and cytosin specimens were fixed with acetone at −20°C for 10 min and allowed to air dry. For double immunostaining with Alb and AFP, cytosin specimens were fixed with 4% PFA in PBS at 4°C for 30 min and washed with PBS. Cells cultured on dishes were fixed with methanol at −20°C for 1 min. After fixation and air drying, nonspecific binding of antibodies was blocked with 10% normal goat serum or 10% normal monkey serum, depending on the source of secondary antibodies. The samples were incubated with primary antibodies overnight at 4°C. Monoclonal rat anti-mouse E-cadherin (clone ECCD-2) (Takara, Otsu, Japan; dilution 1:20), rabbit anti-mouse Alb (Biogenesis, Poole, UK; dilution 1:100), goat anti-mouse AFP (Santa Cruz Biotech, Santa Cruz, CA; dilution 1:100), mouse anti-human CK19 (Dako, Carpinteria, CA; dilution 1:100), mouse anti-human CK18 (Progen Biotechnik, Heidelberg, Germany; dilution 1:20), and rabbit anti-human desmin (Santa Cruz; dilution 1:100) were used as primary antibodies. After being washed in PBS, the samples were incubated with Alexa 488-conjugated goat anti-rat IgG, Alexa 488-conjugated goat anti-rabbit IgG, Alexa 488-conjugated goat anti-mouse IgG, Alexa 488-conjugated donkey anti-mouse IgG, Alexa 488-conjugated donkey anti-goat IgG (Invitrogen, Carlsbad, CA; dilution 1:500), 7-aminomethylcoumarin-3-acetic acid-conjugated donkey anti-rabbit IgG (Chemicon, Temecula, CA; dilution 1:50), Texas red-conjugated pig anti-goat IgG (EY Laboratories; dilution 1:150) or Cy3-conjugated goat anti-mouse IgG (Amersham Pharmacia Biotech, Piscataway, NJ; dilution 1:1000), and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Nacalai Tesque, Kyoto, Japan) for 2 h at room temperature. After being washed, the sections were viewed by using an Olympus IX50 or Olympus BX51 microscope. In all experiments, immunohistochemistry negative controls were prepared by omitting the antibodies from the first incubation step.

**Culture of E-cadherin+ cells.** The sorted E-cadherin+ cells were plated on 35-mm dishes coated with type I collagen (IWAKI, Chiba, Japan), type IV collagen, laminin, or fibronectin (BD Biosciences, San Diego, CA). Type I collagen-coated dishes were purchased from IWAKI. Mouse type IV collagen, mouse laminin, and human fibronectin were purchased from BD Biosciences and were coated at 5, 5, and 2 µg/cm², respectively, according to the manufacturer’s recommended protocols. Cells were plated at a density of 500 cells/cm² (low-cell-density system) or 4 × 10⁶ cells/cm² (high-cell-density system) in DMEM with 10% fetal bovine serum, penicillin, and streptomycin (Invitrogen). HGF (50 ng/ml; PeproTech, Rocky Hill, NJ) and/or EGF (20 ng/ml; R&D Systems, Minneapolis, MN) were added to the medium in certain cases.

In the high-density culture system, the culture medium was supplemented with mouse OSM (10 ng/ml, R&D Systems) and dexamethasone (Dex, 1 × 10⁻⁷ M, Sigma). During the culture period, cells were incubated at 37°C in an atmosphere containing 5% CO₂. The medium was changed after 1 and 3 days of culture.

**Plating efficiency assay.** E12.5 E-cadherin+ cells were plated at a density of 500 cells/cm² on 35-mm dishes coated with type I collagen, type IV collagen, laminin, or fibronectin and incubated for 10 h at 37°C in 5% CO₂. The cell cultures were then washed three times with PBS and fixed with 4% PFA in PBS at 4°C for 30 min. The fixed
cultures were washed in PBS and stained with Giemsa solution (Merck, Darmstadt, Germany). The number of adherent cells was counted, and the plating efficiency was calculated by using the formula (adherent cells plated cells) × 100 (%).

**RT-PCR.** Total RNA was extracted by using the SV total RNA isolation kit (Promega, Madison, WI) according to the manufacturer’s instructions with DNase treatment. Complementary DNA was synthesized from the total RNA by using Superscript II (Invitrogen) with oligo-dT12–18 primers (Invitrogen), according to the manufacturer’s instruction. The resulting cDNA was used as a template for PCR by using specific primers (Table 1) and Blend Taq (Toyobo, Osaka, Japan). The PCR conditions were as follows: initial denaturation at 97°C for 7 min, followed by 30 cycles of 97°C for 1 min, 60°C for 1 min, extension at 72°C for 1 min, and a final extension step of 7 min at 72°C. GAPDH was used as the internal control and was used to normalize the amount of cDNA.

**Periodic acid-Schiff staining analysis of cultured cells.** The cultured cells and transplanted liver sections were washed in PBS for 5 min three times after immunohistochemistry. Intracellular glycogen was stained with the periodic acid-Schiff (PAS) staining kit (Merck), according to the standard protocol.

**Statistical analysis.** All results were expressed as the means ± SE, and statistical significance was determined by Student’s t-test.

### RESULTS

**Characterization of E-cadherin-positive cells.** We evaluated the expression of E-cadherin in the cells of the E12.5 E-cadherin+ fraction. The cells in E-cadherin+ fraction were stained with E-cadherin (Fig. 1, A and B). We investigated the purity of the E12.5 E-cadherin+ fraction by immunocytochemical analysis of the hepatic and biliary epithelial cell marker, CK18, and the hepatic stellate cell marker, desmin. Double immunostaining with these two markers revealed that more than 97.5% of the cells in the E-cadherin+ fraction expressed CK18 (Fig. 1C), whereas less than 2.5% of these cells expressed desmin (Fig. 1D). Therefore, the purity was high enough for the analysis of the differentiation mechanisms of hepatoblasts.

To characterize the E12.5 E-cadherin+ cells further, we evaluated the expression of several makers, including hepatic cell markers (Alb and AFP), a biliary epithelial cell marker (CK19), and hepatocyte markers (TAT and G6Pase) by immunofluorescence and RT-PCR (Fig. 1, F–K). Immunofluorescence analysis showed that E12.5 E-cadherin+ cells were stained for Alb (Fig. 1F) but not CK19 (Fig. 1G). All Alb-positive cells within the E-cadherin+ fraction were stained for AFP (Fig. 1, I–J). Furthermore, Alb and AFP mRNA were strongly expressed in the E-cadherin+ cells (Fig. 1K). CK19 mRNA was not detected by RT-PCR (Fig. 1K). G6Pase and TAT, which are both expressed in hepatocytes, were not detected in the E-cadherin+ cells (Fig. 1K). Thus these results suggest that the E12.5 E-cadherin+ cells comprise predominantly hepatoblasts.

**Transplantation of E-cadherin+ cells.** To investigate whether E12.5 E-cadherin+ cells have the potential to differentiate into hepatocytes and biliary epithelial cells in vivo, we transplanted PKH26 (red fluorescence)-labeled E-cadherin+ cells into the spleens of recipient mice treated with CCl4 to induce liver damage or DAPM to induce biliary epithelial cell damage (16). PKH26 fluorescent dye is not transferred to surrounding cells but is transferred to daughter cells with no cell toxicity (data not shown) (3, 9).

Five days after implantation in CCl4-treated mice, the PKH26-labeled E-cadherin+ cells were found to surround the veins in the livers (Fig. 2, A and H, arrows). Clumps exhibiting red autofluorescence and immunological nonspecific bindings were seen in the transplanted liver (Fig. 2, A–C, asterisks). The clumps were also seen in both the immunohistochemical negative control (Fig. 2, H–J, asterisks) and the nontransplanted negative control (Fig. 2, E–G, asterisks).

The PKH26+ cells expressed Alb, as did the hepatocytes of the recipient mice, whereas the PKH26+ cells did not express CK19 (Fig. 2, A–C, arrows). Moreover, we performed PAS staining to examine glycogen synthesis and storage in the transplanted PKH26+ cells. The transplanted PKH26+ cells were stained for PAS (Fig. 2D).

In the DAPM-treated mice, we found the PKH26-labeled E12.5 E-cadherin+ cells in the host liver and the bile ducts. The PKH26+ cells formed a bile duct-like structure as shown in Fig. 2K. The transplanted PKH26+ cells expressed CK19 (Fig. 2M). In contrast, we did not observe Alb-positive cells in the PKH26+ cells (Fig. 2L). We did not detect nonspecific fluorescence in either the immunohistochemical negative control (Fig. 2, Q–S) or the nontransplanted negative control (Fig. 2, N–P). Our transplantation studies indicated that E12.5 E-cadherin+ cells have the potential to differentiate into hepatocytes and/or biliary epithelial cells in vivo.

**Proliferative potential of E-cadherin+ cells cultured at low cell density.** We first investigated plating efficiencies of E12.5 E-cadherin+ cells on various extracellular matrix (ECM) components. The E12.5 E-cadherin+ cells were plated at a low density (500 cells/cm²) on dishes coated with either type I collagen, type IV collagen, fibronectin, or laminin and cultured in DMEM +10% FBS in the absence of additional growth factors. These components of the ECM are known to affect the survival, growth, and differentiation of hepatoblasts, and these ECMs are present in the fetal liver (1, 38).

We counted the numbers of adherent cells in the type I collagen, type IV collagen, laminin, and fibronectin cultures 10 h after cells were plated at low density. Plating efficiencies of 5.9 ± 0.4, 3.1 ± 0.5, 3.9 ± 0.3, and 0.9 ± 0.1% were

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**Table 1. Primer sequences used for RT-PCR**

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<th>Gene Name</th>
<th>Strand</th>
<th>Primer Sequence</th>
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<tr>
<td></td>
<td>antisense</td>
<td>5′-cagcgcttgcaacagtga-3′</td>
</tr>
<tr>
<td>AFP</td>
<td>sense</td>
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</tr>
<tr>
<td></td>
<td>antisense</td>
<td>5′-gattccaggggtgcttc-3′</td>
</tr>
<tr>
<td>CK19</td>
<td>sense</td>
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</tr>
<tr>
<td></td>
<td>antisense</td>
<td>5′-cgcctgtttccatggta-3′</td>
</tr>
<tr>
<td>TAT</td>
<td>sense</td>
<td>5′-tccgctgctgtcaggtaa-3′</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>5′-tgtctgccgcatctacc-3′</td>
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<tr>
<td>G6Pase</td>
<td>sense</td>
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<td>antisense</td>
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</tr>
<tr>
<td></td>
<td>antisense</td>
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Alb, albumin; AFP, α-fetoprotein; CK19, cytokeratin 19; TAT, tyrosine aminotransferase; G6Pase, glucose-6-phosphatase; Gys, glycogen synthase; OSMR, oncostatin M receptor.
obtained from type I collagen, type IV collagen, fibronectin, and laminin, respectively (Each mean shows the average of 5 dishes in 3 independent experiments.). Moreover, all of the adherent cells were single cells at 10 h after plating.

Five days after plating at low cell densities, colonies of varying sizes were formed. To confirm that these colonies derived from single cells, we monitored adhering cells cultured on type I collagen-coated dishes. Each adherent cell prolifer-
Fig. 2. Transplantation of E12.5 E-cadherin+ cells into recipient mice. E12.5 E-cadherin+ cells were labeled with PKH26 dye and transplanted into CCl4-treated mice (A–J) or 4–4’-diaminodiphenylmethane (DAPM)-treated mice (K–M). The tissue sections of the recipient liver were subjected to observation of PKH26 fluorescence (A, E, H, K, N, and Q; red), double immunostained for Alb (B, F, I, L, O, and R; blue) and CK19 (C, G, J, M, P, and S; green), and subjected to periodic acid-Schiff (PAS) staining (D). A–D: in the CCl4-treated mice, the PKH26-labeled E-cadherin+ cells (arrows) were detected in the liver parenchyma 5 days after transplantation (A and H). The staining of the transplanted PKH26+ cells was positive for Alb (B, blue) and PAS (D) but negative for CK19 (C, green). E–G: in nontransplanted control mice, PKH26+ cells were not observed (E), but Alb (F) and CK19 (G and data not shown) expression could be detected in the liver and bile ducts. H–J: in the immunochemical negative control, specific fluorescence was not detected (I and J). Asterisks indicate clumps exhibiting red autofluorescence and nonspecific bindings for secondary antibodies (A–C and E–J). K–M: in the DAPM-treated mice, the PKH26+ cells formed bile duct structures at 5 days after transplantation (K). These cells expressed CK19 (M) but did not express Alb (L). N–P: in nontransplanted control mice, PKH26+ cells were not observed (N), but Alb (O and data not shown) and CK19 (P) expression could be detected in the liver and bile ducts. In the immunochemical negative control, PKH26+ cells formed bile duct-like structures (Q), but specific fluorescence of Alb (R) and CK19 (S) was not observed. CV, central vein; HD, hepatic duct. Bars: 50 μm.
ated to form a single colony (Fig. 3, B–F). Similarly, colonies in the laminin, fibronectin, or type IV collagen cultures also each derived from a single adherent cell (data not shown). Our data therefore indicate that an individual colony can be considered to originate from a single cell under conditions of low-cell-density plating.

To determine the proliferative potential of E-cadherin+ cells, we counted the number of colonies and the number of cells in each colony at 5 days after plating. The colonies were categorized into three groups according to their size: small, a colony containing 20–49 cells (4–5 generations); medium, a colony containing 50–99 cells (5–6 generations); and large, a colony containing over 100 cells (over 6 generations). E12.5 E-cadherin+ cells formed colonies of various sizes on fibronectin and type IV collagen and, in particular, on type I collagen (Fig. 3A). Few cells adhered to dishes coated with laminin, giving rise to only small and medium size colonies (Fig. 3A). Moreover, there were no obvious differences in the morphologies of cells cultured on type I collagen compared with other ECM components (Supplemental Fig. 1, A–D) or of cells in the three colony size groups (data not shown). (Supplemental information for this article can be found online at The American Journal of Physiology Gastrointestinal and Liver Physiology website.) These results suggest that type I collagen can support the efficient survival and growth of the E12.5 E-cadherin+ cells when cultured without additional growth factors and that the population of E12.5 E-cadherin+ cells included high proliferative potential cells.

We next examined the influence of HGF, EGF, and HGF + EGF on cell growth by using our low-cell-density culture system. Exposure of the cells to HGF or HGF + EGF induced their proliferation and increased the number of colonies in each group (Fig. 4A). Culture of the cells in the presence of HGF or HGF + EGF, but not EGF alone, significantly increased the numbers of medium and large-size colonies. In particular, HGF and HGF + EGF treatments induced a sevenfold increase in the number of large-size colonies (Fig. 4A). These results are consistent with the previous work by Suzuki et al. (39). Thus our low-cell-density culture system is suited to the assessment of the effect of various growth factors on the proliferation of hepatoblasts. In addition, there were obvious differences in the morphologies of cells grown in different growth factors (Fig. 4, B–E).

Characterization of E-cadherin+ cells at low cell density. We examined whether E12.5 E-cadherin+ cells were maintained as hepatoblasts or differentiated into hepatocytes or biliary epithelial cells when cultured on type I collagen in the absence of additional growth factors. Colonies that formed from E12.5 E-cadherin+ cells at low cell density were subjected to double immunofluorescent staining for Alb and CK19. We quantified the proportion (%) of colonies that contained Alb+CK19+ cells by using the formula (number of colonies containing Alb+ and Alb+CK19+ cells in each group/total number of colonies in each group) × 100. Five days after culture, most, if not all, colonies contained only Alb+ cells. All of the large colonies were composed solely of

Fig. 3. The proliferative potential of E-cadherin+ cells grown on extracellular matrix components. A: E12.5 E-cadherin+ cells cultured at low cell density (500 cells/cm²) on culture dishes coated with fibronectin (dark gray bars), type I collagen (white bars), type IV collagen (light gray bars), or laminin (black bars). Colony size was estimated by counting the number of cells per colony by use of DAPI staining after 5 days of culture. Data show the average of 15 dishes in 3 independent experiments. B–F: time-lapse phase contrast images of the cultured cells at 10 h (B and C), 34 h (D), 58 h (E), and 120 h (F) after plating on type I collagen are shown. C shows an enlargement of the boxed area in B. The single cell proliferated and formed a colony. Scratches were made on the bottom surface of the culture dish and were used for orientation when the cells were observed. Bars: 200 μm.
Alb+ cells (Fig. 5D). In contrast, only 1.4% of the small colonies and 2.9% of the medium-size colonies contained a few Alb+CK19+ cells within the colony (Table 2 and Fig. 5, A–C, arrowheads). In addition, the accumulation of glycogen was not observed in any of the cultured cells (data not shown). These data demonstrate that the majority of the colonies derived from the E12.5 E-cadherin+ cells were composed of undifferentiated hepatoblasts (Alb+CK19−) when cultured at low cell density.

It has been reported that over 60 and 20% of the colonies derived from Dlk+ and CD45−TER119−c-kit−CD49f+/low CD29− cells, respectively, expressed both Alb and CK19 when cultured with HGF + EGF (39, 42). We thus evaluated expression of Alb and CK19 in colonies of various sizes derived from E12.5 E-cadherin+ cells in the presence of HGF + EGF. One percent of the small colonies contained Alb+CK19+ cells in colonies of various sizes derived from E12.5 E-cadherin+ cells cultured in the presence of HGF + EGF. The number of cells in each colony was counted after 5 days. Data show the average of 15 dishes in 3 independent experiments. Dark gray bars, nongrowth factor; white bars, HGF; light gray bars, EGF; black bars, HGF + EGF. The P values were determined using the paired Student’s t-test. *P < 0.01. B–E: cell morphologies in a colony after 5 days of culture without additional growth factors (B), or with HGF (C), EGF (D), or HGF + EGF (E). Phase contrast images of medium-size colonies are shown. Bars: 200 μm.

**Differentiation potential of E-cadherin+ cells cultured at high density.** Hepatoblasts isolated from E14.5 mouse differentiate into hepatocytes when cultured at high density in the presence of OSM and Dex (15, 18). We evaluated whether the E14.5 E-cadherin+ cells could differentiate into hepatocytes when cultured at high cell density (4 × 10^4 cells/cm^2) in the presence of OSM and Dex for 5 days. To assess the hepatic maturation of the cultured cells, we observed glycogen accumulation by staining for PAS. Most of the cells cultured in the presence of OSM+Dex stored glycogen (Fig. 6B), whereas those cultured in the absence of OSM+Dex did not (Fig. 6A). These data indicate that E14.5 E-cadherin+ cells can differentiate into hepatocytes when cultured at high density in our condition.

We investigated whether E12.5 E-cadherin+ cells could differentiate into hepatocytes when cultured for 5 days at high cell density in the presence of OSM+Dex. We examined expression of OSMR and gp130 in the cultured E12.5 E-cadherin+ cells. The expression of OSMR and gp130 was detected in the cultured E12.5 E-cadherin+ cells in the absence and presence of OSM+Dex (Fig. 6E). To evaluate the hepatic maturation of the cultured cells, we investigated the accumulation of glycogen and expression of hepatocyte markers. Cultured E12.5 E-cadherin+ cells did not store glycogen (Fig. 6C) or express TAT and G6Pase when cultured without OSM+Dex (Fig. 6F). Cultured E12.5 E-cadherin+ cells weakly expressed TAT and G6Pase (Fig. 6F) but did not accumulate glycogen when cultured with OSM+Dex (Fig. 6D),
whereas E14.5 E-cadherin+ cells did store glycogen when cultured in the presence of OSM/Dex (Fig. 6B). Moreover, we examined expression of Gys. Gys plays a key role in initiation of glycogen synthesis and the accumulation of hepatic glycogen (7). In the fetal mouse liver, Gys expression was detected in E14.5 E-cadherin+ cells (11). We found that the Gys expression in the E14.5 E-cadherin+ cells was about three times higher than that in the E12.5 E-cadherin+ cells (Supplemental Fig. 2B). When cultured in the absence or presence of OSM/Dex, the expression of Gys was not detected (Fig. 6F).

These data indicate that OSM cannot fully induce E12.5 E-cadherin+ cells to differentiate into mature hepatocytes. These results suggest that OSM treatment is not sufficient for hepatic maturation and imply that unidentified factors could participate in the differentiation of mature hepatocytes.

**DISCUSSION**

**Cell sorting by autoMACS.** As shown previously, E-cadherin is specifically expressed in hepatoblasts (22, 23). Nitou et al. (23) isolated E-cadherin+ cells from E12.5 mouse liver by using Dynabeads and enriched E-cadherin+ cells to 98% purity. Nierhoff et al. (21) purified fetal liver epithelial cells (FLEC) from E12.5 livers by FACS sorting by using E-cadherin as the marker. The purity of the cells isolated was similar to that shown here by using autoMACS. Nierhoff et al. (21), Nitou et al. (23), and we demonstrated that E-cadherin+ cells possessed the characteristics of hepatoblasts. Therefore, E-cadherin is a useful marker for isolation of hepatoblasts at high purity.

**Table 2.** Percentage of colonies containing Alb + CK19+ cells in colonies of various sizes

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<th>Nongrowth Factor</th>
<th>HGF + EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small</td>
<td>1.4±0.5</td>
<td>1.0±0.5</td>
</tr>
<tr>
<td>Medium</td>
<td>2.9±1.5</td>
<td>1.5±0.6</td>
</tr>
<tr>
<td>Large</td>
<td>0</td>
<td>8.0±2.8</td>
</tr>
</tbody>
</table>

E-cadherin+ cells were cultured at low cell density on type I collagen in the presence or absence of hepatocyte growth factor (HGF) + epidermal growth factor (EGF). After 5 days, the cultured cells were immunostained for Alb, CK19, and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). The number of cells in each colony was counted by DAPI staining, and the colonies were categorized into 3 groups based on their colony size: small, a colony containing 20–49 cells (4–5 generations); medium, a colony containing 50–99 cells (5–6 generations); and large, a colony containing over 100 cells (over 6 generations). The percentage was calculated as follows: (number of colonies that contained both Alb+ cells and Alb+CK19+ cells in each group/total number of colonies in each group) × 100. Values are means ± SE.
Transplantation studies. We demonstrated that E12.5 E-cadherin+ cells differentiated into mature hepatocytes in mice subjected to liver injury. Tanimizu et al. (42) showed that transplanted Dlk+ cells differentiated into hepatocytes in the Jo1/Fas model. Transplanted CD45+TER119+c-kit+CD49flowCD29− and FLEC cells differentiated into hepatocytes, proliferated, and reconstituted the hepatic lobules in retroresin/CCl4-treated mice (21, 40). The results reported here are therefore similar to previous reports. However, we did not show that the transplanted cells formed hepatic lobules after 5 days of transplantation. Our method using the PKH26-labeling system does not allow long-term tracing of cells, so additional long-term follow-up examination is required.

Cultured CD45TER119c-kit−CD49floWCD29− cells formed bile duct-like structures in the spleen following transplantation to the spleen but not the liver (40). Transplanted BMEL cells and rat FLEC cells differentiated into biliary epithelial cells and formed intrahepatic bile ducts in the livers of the recipient mice (27, 36). In contrast, we have shown here that E12.5 E-cadherin+ cells differentiate into biliary epithelial cells and formed bile duct-like structure in the hepatic ducts following transplantation into the spleen. These structures may result from the aggregation of transplanted cells at the hepatic ducts, leading to the formation of bile duct-like structures. It is unclear at this time what may be giving rise to the differences in the location of transplanted cells in the recipient mice, although it may be due to the differences in the methods of biliary epithelial injury or the injected sites of engrafted cells.

Low-cell density culture. Recent studies have indicated that HGF, EGF, and several additional factors are required to maintain hepatoblasts in culture (23, 37, 40). The presence of HGF + EGF in the medium renders it difficult to assess the effects of other growth factors on hepatoblasts. We have demonstrated here that E-cadherin+ cells isolated by autoMACS can survive when cultured in the absence of additional growth factors at low cell density. Therefore, our culture
conditions may be more useful than other systems to evaluate the effect of various growth factors on hepatoblasts. On the other hand, Nitou et al. (23) showed that the survival of cultured E-cadherin + cells in vitro was dependent on factors derived from an E-cadherin-negative cell fraction that included endothelial and mesenchymal cells. It is unclear at this time what may be giving rise to this difference, although it may be due to differences in plating density or the cell sorting method.

ECM components affect the survival, growth, and differentiation of hepatoblasts (1, 38). We tested the growth of E-cadherin + cells on culture dishes covered with four different ECM components. The survival and proliferation of the E-cadherin + cells was greatest when grown on type I collagen and somewhat less so on type IV collagen. CD45 TER119 − c-kit CD49f low CD29 − cells exhibited high proliferation potential when cultured on laminin and type IV collagen (39, 40), and Dlk+ cells showed high proliferation potential on type IV collagen (42). Thus the optimal ECM component for hepatoblast proliferation may differ depending on the marker(s) used to isolate the hepatoblasts, although this remains to be investigated further.

It has been reported that purified hepatoblasts (Alb + CK19 − ) differentiate into Alb + CK19 + cells at a relatively high frequency when cultured at low density. Over 60% of colonies derived from Dlk + cells expressed both Alb and CK19 (42), and 20% of colonies derived from CD45 TER119 − c-kit CD49f low CD29 − cells expressed both Alb and CK19 (39). In contrast, hepatoblasts isolated by using the E-cadherin rarely differentiate into Alb + CK19 + and are maintained as undifferentiated hepatoblasts when cultured at low density in our culture conditions, regardless of the presence of HGF + EGF. In addition, E-cadherin + cells do not differentiate into Alb + CK19 + cells when cultured on type IV collagen or fibronectin, and colonies containing only Alb − CK19 + cells were observed when such cells were cultured in the presence or absence of HGF + EGF. These results indicate that the percentage of colonies containing Alb + CK19 + cells within the groups of colonies of each size was lower than that reported by others. Thus our low-cell-density culture system is suited to identify factors that can induce differentiation of hepatoblasts into biliary epithelial cells under low-cell-density conditions in the presence or absence of HGF + EGF.

It has been reported that 2.5% of the Dlk + cells and 0.3% of the CD45 TER119 − c-kit CD49f low CD29 − cells have a high proliferation potential (39, 42). In this study, we observed that 0.3% of plated E-cadherin + cells proliferated rapidly and therefore formed large colonies in the presence of HGF + EGF. The percentage of highly proliferating cells within the E-cadherin + cells is nearly identical to that observed for the CD45 TER119 − c-kit CD49f low CD29 − cells. In contrast, the percentage of cells with a high proliferative potential within the Dlk + cells was greater than that observed within the E-cadherin + cell population. Furthermore, 60% of the Dlk + cells and 20% of CD45 TER119 − c-kit CD49f low CD29 − cells differentiated into Alb + CK19 + cells, even though most of the E-cadherin + cells did not differentiate into Alb + CK19 + cells. It will thus be important to clarify the differences between the differentiation and proliferation potential of E-cadherin +, Dlk +, and CD45 TER119 − c-kit CD49f low CD29 − cells. In addition, we conclude that additional cell markers are necessary to enrich further the highly proliferative E-cadherin + cells. Toward this aim, we investigated the expression of Dlk in E-cadherin + cells by double immunochemistry. The percentage of E-cadherin + cells positive for Dlk was 62.7% (Supplemental Fig. 3). However, it is unclear whether the percentage of cells with high proliferative potential in the E-cadherin + Dlk + cell population is greater than the percentage of such cells in the E-cadherin + Dlk − cell population or whether E-cadherin + Dlk + cells have a greater tendency to differentiate into Alb + CK19 + cells than do E-cadherin + Dlk − cells. Finally, it will be necessary to evaluate whether the differentiation and proliferation potential of E-cadherin + Dlk + cells is similar to or different from that of E-cadherin + Dlk − cells.

High-cell density culture. In the E14.5 hepatoblasts, glycoen accumulation is induced by OSM, and maturation of hepatoblasts is promoted by OSM in the presence of Dex (14, 15). In high-cell-density culture, we found here that, in the presence of OSM-Dex, E14.5 E-cadherin + cells exhibited glycogen accumulation, but the E12.5 E-cadherin + cells did not (Fig. 6, A–D). What caused this difference in glyconeogenesis between E14.5 and E12.5 E-cadherin + cells?

These two populations were morphologically indistinguishable. Moreover, in cultured E12.5 E-cadherin + cells, OSMD and gp130 were expressed (Fig. 6F), and hepatocyte markers (TAT and G6Pase) were induced when cultured in the presence of OSM-Dex (Fig. 6F) like E14.5 hepatoblasts (14, 15), suggesting E12.5 E-cadherin + cells seem to have the latent potential to respond to OSM, as well as E14.5 E-cadherin + cells. In contrast, we found that there are obvious differences between the two populations. The E14.5 E-cadherin + cells weakly expressed TAT and G6Pase (Supplemental Fig. 2A), whereas the E12.5 E-cadherin + cells did not (Fig. 1K). Moreover, the amount of Gys in the E14.5 E-cadherin + cells was about three times higher than in the E12.5 E-cadherin + cells (Supplemental Fig. 2B). Totally, these results suggest that the differentiation potential of the E14.5 E-cadherin + cells is different from that of the E12.5 E-cadherin + cells. Probably, hepatic maturation of E12.5 hepatoblasts would require (an) unidentified signal(s) besides OSM signaling. Additional evidence will be necessary to prove this hypothesis. Our culture system using E-cadherin + cells derived from E12.5 and E14.5 may be of use to evaluate this hypothesis.

In conclusion, we have demonstrated that E-cadherin + cells isolated from the livers of E12.5 mice can be maintained as hepatoblasts in vitro and that they can differentiate into mature hepatocytes and biliary epithelial cells in vivo. Furthermore, our low-cell-density culture system may allow the identification of growth factors that regulate the proliferation and differentiation of hepatoblasts, and it may therefore be useful to gain a better understanding of the mechanism of liver development. We have found that exposure to OSM is not sufficient for differentiation of hepatoblasts into mature hepatocytes. These results suggest that (an) unknown factor(s) is/are involved in the process of hepatic maturation. The high-density culture system reported here may be useful to identify these unknown factors.

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REFERENCES