The effect of recombinant E-cadherin substratum on the differentiation of endoderm-derived hepatocyte-like cells from embryonic stem cells

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ABSTRACT

Generation of specific lineages of cells from embryonic stem (ES) cells is pre-requisite to use these cells in pre-clinical applications. Here, we developed a recombinant E-cadherin substratum for generation of hepatic progenitor populations at single cell level. This artificial acellular feeder layer supports the stepwise differentiation of ES cells to cells with characteristics of definitive endoderm, hepatic progenitor cells, and finally cells with phenotypic and functional characteristics of hepatocytes. The efficient differentiation of hepatic progenitor cells (approximately 55%) together with the absence of neuroectoderm and mesoderm markers suggests the selective induction of endoderm differentiation. The co-expression of E-cadherin and alpha-fetoprotein (approximately 98%) suggests the important role of E-cadherin as a surface marker for the enrichment of hepatic progenitor cells. With extensive expansion, approximately 92% albumin expressing cells can be achieved without any enzymatic stress and cell sorting. Furthermore, these mouse ES cell-derived hepatocyte-like cells showed higher morphological similarities to primary hepatocytes. In conclusion, we demonstrated that E-cadherin substratum can guide differentiation of ES cells into endoderm-derived hepatocyte-like cells. This recombinant extracellular matrix could be effectively used as an in vitro model for studying the mechanisms of early stages of liver development even at single cell level.

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1. Introduction

Embryonic stem (ES) cell-derived functionally mature hepatocytes may hold the key to replacing the cells lost in many devastating liver diseases [1–4]. Several recent studies have reported the differentiation of hepatocyte-like cells from human and mouse ES cells [5–8]. Most of the studies demonstrating hepatic differentiation from ES cells have been based on traditional culture techniques, such as embryoid body formation, aggregated colony formation on gelatin-coated plates or feeder layers. However, there are some problems with these current protocols, including spontaneous differentiation, low yield, the presence of undefined and xenogenetic compounds, necessity of cell sorting for specific lineages of cells, and considerable enzymatic stress during repeated culture [9,10]. Moreover, the cellular heterogeneity in the culture limits the diffusion of growth factors and nutrients from the culture medium and therefore, not suitable for differentiation particularly where frequent media changes are required [11,12]. The use of defined and selective culture condition considering biomaterial design can be an alternative to overcome these problems and to make the ES cell system practical for routine research and clinical applications.

Extracellular matrix (ECM), soluble factors and neighboring cells are most important effectors of stem cell behavior and function. Among them, cell–matrix interaction plays a fundamental role in regulating cellular differentiation [13,14]. The calcium-dependent cell–cell adhesion molecules, cadherins, are essential for intercellular adhesion, colony formation and the differentiation of ES cells [15–18]. The predominant cadherin of most epithelia, including endodermal cells and liver cells is E-cadherin. This transmembrane molecule plays an important role at the stage of hepatic differentiation, including stimulation of hepatic morphogenesis and hepatospecific maturation [15,16,19]. However, E-cadherin expression is restricted in mesodermal cells and early ectodermal cells [15,20,21], indicating essential relationship between E-cadherin and endoderm-derived hepatospecific differentiation. Moreover, mouse ES cell-derived hepatocyte developmental pathway involves
consistent expression of E-cadherin throughout all stages of differentiation including mesendoderm [22] definitive endoderm [20] and hepatic progenitor cells [15], indicates the promise of E-cadherin as a marker for the matrix-mediated enrichment of endoderm/hepatic cells.

In our previous studies, we established artificial ECM with a fusion protein of E-cadherin extracellular domain and IgG Fc region (abbreviated as E-cad-Fc) in maintaining pluripotency of ES cells without colony formation [11]. Here, we focused on the efficiency of E-cad-Fc-coated ECM to induce differentiation and enrich mouse embryonic stem (mES) cell-derived hepatocyte-like cells. The purpose of using E-cad-Fc-immobilized ECM has many folds, including efficient differentiation of cells at single cell level, development of defined culture condition, elimination of enzymatic stress on differentiating cells, and E-cadherin-dependent selective enrichment of cells at different stages of the developmental pathway.

2. Materials and methods

2.1. Preparation of E-cad-Fc-coated dishes

Expression and purification of E-cad-Fc fusion proteins and preparation of E-cad-Fc coated dishes have been described in detail elsewhere [11]. Briefly, E-cad-Fc fusion protein was generated using the E-cadherin extracellular domain cDNA from mouse E-cadherin full-length cDNA provided by the RIKEN BRC DNA Bank (code 1184), and mutated mouse IgG1 Fc domain cDNA (T252M/T254S). To prepare the E-cad-Fc-coated surface, 10 μg/ml purified E-cad-Fc solutions was directly added to non-treated polystyrene plates. After 2 h incubation at 37 °C, plates were washed with phosphate buffered saline (PBS) once, and then cells were seeded onto E-cad-Fc-coated plates. The characteristic single cell morphology was observed after 20 h of incubation at 37 °C.

2.2. Cell culture

For all cultures, feeder-free mouse embryonic stem cell line (EB), mouse primary hepatocytes and human hepatoma cell line HepG2 cells (RIKEN, Japan) were used. Mouse embryonic stem cells (mES) were maintained on 0.1% gelatin-coated dishes in Glasgow minimum essential medium (GMEM; Sigma–Aldrich), supplemented with 10% (v/v) fetal bovine serum (FBS), 1 mm l-glutamine (Millipore), 1% nonessential amino acids (Gibco, Invitrogen), 0.1% mouse δ-mercaptooethanol (Sigma Chemical), 1000 units/ml recombinant leukemia inhibitory factor (LIF; Chemicon), 50 μg/ml penicillin and 50 μg/ml streptomycin (nacalai tesque). mES cells were passaged every third day with daily media change for at least three passages (9 days) using Accutase (Millipore) prior to initiation of differentiation studies.

2.3. In vitro differentiation of mES cells

The differentiation medium was identical to that described above for ES cell culture medium except that FBS concentration was reduced to 1%, stage specific differentiation induction factors and 10% (v/v) knockout serum replacement (KSR; Invitrogen) were added and LIF was omitted. Three different combinations of growth factors, soluble factors and small molecules were used: optimized concentration (10 ng/ml) of activin A (R&D systems) in differentiation medium I, 10 ng/ml activin A and 50 ng/ml basic fibroblast growth factor (bFGF; Promega) in differentiation medium II, and 10 ng/ml hepatocyte growth factor (HGF, Sigma), 10 ng/ml oncostatin M (OSM, Sigma) and 1 μg dexamethasone (DEX, Sigma) in differentiation medium III.

The detailed method of differentiation is illustrated in Fig. 1. Briefly, before the induction of differentiation, mES cells were cultured on 0.1% gelatin-coated plates for 3 days. The cells were dissociated with Accutase before confluent, washed two times with PBS to remove LIF. 9000 cells were plated onto 35 mm E-cad-Fc-coated culture dishes in differentiation medium I. By convention, this time point was designated ”day 0” or “d0” post differentiation. After 3 days, the differentiating cells were further induced to endoderm cells in differentiation medium II for 3 more days. For further maturation, cells at this stage were dissociated with enzyme free cell dissociation buffer (CDB; Gibco, Invitrogen) and reseeded again onto 35 mm E-cad-Fc-coated dishes at a density of 9000 cells in differentiation medium III for another 2 weeks.

For optimization of activin A concentration to induce endoderm differentiation, varying concentrations of activin A were used for the first five days of differentiation (d1 to d5). The following concentrations of activin A were selected: 5, 10, 15, and 20 ng/ml. Control cultures without activin A were always grown in parallel.

For comparative study to conventional culture techniques, embryoid body (EB) and cells on 0.1% gelatin-coated dishes were induced to differentiate. For mES cells on 0.1% gelatin-coated matrices, cells were cultured for 3 days in presence of LIF to form tight shiny colonies and then switched into differentiation medium following the same protocol as discussed previously. Differentiation in embryoid bodies (EBs) was carried out using the hanging drop method. Briefly, cells on gelatin-coated dishes were dissociated with Accutase and diluted to 9.9x10^4 cells/ml in differentiation medium I; 30 μl drops were placed inside of a polystyrene petri dish lid. On day 3, three hanging drops containing embryoid bodies were transferred to each 35 mm 0.1% gelatin-coated dish and incubated for three more days in differentiation medium II. At day 7, media was switched to differentiation medium III for 2 more weeks. EBs without any added growth factors were used for spontaneously differentiated cells. Growth and changes in morphology were monitored daily. At different differentiation time points, cells were collected for the analysis of stage specific markers.

2.4. Culture of control cells

Mouse primary hepatocytes and hepatoma cell line HepG2 cells were used as control. Primary hepatocytes were isolated from male ICR (6–8 weeks) mice (SLC, Shizuoka) by the modified in situ collagenase perfusion method as described elsewhere [23]. The viable parenchymal hepatocytes were suspended in Williams’ E medium (Invitrogen) containing 10% (v/v) FBS and seeded at 3 x 10^4 cells/cm² in
2.5. Adhesion assays

For adhesion assay, cells were seeded on E-cad-Fc or gelatin substrate. After 24 h culture in a 96 well-plate, medium and non-adhered cells were removed, and washed three times with PBS. Adhered cells were then fixed with Mildform 20N (4% formaldehyde, pH 7.0—7.5; Wako Pure Chemical) for 15 min. After washing with PBS, cells were stained with 0.1% (w/v) Crystal Violet for 10 min and washed again. The absorbency at 570 nm was measured by using a micro-plate reader following incubation for 30 min in presence of 2% SDS (nacalai tesque). For inhibition experiment, cells were pre-cultured with monoclonal anti-mouse E-cadherin antibody (ECD-1; Takara) at 4 °C for 1 h and seeded to E-cad-Fc-coated plates.

2.6. Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen). RNA was reverse-transcribed into cDNA with an oligo-dt primer using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen). PCR was performed with TopTaq polymerase (Qigen) in PCR buffer containing 0.2 mM dNTPs (Takara). Primers and virus (M-MLV) reverse transcriptase (Invitrogen). PCR was performed with TopTaq polymerase (Qigen) in PCR buffer containing 0.2 mM dNTPs (Takara). Primers and PCR conditions used are listed in Supplementary Table 1. PCR products were resolved by 2% agarose gel electrophoresis and scanned by Typhoon 8600 Imager. The amount of mRNA for each marker was deduced from the PCR products using ImageQuant image analysis software (Version 5.2, Molecular Dynamics).

2.7. Flow cytometry

The cultured cells were harvested with cell dissociation buffer for E-cad-Fc matrix or with Accutase for gelatin-coated plates and analyzed. The dissociated 1 × 10⁶ cells/ml were resuspended in cold PBS with 2% FBS for 30 min to block nonspecific antibody binding. The cells were incubated with FITC-conjugated anti-mouse (BD Transduction Laboratories), goat anti-mouse Sox17 (V-20, Santa Cruz Biotechnology) rabbit anti-human s-fetoprotein (AFP; H-140, Santa Cruz Biotechnology), goat anti-mouse albumin (ALB, Abcam), rabbit anti-human ASGPR1/2 (FL-291, Santa Cruz Biotechnology). The samples were washed twice and resuspended in PBS buffer for analysis using EPICS XL flow cytometer (Beckman Coulter).

2.8. Immunofluorescence

Cells were fixed with Mildform 20N (8% formaldehyde) for 30 min and permeabilized with 0.2% Triton X-100 (nacalai tesque) for 5 min. Fixed cells were incubated with Image IT-FX signal enhancer (Invitrogen) in PCR buffer containing 0.2 μM dNTPs (Takara). Primers and PCR conditions used are listed in Supplementary Table 1. PCR products were resolved by 2% agarose gel electrophoresis and scanned by Typhoon 8600 Imager. The amount of mRNA for each marker was deduced from the fluorescent signal of PCR products using ImageQuant image analysis software (Version 5.2, Molecular Dynamics).

2.9. Western-blot analysis

The total cellular protein was extracted with lysis buffer (10 mM Tris—HCl, 150 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, 2 mM MgSO₄, and protease inhibitor cocktail; PH 7.4), and cell lysates were centrifuged at 15,000 g for 15 min at 4 °C. Samples were separated by electrophoresis on 7.5% polyacrylamide gels and electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). The primary antibodies were as follows: rabbit anti-mouse STAT5 (Cell Signaling Technology), rabbit anti-mouse pSTAT5 (Cell Signaling Technology), mouse anti-E-cadherin (BD Transduction Laboratories), rabbit anti-human N-cadherin (H-63, Santa Cruz Biotechnology), goat anti-mouse albumin (Abcam), and mouse anti-β-actin (Sigma). The membranes were then reacted with horseradish peroxidase (HRP)-conjugated secondary antibody (1: 10,000 dilution; Jackson Immunoresearch Laboratories) for 1 h. HRP activity was assessed using Immobilon Western detection reagents (Millipore) according to the manufacturer’s instruction.

3. Results

We previously established E-cad-Fc-immobilized extracellular matrix (ECM) in maintaining pluripotency of ES cells without colony formation. ES cells on this recombinant ECM showed higher proliferative ability, lower dependency on LIF, and higher transfection efficiency than in conventional culture conditions [11]. Here, we focused on the efficiency of E-cad-Fc-coated ECM to induce differentiation of mouse ES cell line (EB3) to hepatocyte-like cells. As previously reported, all undifferentiated cells on E-cad-Fc-coated plates showed scattering single cell morphology with the expression of Oct3/4, Nanog and E-cadherin.

3.1. Dosage effect of activin A signaling on endoderm differentiation

Hepatocytes are definitive endoderm-derived population. In recent studies, ES cells were differentiated to mesendoderm and subsequently to definitive endoderm cells by the treatment with activin [3,20,22]. However, the efficiency of endoderm production was lower without cell sorting and required relatively higher concentration of activin due to heterogeneous condition in EB culture system [5,24]. It was shown that homogeneous population of undifferentiated cells on E-cad-Fc-coated plates is less dependent on growth factors to maintain their undifferentiated state [11]. Considering this phenomenon, we optimized the concentration of activin A for endoderm cell differentiation on E-cad-Fc-coated plates. The scheme of ES cell differentiation into endoderm cells is illustrated in Fig. 1. 20—50 ng/ml activin A was most commonly used in mouse ES cell differentiation schemes [5,24]. Here, we selected four different concentrations of activin A (5, 10, 15 and 20 ng/ml) and monitored the relative expression of endoderm cell
Fig. 2. Differentiation of mES cells into endoderm cells on E-cadherin and gelatin substratum. Bright field microscopic image shows the morphological changes of mES cells (EB3) on 0.1% gelatinized or 10 μg/ml E-cad-Fc-coated surfaces on first (d1) and fifth (d5) days of differentiation. The most prominent morphological changes of EB3 cells on E-cad-Fc-coated surface were observed on day 5. Undifferentiated mES cells were used as control (A). By day 5, expression of endoderm markers, Sox17 (B) and Foxa2 (C) was monitored at different concentrations of activin A using RT-PCR analysis and relative expression was quantified using ImageQuant software. β-actin was used to normalize the expression level and differentiated cells on gelatin produced by embryoid body culture system were used for comparative study. Differentiated cells on day 5 were stained with Alexa Fluor 488 phalloidin (green) to observe F-actin. Immunofluorescence images showing cells with scattering distribution could express E-cadherin, and Sox17 at the same stage of differentiation. DAPI was used for nuclear staining (blue) (D). Flow cytometric analysis of differentiated ES cells for the definitive endoderm progenitor marker, CXCR4 (E). mES cells and differentiated cells at d1 and d5 could adhere to E-cad-Fc-coated dish as well as gelatin-coated surface after 24 h culture. Inhibition assay shows that in presence of E-cadherin neutralizing antibody (α-ECD) the cells can adhere on gelatin-coated surface but not on E-cadherin substratum. Blocking one was used as negative control (F). Scale bar: 50 μm.
markers on day 5. Exposure of mES cells to activin A (5–20 ng/ml) induced scattering single cell morphology on E-cad-Fc-immobilized matrix, which was not appeared in undifferentiated mES cells and spontaneously differentiated cells (Fig. 2A). Moreover, differentiated cells on 0.1% gelatin-coated plate demonstrated a clear heterogeneous population with elongated cells only at the surfaces of the colonies. We also analyzed endoderm cell differentiation using RT-PCR for Foxa2 and Sox17. Differentiated cells in the presence of 10 ng/ml activin A showed most efficient expression of Foxa2 and Sox17 (Fig. 2B,C). Therefore, in addition to unique changes in cell morphology, mES cell differentiation on E-cadherin substratum was advantageous with regard to reagent conservation. Compared to conventional culture systems, artificial acellular feeder layer allowed us to use 2–5 times less of this expensive growth factor during a 5 day experiment. Considering this dosage effect, we selected 10 ng/ml activin A for endoderm differentiation in next stages of this study.

3.2. Selective induction of endoderm gene and protein expression

Undifferentiated mES cells were exposed to optimized concentration of activin A (10 ng/ml) for three days to induce mesendoderm formation, and then were treated with bFGF (50 ng/ml) in the presence of activin A for another three days to induce definitive endoderm cells. To determine the ability of the E-cad-Fc-coated matrix to induce an endoderm phenotype, we cultured mES cells at a seeding density of 9000 cells per 35 mm tissue culture dish in low serum condition. As we mentioned previously, all the single cells on E-cad-Fc-coated matrix acquired a characteristic scattering morphology within 5 days of differentiation (Fig. 2A). To determine whether the cells on E-cad-Fc with scattering morphology express endoderm-specific markers, we performed immunofluorescence staining for the major endoderm transcription factors Sox17. We found that all most all cells with scattering morphology expressed Sox17 (Fig. 2D). To determine the relative fraction of these endoderm-like cells, we used flow cytometry, demonstrating that approximately 54% of the cells were positive for CXCR4 by day 5 of culture on E-cad-Fc, whereas only about 6% cells were positive on gelatin-coated plates (Fig. 2E).

E-cadherin is the key molecule for the attachment of differentiated cells on E-cad-Fc-coated plates. We confirmed the expression of E-cadherin at first and fifth days of differentiation by evaluating the ability of dissociated single cells to adhere on E-cad-Fc-coated plates (Fig. 2F). To further explore the possibility of E-cadherin-dependent attachment of differentiated cells, a neutralizing antibody targeting extracellular domain of E-cadherin (ECCD-1) was used to block the hemophilic interaction of E-cadherin present on substratum and cell surface (Fig. 2F). The attachment efficiency of mES cells on gelatin was not changed by the anti-ECCD-1 antibody, whereas both undifferentiated and differentiated cells failed to attach on E-cad-Fc-coated ECM in presence of E-cadherin neutralizing antibody, suggesting the E-cadherin-dependent differentiation of mES cells on E-cad-Fc-coated ECM. Immunofluorescence images, Western blotting and RT-PCR analysis further confirmed the presence of E-cadherin on activin treated endodermal cells (Figs. 2D, 3A–C).

![Fig. 3](image-url)

Fig. 3. The effects of different culture conditions on mRNA expression level of lineage genes on the differentiation of mES cells. mES cells cultured in embryoid body (EB) system or on gelatin and E-cad-Fc extracellular matrix were treated with activin A (10 ng/ml) and bFGF (50 ng/ml). The mRNA expression level of E-cadherin and N-cadherin was measured on 3 and 5 days of differentiation (A). Markers for primitive (Oct3/4), early ectoderm (Sox1), mesendoderm (Brachyury, Goosecoid), endoderm (Sox17, Fox2, Gata6), and mesoderm (Gata1) were used for transcription analysis (B). Western-blot analysis showing protein expression level of E-cadherin and N-cadherin, and phosphorylation level of STAT3. The protein expression level was normalized using β-actin (C). Semi-quantitative RT-PCR was used to evaluate the time point of maximum expression of transcripts of genes expressed in endoderm cells on E-cad-Fc- and gelatin-coated plates (D). Gene expression was measured by RT-PCR and quantified using ImageQuant software by measuring the fluorescent intensity of amplified bands (E). The expression level was normalized using housekeeping gene, β-actin or GAPDH. Abbreviation: ES, embryonic stem cell; PH, primary hepatocyte; G, gelatin-coated matrix; E, E-cad-Fc-coated matrix.
To determine particular gene expression pattern of mesendoderm and definitive endoderm, we studied differentiated populations on two different days (d3 and d5) using RT-PCR. Spontaneously differentiated cells in embryoid body RNA was used as positive control for all primers. We detected mesendoderm (Goosecoid, Brachyury, E-cadherin and N-cadherin) and endoderm (E-cadherin, Foxa2, Sox17 and Gata6) but not ectoderm (Sox1) and mesoderm (Gata1) genes in differentiated cells on E-cad-Fc-coated matrix (Fig. 3A,B) suggesting the selective induction of differentiation on E-cad-Fc-coated matrix. In accordance with previous reports, differentiated cells in EB condition or on gelatin-coated matrix expressed transcripts for Sox1 and Gata1[5,25]. Compared to these two conventional culture systems, the relative gene expression pattern of differentiated cells on E-cadherin substratum showed most efficient expression of mesendoderm and endoderm genes. Moreover, with differentiation time the rapid decrease of STAT3 activation and Oct3/4 expression with onset of N-cadherin expression suggests the less chance of contamination of endoderm cells with ES cells on E-cad-Fc matrix (Fig. 3A–C).

We also determined the time course of Sox17 and Foxa2 for 3, 5, 6, 10 and 15 days of differentiation. mES cells and primary hepatocytes were used as control. By 5 and 6 days of differentiation, the highest expression was observed for Foxa2 and Sox17, respectively, and the expression was decreased afterwards suggesting the differentiation is closely related with the normal developmental pathway for hepatocytes (Fig. 3D,E). The expression of Sox17 and
Foxa2 on E-cad-Fc-coated matrices kept higher than on gelatin throughout all stages of differentiation.

3.3. Generation and enrichment of hepatic progenitor cells

In the next stage of differentiation, we used HGF, OSM and DEX to promote the generation of early hepatic cells. In previous studies, it was shown that endoderm and hepatocytes showed consistent expression of E-cadherin on their cell surfaces, whereas ectoderm and mesoderm cells were lack of this surface molecule [22,24]. To enrich the relative fraction of hepatic progenitor cells and to maintain the single cell scattering morphology under homogeneous condition we dissociated the cells on 7 days of differentiation using enzyme free cell dissociation buffer (CDB, Invitrogen) that minimize the enzymatic disruption of E-cadherin [26]. To determine whether the cells on E-cad-Fc with scattering morphology expressed hepatic progenitor markers, we performed immunofluorescence staining for the major early hepatic progenitor marker AFP on day 9. We found that nearly all cells (96%) with scattering morphology expressed AFP (Fig. 4A), an indication of matrix mediated selective enrichment of hepatic progenitor cells. These cells also showed its potentiality to produce ALB at next stages of differentiation (Fig. 4A). To quantify the level of cell–matrix interaction of AFP-positive cells after repeated culture, subcellular localization of E-cadherin was detected on 9 days of differentiation (Fig. 4B). Immuno-fluorescence using antibodies specific for E-cadherin revealed no AFP-expressing cells lacked E-cadherin labeling. This result was confirmed by repeated tests using laser scanning confocal microscopy (Fig. 4C). The representative
images were evaluated based on the accumulation of E-cadherin in AFP-positive cells (co-expression index; CEI). It was found that approximately 98% AFP-positive cells also expressed E-cadherin on their surfaces (Fig. 4D). Day 5 and 15 were selected to determine the relative fraction of immunoreactive cells for Sox17 and ALB, respectively. Fig. 4E shows that relatively higher proportion of differentiated cells on E-cad-Fc-coated plates were positive for Sox17 (approximately 93%) and ALB (approximately 92%), whereas very small fraction of populations were positive for Sox17 (approximately 11%) and ALB (approximately 21%) on gelatin-coated plates.

To determine whether the mES cell-derived hepatic progenitor cells have typical markers of hepatic progenitor cells and distinguish the phenotypes from ES cells, we evaluated E-cadherin, AFP, ALB, hepatic nuclear factor-4α (HNF-4α), and cytokeratin 18 (CK18) by RT-PCR. The highest expression of AFP was found on day 12 with gradual decrease at next stages of differentiation (Fig. 5A,B). The expression of HNF-4α was elevated on 15 days of differentiation and kept increasing on late stages of differentiation (Fig. 5D,E). Although most profound CK18 transcription level was found on d9 for mES cells cultured on E-cad-Fc, the expression was decreased at 12 days of differentiation and kept constant afterwards, which was much more comparable with the CK18 transcription level of primary hepatocytes (Fig. 5F). Compared to differentiated cells on gelatin-coated plates, the transcription level of all hepatic progenitor marker genes was higher on E-cad-Fc-coated matrix. mES cells, primary hepatocytes, and HepG2 cells were used as control.

### 3.4. Differentiation of hepatic progenitor cells into hepatocyte-like cells

To promote the maturation of hepatic progenitor cells into hepatocyte-like cells, we continued differentiation under HGF, OSM and DEX containing conditions up to day 24. The cell growth and morphology of differentiated cells were monitored every day. In
contrast to the cells on gelatin-coated plates, the differentiated cells on E-cad-Fc showed efficient expression of ALB and asialoglyco-protein receptor (ASGPR) at protein level (Fig. 6A). With the exception of AFP expression, RT-PCR analyses revealed that the expression of genes characteristic of hepatocytes, including HNF-4a, CK18, N-cadherin, E-cadherin, ALB, ASGPR and TO was increased in the differentiated hepatocyte-like cells comparing with the hepatic progenitor cells on day 21 (Figs. 5E,F and 6B).

PAS reaction was performed to evaluate the capability of glycogen storage in the differentiated hepatocytes. Glycogen storage is an important metabolic function of hepatocytes, which was manifested as the accumulation of dark red staining in the cytoplasm of cells. Our results demonstrated that by 21 days of differentiation, ES-derived hepatocytes had an accumulation of cytoplasmic deposits with a dark red color (Fig. 6C), as compared with that of spontaneously differentiated cells indicate that the mES cell-derived hepatocyte-like cells have the ability to accumulate glycogen.

Furthermore, immunofluorescence staining (Fig. 7A) and statistical analyses (Fig. 7B,C) were conducted to measure the extent of morphological similarities among mES cell-derived hepatocyte-like (mES-H) cells and mouse primary hepatocytes (PH) on E-cad-Fc and gelatin-coated matrix. By day 24, the spreading area and bipolarity index (BI) were assessed. The mES-H cells on E-cad-Fc-coated surfaces showed larger spreading area (168 ± 6.38 μm²) and BI (approximately 1.7) which were more comparable to cell area (135 ± 14 μm²) and BI (approximately 1.4) of PH on E-cad-Fc-coated surfaces (Fig. 7B,C). In contrast, mES-H cells on gelatin-coated ECM formed aggregated cells with scattering distribution on the surrounding of colonies (Fig. 7A). The single spreading cells on the edges of colonies were selected for the measurements of spreading area (58 ± 18 μm²) and BI (approximately 2.6), which showed significant differences to PH on gelatin-coated surfaces (area: 243 ± 20 μm² and BI: approximately 1.5) indicate the evidence of higher percentage of hepatocyte-like cells on E-cad-Fc-coated matrices.

4. Discussion

Preparation of specific lineages of endoderm-derived hepatocytes at high purities from embryonic stem (ES) cells is a complex and dynamic process and requires both selective culture conditions and markers to guide and monitor the differentiation. Therefore, the generation of a simple ES cell culture system that could address fundamental questions, without the need for complex medium formulation or cell sorting, is a major goal in the field. In this study, we established a new culture system involving a marker (E-cadherin) to guide the mouse ES cells towards homogeneous population of endoderm-derived hepatocyte-like cells. Our work demonstrates that an endoderm-like cell population can be induced by culture on E-cad-Fc-coated extracellular matrices, without any requirement for enzymatic treatment, higher concentration of complex serum or activin supplements, or serial cell sorting, which were previously thought to be essential for endoderm induction. Moreover, we successfully induced endoderm cells under single cell level that showed its potentiality to differentiate into hepatocyte-like cells.

Previously, we demonstrated that mouse ES cells cultured on E-cad-Fc-coated surface could maintain unique single cell morphology and complete ES cell features [11]. The single cell morphology might be due to reduced Rho-ROCK signaling which was...
observed for undifferentiated state of mES cells [27,28]. Since F9 cells differentiate into two different endoderm derivatives by addition of all-transretinoic acid [29,30], and parietal endoderm derivatives also exhibit scattering morphology, we checked the differentiation status of mES cells with scattering morphology on E-cadherin substratum. Prominent morphological differences from mES cells and expression of endoderm marker genes suggest that the cell scattering activity was correlated with differentiation in presence of activin/bFGF (Fig. 2A–D). Although we did not compare the actual amount of activin A interacting with cells, we found that a reduced amount of activin A was required for induction of definitive endoderm differentiation on an E-cad-Fc-immobilized surface, probably due to homogeneous exposure to activin A that was achieved in this culture system (Fig. 2B,C) [31].

In this study, we also found consistent expression of E-cadherin throughout all stages of differentiation pathway. These results are consistent with previous reports describing the E-cadherin expression pattern during mouse and human liver development [15]. In the mouse liver, E-cadherin is expressed during the fetal stage and continues to be expressed in adult hepatocytes. In addition, E-cadherin is uniquely expressed in hepatic endoderm cells and undifferentiated mES cells. Therefore, compared with other reported surface markers of fetal liver such as N-cadherin, which is not expressed in undifferentiated ES cells (Fig. 6B), E-cadherin can be used as a surface marker for mES cell differentiation to hepatocyte-like cells to exclude ectoderm and mesoderm cells. Considering this phenomenon, E-cad-Fc-immobilized ECM would be suitable for culture and differentiation of mES cells and to achieve matrix mediated selective enrichment of hepatic endoderm cells without any necessity to introduce new culture matrix throughout all stages of differentiation. In accordance with this hypothesis we found selective induction of endoderm cell differentiation with absence of early ectoderm and mesoderm markers (Fig. 3A,B). Since, attachment of cells on E-cad-Fc is Ca$^{2+}$-dependent hemophilic interaction between E-cadherin molecules, we used enzyme free chelating agent (EDTA containing cell dissociation buffer, CDB) for single cell dissociation to keep E-cadherin on the cell surface intact [26] and re-cultured the cells on E-cad-Fc matrix in order to exclude remaining mesoderm or ectoderm cells which lack E-adherin [22]. In contrast to conventional culture condition, we found that after reseeding approximately 94% cells on E-cad-Fc-coated surface expressed AFP. Moreover, E-cadherin expression specifically matched AFP expression after the generation of hepatic endoderm cells with very high co-expression index (nearly 98%) (Fig. 4D).

After expansion, hepatic endoderm cell cultures could undergo differentiation into hepatic progenitor cells that expressed AFP, ALB and HNF-4$\alpha$ but not ASGPR or TO (Fig. 5A–D). Late stage of differentiation for hepatocyte-like cells showed stored glycogen, expression of ALB, HNF-4$\alpha$, TO, ASGPR, E-cadherin and N-cadherin (Fig. 6). We utilized the advantages of single cell morphology on E-cadherin substratum and showed that more of these hepatocyte-like cells had higher similarity to primary hepatocytes than cells on gelatin-coated surfaces (Fig. 7).

5. Conclusions

We successfully established E-cadherin substratum for selective differentiation and enrichment of hepatocyte-like cells. This recombinant substratum facilitated the generation of homogeneous population of differentiated cells without any enzymatic stress for dissociated single cells. Moreover, this matrix can also be used as a tool to confirm the important role of E-cadherin in cell survival, proliferation and differentiation. In addition, the single cell scattering morphology will facilitate the studies of the molecular mechanisms of hepatic stem/progenitor cell origin, self-renewal and differentiation in vitro. Further studies along this differentiation scheme will provide additional insights to monitor into complex regulatory networks at every stages of differentiation even at single cell level.

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Appendix

Figures with essential color discrimination, Figs. 3 and 5 in this article are difficult to interpret in black and white. The full color images can be found in the online version, at doi:10.1016/j.biomaterials.2010.11.045

Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.biomaterials.2010.11.045

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