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Original article

A heparin-modified thermoresponsive surface with heparin-binding epidermal growth factor-like growth factor for maintaining hepatic functions *in vitro* and harvesting hepatocyte sheets

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ABSTRACT

A heparin-modified thermoresponsive surface bound with heparin-binding epidermal growth factor-like growth factor (HB-EGF) was designed to allow creation of transferrable and functional hepatocyte sheets. A heparin-modified thermoresponsive surface was prepared by covalently tethering heparin onto poly(*N*-isopropylacrylamide-*co*-2-carboxyisopropylacrylamide)-grafted tissue culture polystyrene surfaces (Heparin-IC). HB-EGFs were able to stably bind to heparin-IC via affinity interaction. The survival of primary rat hepatocytes was maintained through HB-EGF-bound heparin-IC (HB-EGF/heparin-IC). Moreover, cultured rat primary hepatocytes on HB-EGF/heparin-IC exhibited higher albumin-secretion than hepatocytes cultured on PIPAAm-grafted and collagen-coated surfaces with soluble HB-EGF in the culture medium, regardless of whether soluble EGF was added. These results suggested that HB-EGF/heparin-IC is able to effectively maintain hepatic function via continuous signaling of HB-EGF. After a 4-day cultivation, the cultured hepatocytes on HB-EGF/heparin-IC detached as a cell sheet with fibronectin and HB-EGF only after the temperature was lowered to 20 °C. In addition, higher expression of hepatocyte-specific genes (albumin, hepatocyte nuclear factor 4 alpha, coagulation factor VII, and coagulation factor IX) in hepatocyte sheets was detected on HB-EGF/heparin-IC than on a PIPAAm surface with soluble HB-EGF, indicating that HB-EGF/heparin-IC suppressed the dedifferentiation of cultured hepatocytes. Hence, heparin-modified thermoresponsive surfaces bound with HB-EGF facilitate the fabrication of transferrable hepatocyte sheets with intact hepatic functions and have the potential to provide an *in vitro* culture system using functional hepatocyte sheet tissues, which may serve as an effective hepatocyte-based tissue engineering platform for liver disease treatments.

Abbreviations: IPAAm, *N*-isopropylacrylamide; CIPAAm, 2-carboxyisopropylacrylamide; TCPS, tissue culture polystyrene dishe; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; HB-EGF, heparin-binding EGF-like growth factor; PIPAAm, poly(*N*-isopropylacrylamide) on TCPS; IC, poly(*N*-isopropylacrylamide-*co*-2-carboxyisopropylacrylamide) on TCPS; heparin-IC, heparin-modified IC; HB-EGF$_X$/heparin-IC, HB-EGF-bound heparin-IC; PIPAAm + HB-EGF$_Y$, PIPAAm with soluble HB-EGF; NHS, *N*-hydroxysuccinimide; EDC, 1-ethyl-3-(3-dimetylaminopropyl)-carbodiimide hydrochloride; MES, morpholinoethanesulfonic acid monohydrate; ECM, extracellular matrix; DMEM, Dulbecco's modified Eagle's medium; PBS, Dulbecco's phosphate buffered saline; EDTA, trypsin/ethylenediaminetetraacetic acid; FBS, fetal bovine serum; Alb, albumin; Hnf4α, hepatocyte nuclear factor 4 alpha; F7, coagulation factor VII; F9, coagulation factor IX; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription polymerase chain reaction.

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1. Introduction

Cellular responses including survival, division, differentiation, and apoptosis are regulated by extracellular signaling molecules via receptors on the cellular membrane [1]. Stimulation with the extracellular signaling molecules such as extracellular matrices (ECMs), growth factors, and cytokines triggers intracellular signal transduction and subsequent cellular responses. In conventional cell culture method, soluble signaling molecules including growth factors and cytokines are supplemented in culture medium. However, the frequent dosing of the soluble molecules is required for maintaining the stimulation of cells because of the down-regulation through the endocytosis of the receptors [2]. Covalent immobilization of growth factors and/or ECMs on cell culture surfaces facilitates the long-term stimulation of the receptors without the down-regulation of the receptors [2,3], leading to improved cell growth and maintenance of differentiated state. However, isolation and passage processes using enzymatic treatments such as trypsinization may induce irreversible damage of the receptors, resulting in the reduction of cellular functions [4].

In order to harvest cultured cells nonenzymatically with maintaining cellular functions, our laboratory reported a new type of stimuli-responsive surface that was co-immobilized with ECMs and growth factors [5]. Recently, we designed thermoresponsive poly(*N*-isopropylacrylamide) (PIPAAm)-grafted surfaces modified with heparin molecules for switching of sustained stimulation and detachment of cultured cells [6,7]. Heparin has affinity interactions with bioactive molecules such as growth factors, ECMs, and protease [8]. Immobilized heparin on shrunken PIPAAm chains at 37 °C was able to bind basic fibroblast growth factor (bFGF), a heparin-binding growth factor. This heparin-modified surface stabilizes and reinforces the formation of a complex between immobilized heparin and bFGF, thus leading to sustained stimulation of cultured fibroblasts by bFGF and rapid formation of a fibroblast sheet. At 20 °C, by contrast, bound bFGF were released with the cultured fibroblast sheet due to the dynamic motion of heparin accompanied with the swelling of hydrated PIPAAm chains. Thus, the switch from cell proliferation to cell detachment occurred only after the temperature was changed [6].

In present paper, we focused on the cultivation of hepatocytes on the heparin-modified thermoresponsive surfaces. We have previously succeeded in the fabrication and subcutaneous transplantation of hepatocyte sheets [9,10]. However, it is difficult to culture functional primary hepatocytes because the hepatocytes rapidly lose their specific function as the cultivation time is increased. In previous studies, the long-term maintenance of hepatic morphologies and functions have been achieved by co-culturing with nonparenchymal cells [11] and using ECMs such as collagen [12] and Matrigel [13]. However, the conventional culture systems for hepatocytes are not suitable for manipulation and transplantation. In contrast, co-culturing of hepatocyte sheets with nonparenchymal cells, including endothelial cells, on thermoresponsive cell culture surfaces with two-dimensional micropatterning [14] or three-dimensional layering techniques [15] facilitates both the transfer and the functional maintenance of hepatocytes. One of the reasons for this maintenance of hepatic functions is considered to be the continuous stimulation of cellular signaling by bioactive molecules containing growth factors, which are continuously secreted from nonparenchymal cells. If the signaling for the activation of hepatocytes were to continuously be supplied from immobilized growth factors on thermoresponsive cell culture surfaces, the cultured hepatocyte sheet would be able to effectively maintain its functions. In addition, the creation of transplantable hepatocyte sheets with high hepatic functionality would lead to an improvement in the quality of cell-sheet based therapies for liver diseases.

The purpose of this study was the development of a functional thermoresponsive cell culture system that maintains hepatocyte specific functions without non-parenchymal cells and allows for the recovery of a cell sheet. Under typical culture conditions of hepatocytes and their sheets, the addition of soluble EGF, a potent hepatocyte mitogen [16,17], to the culture medium is essential for hepatocyte survival. Here, we utilize affinity-immobilization of heparin-binding epidermal growth factor-like growth factor (HB-EGF), which has the ability to bind to heparin with a specific affinity interaction [18–20] on a heparin-modified thermoresponsive surface for the maintenance of hepatic function. HB-EGF activates EGF receptors (ErbB1 and ErbB4) and, similarly to EGF, acts as a potent hepatocyte mitogen [21–24]. In this study, rat primary hepatocytes were cultured on a heparin-modified thermoresponsive surface with HB-EGF, and the hepatic function was investigated. To demonstrate the effectiveness of affinity-bound HB-EGF in a hepatocyte culture, two culture conditions were examined as follows: 1) hepatocytes on an HB-EGF-bound heparin-modified thermoresponsive surface (Fig. 1a) and 2) hepatocytes on a thermoresponsive surface in a culture medium containing heparin and soluble HB-EGF (Fig. 1b). The albumin secretion of hepatocytes under each condition was determined by enzyme-linked immunosorbent assay (ELISA). After a 4-day cultivation, the hepatocytes were detached as a cell sheet from each of the surfaces. To evaluate the cell function of each hepatocyte sheet, several hepatic mRNA expression levels were determined by quantitative reverse transcription polymerase chain reaction (RT-PCR) analyses.

2. Experimental section

2.1. Chemicals and materials

N-Isopropylacrylamide (IPAAm) was obtained from Kohjin (Tokyo, Japan) and purified by recrystallization from *n*-hexane. 2-Carboxyisopropylacrylamide (CIPAAm) was synthesized using the method described previously [25]. Tissue culture polystyrene dishes (TCPS) (Nunc™, culture area: 8.8 cm^2) were purchased from Thermo Fisher Scientific (Roskilde, Denmark). *N*-Hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimetylaminopropyl)-carbodiimide hydrochloride (EDC), Triton-X 100, 2-propanol, dexamethasone, and insulin were purchased from Wako Pure Chemicals (Osaka, Japan) and used as received. 2-Morpholinoethanesulfonic acid monohydrate (MES) was purchased from Dojindo (Kumamoto, Japan). Heparin sodium salt from porcine intestinal mucosa (grade I-A, 180 USP units/mg), Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffered saline (PBS), and trypsin/ethylenediaminetetraacetic acid (EDTA) solution were obtained from Sigma–Aldrich (St. Louis, MO, USA). Heparin-binding epidermal growth factor-like growth factor (HB-EGF) was purchased from R&D Systems (Minneapolis, Minnesota, USA). L-Proline was obtained from ICN Biomedicals (Aurora, OH, USA). Nicotinamide was obtained from Kanto Chemicals (Tokyo, Japan). Epidermal growth factor (EGF) and penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from Japan Bioserum (Nagoya, Japan). [^{125}I]-labeled HB-EGF (191 Ci/μg) was obtained from Anawa (Wangen, Switzerland). The ultrapure water used for all experiments was prepared by an ultrapure water purification system (synthesis A10; Millipore, Billerica, MA, USA). Male Wistar rats from 5 to 6 weeks of age were purchased from Japan Laboratory Animals (Tokyo, Japan) and used for isolating primary hepatocytes. The rats were housed under temperature-controlled conditions with a 12-h light/dark cycle and also had *ad libitum* access to rat chow and water. All animal experiments were approved by the Animal Care and Use Committee

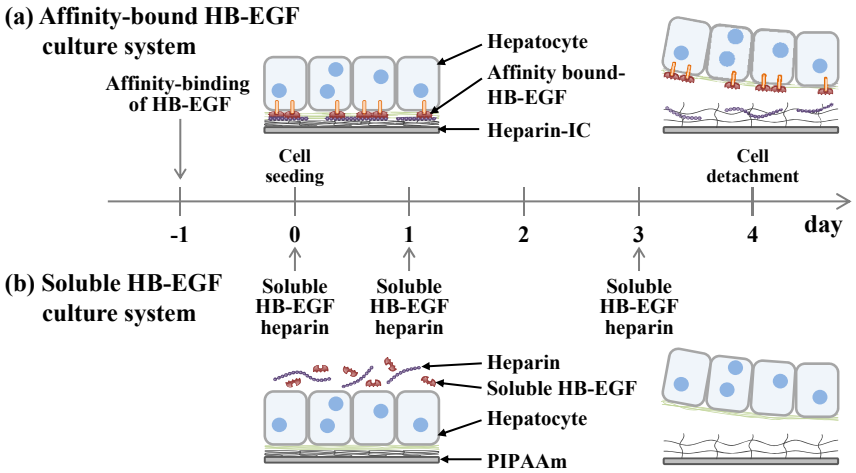

Fig. 1. Schematic illustration of the process for fabricating hepatocyte sheets in (a) affinity-bound HB-EGF and (b) soluble HB-EGF culture systems. HB-EGF indicates heparin-binding EGF-like growth factor. Primary rat hepatocytes were cultured on an HB-EGF-bound heparin-modified thermoresponsive surface and a PIPAAm surface with the addition of soluble HB-EGF and heparin at 37 °C. The culture medium was replaced with new medium on the 1st and 3rd days. After a 4-day incubation, cultured hepatocytes were harvested from theses surfaces after lowering the temperature to 20 °C.

of Tokyo Women's Medical University and performed in accordance with the institutional guidelines for Tokyo Women's Medical University on Animal Use.

2.2. Affinity-binding of HB-EGF on heparin-modified thermoresponsive surfaces

Heparin-modified thermoresponsive surfaces were prepared as previously described [6]. First, a 55% wt monomer solution (28 μL) containing IPAAm and ClPAAm (99:1 M ratio) in 2-propanol was spread on TCPS (culture area: 8.8 cm^2). Then, polymerization and covalent grafting of the polymer on the TCPS surface was carried out by electron beam irradiation from an area beam electron-processing system (Curetron EBC-200-AA2; Nissin High Voltage, Kyoto, Japan), resulting in the grafting of poly(IPAAm-*co*-ClPAAm) on TCPS (IC). After washing with water and drying, heparin was covalently tethered onto IC surfaces through a condensation reaction with carboxyl groups [26,27]. Finally, 2 mL of HB-EGF solution (10, 50, 100, or 1000 ng/cm^2) was added on the heparin-modified surfaces (Heparin-IC), which were then incubated at 37 °C for 24 h to obtain HB-EGF affinity-bound heparin-IC (HB-EGF/heparin-IC). All handling of the HB-EGF solution, such as dilutions, was carried out using Sapphire low retention pipette tips (Greiner, Frickenhausen, Germany) in Sumilon Proteosave SS microtubes (Sumitomo Bakelite, Tokyo, Japan) to reduce nonspecific adsorption.

2.3. Quantitative analysis of affinity-bound HB-EGF on thermoresponsive surfaces

The amount of affinity-bound HB-EGF on heparin-IC was quantified with [^{125}I]-labeled HB-EGF. A mixture of traces of [^{125}I]-labeled HB-EGF and unlabeled HB-EGF was diluted with PBS using Sapphire low retention pipette tips (Greiner) in Sumilon Proteosave tubes (Sumitomo Bakelite) to final concentrations of 10, 50, 100, or 1000 ng/mL and then added to heparin-IC for 24 h at 37 °C. After being washed with an excess volume of PBS, the radioactivity of affinity-bound [^{125}I]-labeled HB-EGF on heparin-IC was measured by a gamma counting instrument (50-Well gamma system ARC-950) (Hitachi-Aloka Medical, Tokyo, Japan). To quantify the levels of affinity-bound HB-EGF, a calibration curve of [^{125}I]-labeled HB-EGF standards was established with known-concentrations of HB-EGF.

2.4. Hepatocyte isolation and cultivation

Isolation of hepatocytes from a rat liver was carried out by a modified two-step collagenase perfusion method as previously described [15] and was followed by purification with 45% Percoll density centrifugation. Hepatocytes were seeded on IC, heparin-IC, and HB-EGF/heparin-IC at a cell density of 1.0 × 10^5 cells/cm^2. Hepatocytes were cultured with DMEM supplemented with 1 × 10^{-7} mol/L dexamethasone, 0.5 μg/mL insulin, 30 μg/mL L-proline, 10 mmol/L nicotinamide, 0.2 mmol/L ascorbic acid-2 phosphate, 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. The culture medium was refreshed with new medium on days 1 and 3.

Cell morphologies were recorded with a phase contrast microscope (Eclipse TE2000-U; Nikon, Tokyo, Japan) equipped with a digital camera (DXM1200C; Nikon). The hepatocyte density on each cell culture surface was determined at one day intervals with a disposable cell counter after removal of the cells from the surface by trypsin/EDTA treatment. The detachment time of hepatocytes from the culture surfaces was measured by monitoring attachment at 10 min intervals after the surfaces were transferred to a 5% CO_2 incubator at 20 °C.

2.5. Fluorescence staining of a hepatic cell-sheet detached from an HB-EGF/heparin modified thermoresponsive cell culture surface

After the temperature was reduced to 20 °C from 37 °C, hepatocyte sheets were harvested from HB-EGF/heparin-IC and were fixed in PBS containing 4% paraformaldehyde for 15 min. The fixed cell sheets were blocked with TNB buffer (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) for 30 min at room temperature and washed with a washing buffer of 0.05% Tween-20 in PBS. The cell sheets were immunostained with a TSA system (PerkinElmer Life and Analytical Sciences) to facilitate the visualization of an extremely small amount of HB-EGF. The cell sheets were labeled with biotinylated anti-human HB-EGF goat antibody (1:1000) (R&D system, Minneapolis, USA) as a primary antibody in TNB buffer overnight at 4 °C and washed with washing buffer.

Biotin-labeled cell sheets were incubated in horseradish peroxidase-conjugated streptavidin (1:2000 in TNB buffer, 30 min at room temperature) and washed with washing buffer. The cell sheets were treated with biotin-labeled tyramide derivative solution (1:50 in amplification diluent, 8 min at room temperature). After being washed with washing buffer, the cell sheets were stained with AlexaFluor® 488-conjugated streptavidin (1:500) (Invitrogen, Carlsbad, CA, USA) in TNB buffer for 30 min at room temperature and washed with the washing buffer. For fibronectin staining, the cell sheets were incubated with rabbit polyclonal anti-bovine fibronectin (1:200, Abcam, Cambridge, MA, USA) at room temperature for 2 h. After being washed with washing buffer, the cell sheets were incubated with AlexaFluor® 568-conjugated goat anti-rabbit IgG (1:800, Invitrogen) for 1 h at room temperature and washed with washing buffer. After the cell sheets were washed with washing buffer, the fluorescence images of the stained cell sheets were obtained with a fluorescence microscope (Eclipse TE2000-U) and processed with AxioVision 4.6 software (Carl Zeiss, Jena, Germany).

2.6. Quantitative determination of albumin secretion

Albumin secretion from cultured hepatocytes was measured over the four days of cultivation. On HB-EGF affinity-bound heparin-IC (HB-EGF 76.2 ng/cm^2) and collagen type I coated TCPS (collagen-TCPS; Iwaki Glass, Chiba, Japan), hepatocytes were cultured in a culture medium with or without soluble EGF (10 ng/mL). Hepatocytes on PIPAAm were cultured in culture medium containing both soluble heparin (1.0 μg/cm^2) and soluble HB-EGF (100 ng/cm^2) with or without soluble EGF (10 ng/mL). Albumin secreted into the culture medium was collected at 24 h intervals and quantified with the rat albumin ELISA Quantitation kit (Bethyl Laboratories, Montgomery, TX, USA).

2.7. Quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA from each hepatocyte sheet was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was performed with an Omniscript RT Kit (Qiagen). Quantitative RT-PCR was performed with TaqMan® Fast Advanced Master Mix and TaqMan® Gene Expression Assay (Applied Biosystems, Foster City, CA, USA) using ViiA7 Real-Time PCR System (Applied Biosystems). The TaqMan® Gene Expression Assay probes were used as follows: rat β-actin (*Actb*, Rn00667869_m1), rat albumin (*Alb*, Rn00592480_m1), rat hepatocyte nuclear factor 4, alpha (*Hnf4α*, Rn00573309_m1), rat coagulation factor VII (F7, Rn00596104_m1), and rat coagulation factor IX (F9, Rn01451633_m1). Relative mRNA expressions in each sample were determined by comparative C_T ($\Delta\Delta C_T$) method and normalized to *Actb* expression. The PCR cycling conditions for all reactions were 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C.

3. Results

3.1. Estimation of the quantity and stability of bound HB-EGF on the heparin-modified thermoresponsive surface

HB-EGF was introduced through an affinity interaction with heparin, which was covalently immobilized on poly(IPAAm-*co*-CIPAAm)-grafted TCPS (IC). The levels of affinity-bound HB-EGF on heparin-modified IC (heparin-IC) were determined with [^{125}I]-labeled HB-EGF and were found to be 241.2 ± 36.1, 76.2 ± 3.8, 45.4 ± 7.7 10.3 ± 2.3 ng/cm^2 when 1000, 100, 50, 10 ng/cm^2 soluble HB-EGF, respectively, was added (Fig. 2a). These surfaces are denoted "HB-EGF$_X$/heparin-IC", where *X* indicates the amount of affinity-bound HB-EGF. To evaluate the stability of affinity-bound HB-EGF on heparin-IC, the amounts of HB-EGF were estimated by monitoring the levels of HB-EGF eluted into the 10% FBS-containing medium at one-day intervals. After incubation for four days, the amount of affinity-bound HB-EGF on heparin-IC was maintained at more than 85.5% of the levels on day 0, excluding the HB-EGF$_{241}$/heparin-IC surface, which lost 21.3% of its immobilized HB-EGF (Fig. 2b).

3.2. The effect of affinity-bound HB-EGF on the heparin-modified thermoresponsive surface and hepatocyte culture

Typically, the *in vitro* cultivation of primary rat hepatocytes requires ECMs, such as collagen type I, on the culture surface and soluble epidermal growth factor (EGF) supplementation of the culture medium [15,28]. To evaluate the effects of tethered growth factor on hepatocytes, the hepatocytes were cultured on HB-EGF/heparin-IC without soluble growth factors and on PIPAAm-grafted surfaces with both soluble heparin (1.0 μg/cm^2) and soluble HB-EGF as a replacement for EGF. The PIPAAm surfaces with soluble heparin and HB-EGF are denoted "PIPAAm + HB-EGF$_Y$", where *Y* indicates the amount of soluble HB-EGF. The amount of soluble heparin was almost comparable to that of covalently immobilized heparin on the IC surface (i.e., 0.8 ± 0.4 μg/cm^2 [6]). As controls, collagen-TCPS, PIPAAm, IC, and heparin-IC were used under normal culture conditions without growth factors. The seeding density necessary for achieving the maximum level of adhered hepatocytes was determined to be 1.0 × 10^5 cells/cm^2 (Fig. S1 in Supporting Information). All HB-EGF$_X$/heparin-IC and PIPAAm + HB-EGF$_{100}$ surfaces were completely covered by adhered hepatocytes, and the morphology of the hepatocytes showed less spread on these surfaces compared with other surfaces (Fig. 3). Firstly, initial adhesion of hepatocytes on HB-EGF$_{76}$/heparin-IC was investigated when adding soluble EGF in the medium (Fig. S2 in Supporting Information). Hepatocytes exhibited low adhesiveness on HB-EGF$_{76}$/heparin-IC (the percentage of adhered, 8.0%) after 1.5 h-incubation. The percentage was decreased to 3.8% in the medium containing 1000 ng/mL of EGF while the results of one-way analysis of variance (ANOVA) were not significant. Therefore, initial adhesion of hepatocytes on HB-EGF bound heparin-modified thermoresponsive surfaces was partially inhibited by the presence of soluble EGF. In addition, the densities of the adhered hepatocytes on each surface were investigated at one-day intervals for four days. After one-day incubation, the percentage of adhered hepatocytes on all surfaces was increased to approximately 60–70% (Fig. 4). These results implied that the adhesion of hepatocytes on all surfaces was mediated mainly through physisorbed serum proteins, not through the affinity binding between immobilized HB-EGF and EGF receptor. Although the number of adhered hepatocytes on collagen-TCPS, PIPAAm, IC, and heparin-IC, which contained no HB-EGF, was drastically decreased (Fig. 4a), the number on HB-EGF$_{76}$/heparin-IC and PIPAAm + HB-EGF$_{100}$ was maintained for four days (Fig. 4b). Therefore, tethered or soluble HB-EGF maintained the survival of hepatocytes during cultivation.

3.3. Cell detachment of hepatocytes from HB-EGF/heparin-modified thermoresponsive surfaces is accomplished by reducing the temperature

After 4 days, cultured rat primary hepatocytes grown at 37 °C on HB-EGF$_X$/heparin-IC and PIPAAm + HB-EGF$_Y$ were transferred to a 20 °C CO_2 incubator to stimulate detachment of the cells

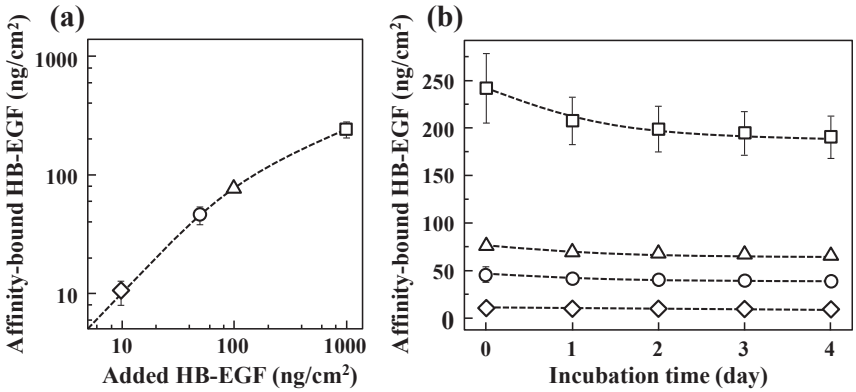

Fig. 2. (a) The relationship between added HB-EGF and affinity-bound HB-EGF on heparin-IC surface after incubation at 37 °C for 24 h. The amounts of affinity-bound HB-EGF on heparin-IC were 241.2 ± 36.1 (open square), 76.2 ± 3.8 (open triangle), 45.4 ± 7.7 (open circle), and 10.3 ± 2.3 ng/cm^2 (open diamond), when a PBS solution with 1000, 100, 50, and 10 ng/cm^2 of soluble HB-EGF was added to these surfaces, respectively. The data are expressed as the mean ± S.D. of triplicate experiments. (b) Time-courses of affinity-bound HB-EGF on each heparin-IC surfaces in 10% fetal bovine serum-containing medium for 4 days at 37 °C. The data are expressed as the mean ± S.D. of triplicate experiments.

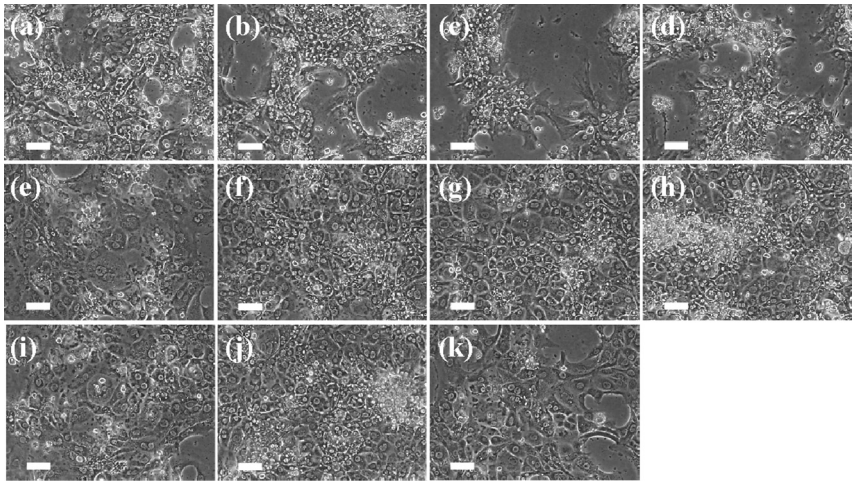

Fig. 3. Phase contrast microscopic images of cultured primary rat hepatocytes after a 4-day incubation at 37 °C. (a) collagen-coated tissue culture polystyrene (collagen-TCPS), (b) PIPAAm, (c) IC, (d) heparin-IC, (e–h) HB-EGF$_X$/heparin-IC, and (i–k) PIPAAm + HB-EGF$_Y$ surfaces. Heparin-IC indicates heparin-modified poly(*N*-isopropylacrylamide-*co*-2-carboxyisopropylacrylamide) (poly(IPAAm-*co*-CIPAAm))-grafted tissue culture polystyrene (TCPS). HB-EGF indicates heparin-binding EGF-like growth factor. HB-EGF-bound heparin-IC is denoted "HB-EGF$_X$/heparin-IC", where *X* indicates the amounts of affinity-bound HB-EGF. The amounts of affinity-bound HB-EGF on heparin-IC are 241.2 ± 36.1 (e), 76.2 ± 3.8 (f), 45.4 ± 7.7 (g), and 10.3 ± 2.3 ng/cm^2 (h). PIPAAm surfaces with added soluble HB-EGF and heparin (1.0 μg/cm^2) are denoted "PIPAAm + HB-EGF$_Y$", where *Y* indicates the amount of soluble HB-EGF per addition. The amounts of soluble HB-EGF are 100 (i), 50 (j), and 10 ng/cm^2 (k). Scale bars: 100 μm.

from their respective surfaces. No cellular sheet formation was observed on PIPAAm, IC, and heparin-IC control surfaces (Fig. 5b–d) because adhered hepatocytes were sparse on these surfaces after a 4-day incubation (Fig. 4a). Hepatocytes were unable to detach from the collagen-TCPS surface (Fig. 5a). On the affinity-bound HB-EGF surfaces with more than 10 ng/cm^2 of HB-EGF (Fig. 5e–h) and PIPAAm surfaces with more than 50 ng/cm^2 of soluble HB-EGF (Fig. 5i and j), hepatocytes were able to connect to each other and be harvested as a cell sheet within 40 min after lowering of the temperature to 20 °C (Table 1). When the bound HB-EGF was increased, the detachment time of the cell sheets was accelerated to 10 min (Table 1). On the PIPAAm surface with 10 ng/cm^2 of HB-EGF, the fabrication of hepatocyte sheets was difficult and unsuccessful (Fig. 5k) because the stimulation with small amounts of soluble HB-EGF was insufficient for maintaining the survival of the hepatocytes during the four days of cultivation.

To trace the localization of affinity-bound HB-EGF and ECM after cell detachment from heparin-IC, a harvested hepatocyte sheet and HB-EGF$_{76}$/heparin-IC surface were observed by fluorescence microscopy after fluorescent staining of fibronectin and HB-EGF. Fibronectin and HB-EGF were apparent in the detached hepatocyte sheet, although these proteins were not detected on the heparin-IC surface (Fig. 6). The internalization of affinity-bound HB-EGF by the hepatocytes was suppressed during the two days of cultivation (Fig. S3 in Supporting Information), indicating that affinity-bound HB-EGF was located on the cellular surfaces of the hepatocytes. In contrast, soluble HB-EGF was incorporated into hepatocytes via receptor-mediated endocytosis of the EGF receptor (Fig. S3 in Supporting Information) [29].

3.4. Albumin secretion and mRNA expression of hepatocytes on an HB-EGF/heparin-modified thermoresponsive surface

To evaluate the effects of affinity-bound HB-EGF on hepatic functions, the level of albumin secreted into the culture medium on HB-EGF$_{76}$/heparin-IC was determined by ELISA (Fig. 7). Although the albumin secretion from hepatocytes gradually decreased on PIPAAm + HB-EGF$_{100}$ and collagen-TCPS, the secretion on HB-EGF$_{76}$/heparin-IC was almost doubled during the 4-day cultivation.

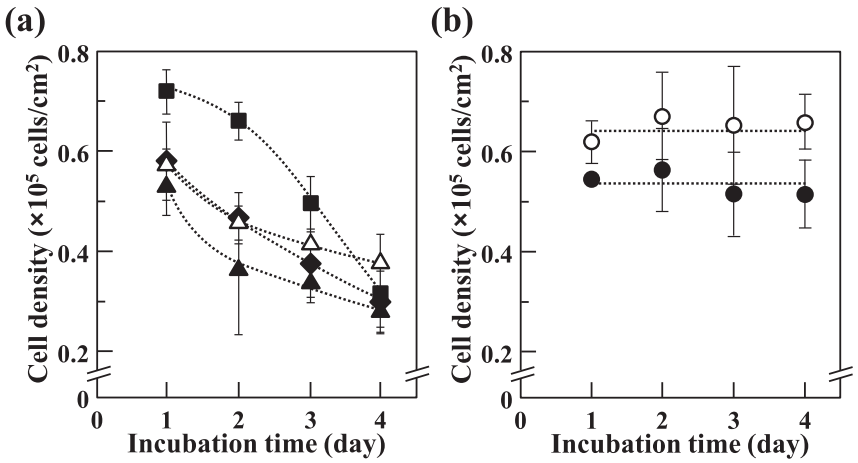

Fig. 4. Time-courses of adhering primary rat hepatocytes on collagen-coated tissue culture polystyrene (Collagen-TCPS, closed square), PIPAAm (closed diamond), IC (closed triangle), heparin-IC (open triangle) (a), and on HB-EGF$_{76}$/heparin-IC (open circle) and PIPAAm + HB-EGF$_{100}$ surfaces (closed circle) (b). Heparin-IC indicates heparin-modified poly(*N*-isopropylacrylamide-*co*-2-carboxyisopropylacrylamide) (poly(IPAAm-*co*-CIPAAm))-grafted tissue culture polystyrene (TCPS). HB-EGF indicates heparin-binding EGF-like growth factor. HB-EGF$_{76}$/heparin-IC indicates heparin-IC surface with surface-bound HB-EGF (76.2 ± 3.8 ng/cm^2). PIPAAm + HB-EGF$_{100}$ indicates PIPAAm surfaces with soluble HB-EGF (100 ng/cm^2) and heparin (1.0 μg/cm^2) per addition. Hepatocytes were cultured for a 4-day incubation at 37 °C. The initial cell density was 1.0 × 10^5 cells/cm^2. The data are expressed as the mean ± S.D. of triplicate experiments.

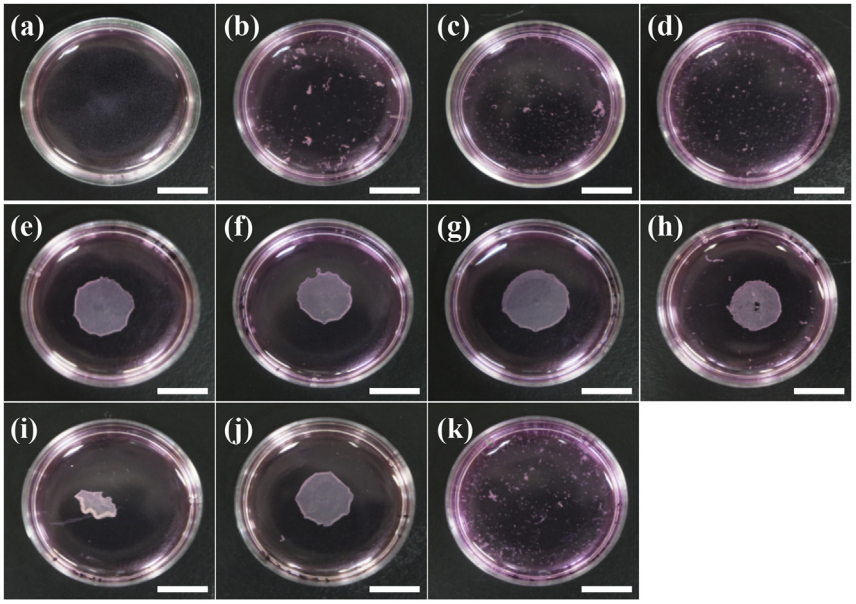

Fig. 5. Photographs of primary rat hepatocytes after reduction of the temperature to 20 °C after a 4-day incubation at 37 °C. (a) collagen-coated tissue culture polystyrene (collagen-TCPS), (b) PIPAAm, (c) IC, (d) heparin-IC, (e–h) HB-EGF$_X$/heparin-IC and (i–k) PIPAAm + HB-EGF$_Y$ surfaces. Heparin-IC indicates heparin-modified poly(*N*-isopropylacrylamide-*co*-2-carboxyisopropylacrylamide) (poly(IPAAm-*co*-CIPAAm))-grafted tissue culture polystyrene (TCPS). HB-EGF indicates heparin-binding EGF-like growth factor. HB-EGF-bound heparin-IC surfaces are denoted "HB-EGF$_X$/heparin-IC", where *X* indicates the amounts of affinity-bound HB-EGF. The amounts of affinity-bound HB-EGF on heparin-IC are 241.2 ± 36.1 (e), 76.2 ± 3.8 (f), 45.4 ± 7.7 (g), and 10.3 ± 2.3 ng/cm^2 (h). PIPAAm surfaces with additive soluble HB-EGF and heparin (1.0 μg/cm^2) are coded as "PIPAAm + HB-EGF$_Y$", where *Y* indicates the amount of soluble HB-EGF per addition. The amounts of soluble HB-EGF are 100 (i), 50 (j), and 10 ng/cm^2 (k). Scale bars: 10 mm.

The addition of soluble EGF failed to show observable effects on albumin secretion of hepatocytes on HB-EGF$_{76}$/heparin-IC, PIPAAm + HB-EGF$_{100}$ and collagen-TCPS. Therefore, the tethering of HB-EGF onto the heparin-modified thermoresponsive surface effectively maintained albumin secretion from hepatocytes, in contrast with soluble HB-EGF and/or EGF.

To further investigate the relationship between the level of HB-EGF and the function of hepatocytes on HB-EGF-bound heparinized thermoresponsive surfaces (HB-EGF$_X$/heparin-IC) and PIPAAm surfaces with soluble HB-EGF (PIPAAm + HB-EGF$_Y$), hepatocyte-specific gene expression in hepatocytes after a 4-day cultivation was analyzed by quantitative RT-PCR (Fig. 8). The hepatocyte-specific genes, albumin (*Alb*), hepatocyte nuclear factor 4 alpha (*Hnf4α*), coagulation factor VII (*F7*), and coagulation factor IX (*F9*) were chosen. When grown on HB-EGF$_{76}$/heparin-IC, the mRNA expression of *Alb* and *F7* was the highest compared with expression in other HB-EGF/heparin-ICs, although the gene expression on PIPAAm + HB-EGF$_Y$ remained constant, irrespective of the amount of soluble HB-EGF. *Hnf4α* gene expression on HB-EGF$_X$/heparin-IC increased with rising levels of affinity-bound HB-EGF and was higher than that observed with PIPAAm + HB-EGF$_Y$. *F9* gene expression on HB-EGF$_X$/heparin-IC was higher, even with small amounts of affinity-bound HB-EGF, than that on PIPAAm + HB-EGF$_Y$. These results indicated that the HB-EGF$_{76}$/heparin-IC surface

Table 1
Amounts of HB-EGF and cell detachment time.

Sample[a]	Amounts of HB-EGF (ng/cm^2)	Detachment time (min)[b]
Collagen-TCPS	–	No detachment[c]
PIPAAm	–	90
IC	–	90
Heparin-IC	–	90
HB-EGF$_{241}$/heparin-IC	241.2 ± 36.1	10
HB-EGF$_{76}$/heparin-IC	76.2 ± 3.8	10
HB-EGF$_{45}$/heparin-IC	45.4 ± 7.7	10
HB-EGF$_{10}$/heparin-IC	10.3 ± 2.3	40
PIPAAm + HBEGF$_{1000}$	1000	10
PIPAAm + HBEGF$_{100}$	100	10
PIPAAm + HBEGF$_{10}$	10	40

[a] As control surfaces, collagen coated-tissue culture polystyrene (collagen-TCPS), poly(*N*-isopropylacrylamide) grafted TCPS (PIPAAm), poly(IPAAm-*co*-CIPAAm)-grafted TCPS (IC), and heparin-immobilized IC (heparin-IC) were used. HB-EGF indicates heparin-binding EGF like growth factor. HB-EGF-bound heparin-IC is denoted "HB-EGF$_X$/heparin-IC", where *X* indicates the amount of affinity-bound HB-EGF. PIPAAm surfaces with additive soluble HB-EGF and soluble heparin (1.0 μg/cm^2) are denoted "PIPAAm + HB-EGF$_Y$", where *Y* indicates the amount of soluble HB-EGF per addition.
[b] Time required for cell detachment from the substrates after reducing the temperature from 37 to 20 °C.
[c] Cells remained attached on the surfaces over the 120 min at 20 °C.

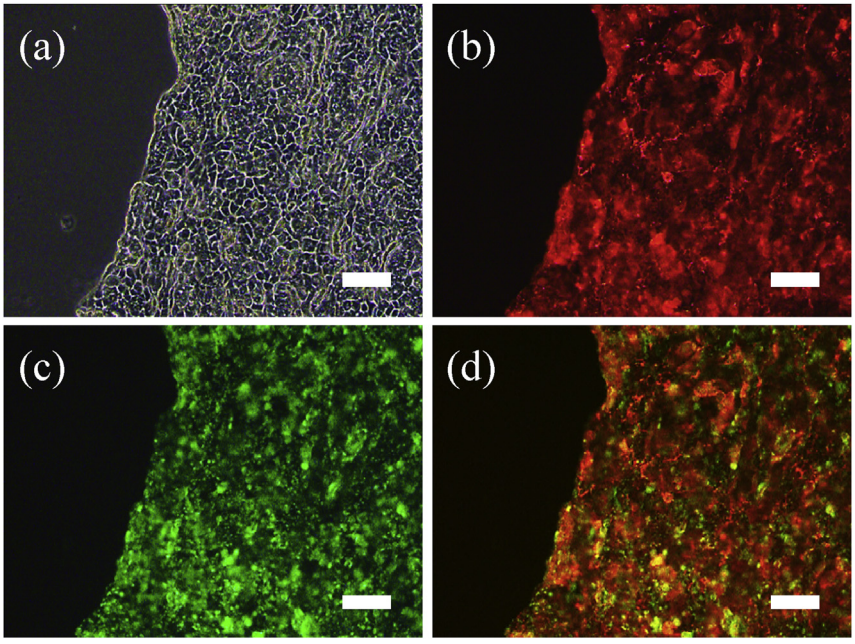

Fig. 6. Phase contrast and fluorescence microscopy images of a harvested primary rat hepatocyte sheet from HB-EGF$_{76}$/heparin-IC surface that was harvested after reduction of the temperature to 20 °C. (a) After a 4-day cultivation at 37 °C, hepatocytes were detached as a monolayer by reduction of the temperature to 20 °C and observed by a phase-contrast microscope. Harvested hepatocyte sheets were fixed with 4% performaldehyde. Fibronectin (FN) and HB-EGF on the cell-sheet and the surface were stained with (b) AlexaFluor® 568 (red) and (c) AlexaFluor® 488 (green), respectively. The merged image (d) was created by the superimposition of FN (red) and HB-EGF (green) fluorescent images. Scale bars: 100 μm. HB-EGF and heparin-IC indicate heparin-binding EGF-like growth factor and heparin-modified poly(*N*-isopropylacrylamide-*co*-2-carboxyisopropylacrylamide) (poly(-IPAAm-*co*-CIPAAm))-grafted tissue culture polystyrene (TCPS), respectively. HB-EGF$_{76}$/heparin-IC indicates heparin-IC surface with surface-bound HB-EGF (76.2 ± 3.8 ng/cm^2).

was able to more effectively maintain hepatic function than other HB-EGF$_X$/heparin-ICs and PIPAAm + HB-EGF$_Y$.

4. Discussion

Mono-cultivation of hepatocytes, while maintaining their survival, is quite difficult on synthetic polymer-based cell culture substrates, including polystyrene and thermoresponsive surfaces. Hepatocytes can rapidly lose their normal cellular morphology and cell-specific functions. In this study, we combined a thermoresponsive surface with bioactive heparin molecules to achieve both the maintenance of hepatic functions and recovery of the hepatocyte sheet. In a previous study, we have reported that a heparin-modified thermoresponsive surface bound with bFGF enhances the proliferation of fibroblasts and enables the cultured cell-sheets to be detached. Moreover, after the temperature is reduced from 37 to 20 °C, this detachment is accompanied by dynamic motion of the heparin molecules and conformational change of the hydrated PIPAAm chains [6].

The present work demonstrated that immobilized heparin on a thermoresponsive surface stably held HB-EGF via affinity-binding (Fig. 9, left) and maintained the survival and functionality of hepatocytes. HB-EGF shows a high affinity interaction with heparin [18–20]. Therefore, HB-EGF was able to bind to heparin immobilized on a thermoresponsive surface in 10% FBS-containing medium for 4 days (Fig. 2b), which was the minimum time required for fabricating hepatocyte sheets. When grown on HB-EGF$_{76}$/heparin-IC and PIPAAm + HB-EGF$_{100}$, the number of hepatocytes was almost

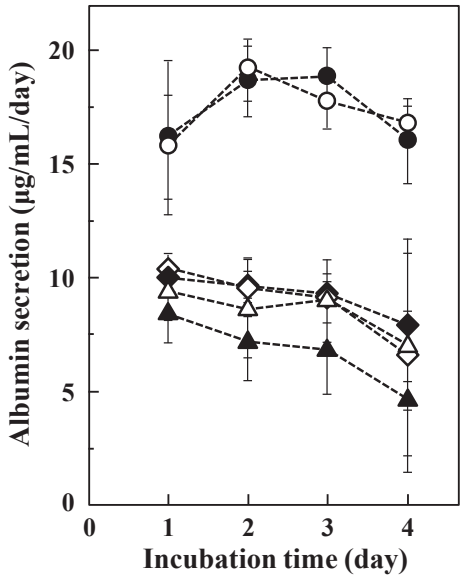

Fig. 7. Albumin secretion from cultured primary rat hepatocytes during a 4-day incubation was measured by enzyme-linked immunosorbent assay (ELISA). Hepatocytes were cultured on HB-EGF$_{76}$/heparin-IC (circle), PIPAAm + HB-EGF$_{100}$ (triangle), and collagen coated tissue culture polystyrene surfaces (diamond) under culture conditions with added epidermal growth factor (EGF, 10 ng/mL, open symbol), and without EGF (closed symbols). To measure rat albumin secretion, culture medium was collected from each sampling tube at 24 h intervals and replenished each time. The data are expressed as the mean ± S.D. from triplicate experiments. HB-EGF and heparin-IC indicate heparin-binding EGF-like growth factor and heparin-modified poly(*N*-isopropylacrylamide-*co*-2-carboxyisopropylacrylamide) (poly(IPAAm-*co*-CIPAAm))-grafted tissue culture polystyrene (TCPS), respectively. HB-EGF$_{76}$/heparin-IC indicates heparin-IC surface with surface-bound HB-EGF (76.2 ± 3.8 ng/cm^2). PIPAAm + HB-EGF$_{100}$ indicates PIPAAm surfaces with soluble HB-EGF (100 ng/cm^2) and heparin (1.0 μg/cm^2) per the addition of the medium.

constant, unlike that on collagen-TCPS, IC, and heparin-IC (Fig. 4). This result indicated that the presence of affinity-bound HB-EGF or soluble HB-EGF could greatly contribute to hepatocyte survival. In addition, HB-EGF tethered onto the cell culture surfaces, compared with soluble HB-EGF in culture medium, strongly stimulated the cultured hepatocytes over a long period. When the medium contained less than 10 ng/cm^2 of soluble HB-EGF, the hepatocytes were not able to adhere and form their cell sheets (Fig. 5k). In contrast, hepatocytes adhered and formed sheets on a heparin-modified thermoresponsive surface containing 10.3 ng/cm^2 of bound HB-EGF (Fig. 5h). Hence, lower amounts of bound HB-EGF, as compared to soluble HB-EGF, on heparin-modified surfaces were sufficient for hepatocyte survival.

With respect to hepatic functions, soluble HB-EGF in the culture medium poorly stimulated albumin secretion from hepatocytes, as compared with the effects of affinity-bound HB-EGF on heparin-IC (Fig. 7). In general, the addition of soluble EGF (10 ng/mL) is absolutely necessary for the survival of primary hepatocytes and the maintenance of their function *in vitro* [15,28]. However, HB-EGF$_{76}$/heparin-IC effectively enhanced the albumin secretion from hepatocyte sheets in the absence of soluble EGF. These results implied that affinity-bound HB-EGFs on a surface more strongly activate the intracellular signal transduction pathways than does the addition of soluble EGF and/or HB-EGF, owing to the inhibition of down-regulation of their signaling via receptor-mediated endocytosis on affinity-bound HB-EGF surfaces (Fig. S2 in Supporting Information). Similarly, hepatocyte mRNA expression of *Alb*, *Hnf4α*, *F7* and *F9* on HB-EGF/heparin-IC surfaces was significantly higher than that on other surfaces with soluble HB-EGF (Fig. 8). These results indicated that dedifferentiation of cultured hepatocytes on HB-EGF/heparin-IC surfaces was suppressed. Therefore, heparin-modified thermoresponsive surfaces bound with HB-EGF contributed to the maintenance of highly differentiated status of cultured hepatocytes.

A culture system for hepatocyte sheets using heparin-modified thermoresponsive surfaces was able to reduce the total amounts of required HB-EGF. Typically, 88 ng of HB-EGF was used for preparing HB-EGF$_{76}$/heparin-IC with the surface area of 8.8 cm^2. No additional HB-EGF in the medium was required. By contrast, the amounts of HB-EGF on PIPAAm + HB-EGF$_{100}$ for a 4-day cultivation was three times than that on HB-EGF$_{76}$/heparin-IC because the frequent exchange of the medium containing soluble HB-EGF was done at days 0, 1, and 3. In addition, the amount of albumin secretion from hepatocytes (Fig. 7) and mRNA expressions of hepatocytes (Fig. 8) on HB-EGF/heparin-IC surfaces were significantly higher than that on PIPAAm surfaces with soluble HB-EGF. Therefore, heparin-modified thermoresponsive surfaces is a cost-effective tool for preparing functional hepatocyte sheets.

Another factor for the maintenance of hepatic function is the interaction with the ECM. In the native liver, the hepatic perisinusoidal space between hepatocytes and endothelial cells contains ECMs including collagen, fibronectin, and proteoglycans such as decorin and perlecan [30,31]. Hepatocytes are unable to synthesize decorin and perlecan, which are provided from endothelial and hepatic stellate cells [31]. In a coculture system of hepatocytes and non-parenchymal cells, decorin contributes to the up-regulation of liver-specific functions [32]. However, the monoculture of hepatocyte sheets on thermoresponsive PIPAAm surfaces lacks proteoglycans. Our previous report has suggested that an endothelial cell sheet layered onto a hepatocyte sheet provides the endothelial cell-derived ECMs between the endothelial cell and the hepatocyte sheets, and the presence of ECMs would preserve the hepatic functions [15]. In the present paper, the presence of heparin molecules on the thermoresponsive surfaces would provide an ECM-mimicking structure in the hepatic perisinusoidal space and contribute to the maintenance of hepatic functions. However, the amount of albumin secretion from hepatocytes on HB-EGF bound surfaces in the current work was less than that from the coculture system of hepatocyte and non-parenchymal cell sheets [15]. Therefore, further investigations of molecular mechanism of maintaining hepatic phenotype and functions are required for achieving a complete monoculture system for hepatocyte sheets using synthetic polymer substrate.

Finally, heparin-modified thermoresponsive surfaces facilitated the fabrication of hepatocyte sheets in which hepatic functions were maintained, and the sheets could be transferred only by reducing the temperature to 20 °C. The adhered hepatocytes were able to be harvested as a hepatocyte sheet from the HB-EGF/heparin-IC by lowering the temperature to 20 °C. We confirmed that affinity-bound HB-EGF and fibronectin were transferred from the HB-EGF/heparin-IC surface to the basal side of the cell sheet during the detachment of the cell sheet (Fig. 6). These results showed that cultured hepatocytes were able to detach from HB-EGF/heparin-IC surfaces through the reduction of affinity-binding between HB-EGF and immobilized heparin when the mobility of heparin and the steric hindrance of the swollen PIPAAm chains were increased by lowering the temperature to 20 °C (Fig. 9, right).

5. Conclusions

Heparin-modified thermoresponsive surfaces bound with HB-EGF (HB-EGF/heparin-IC) were designed for the purpose of creating hepatocyte sheets while maintaining hepatic cell functions. Heparin-modified thermoresponsive surfaces facilitated the stable binding of HB-EGF, which maintained the survival and

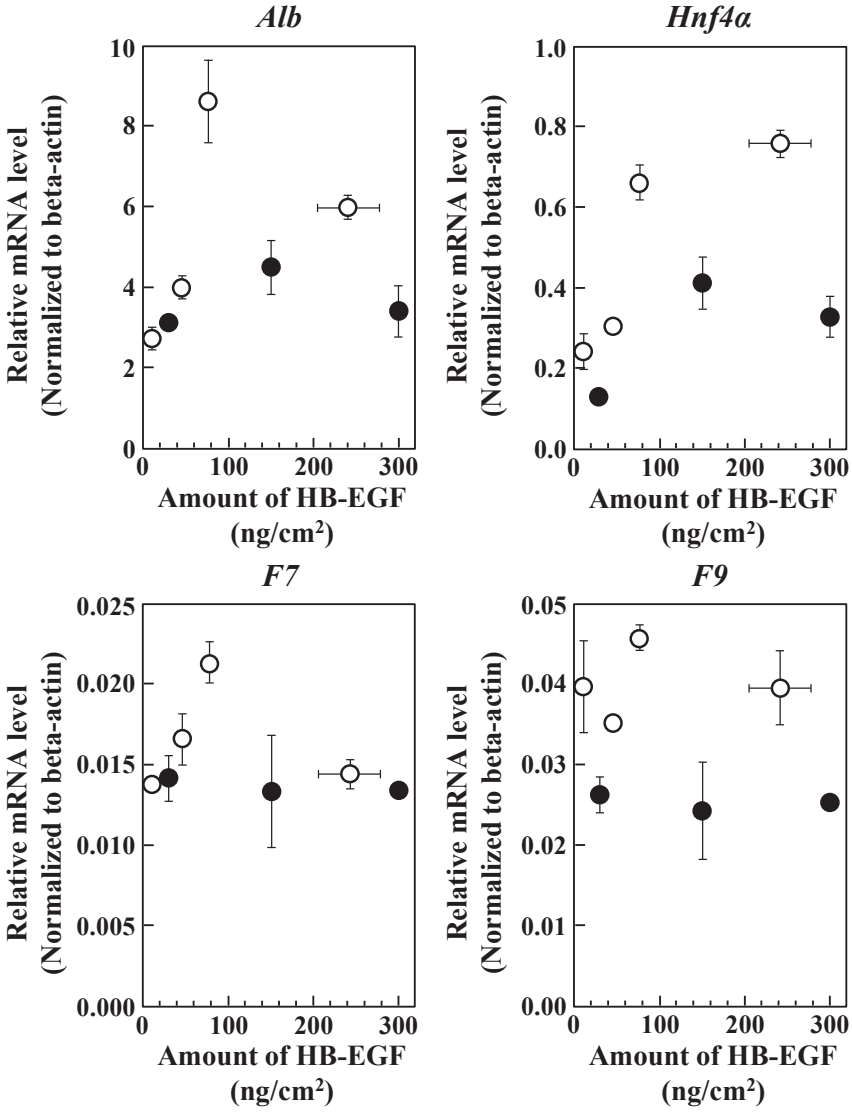

Fig. 8. Hepatocyte-specific gene expression in harvested hepatocyte sheets. The hepatocyte sheets were harvested from HB-EGF$_X$/heparin-IC surfaces (opened circles) and PIPAAm$_Y$ + HB-EGF surfaces (closed circles) after a 4-day cultivation at 37 °C. Gene expression levels of albumin (*Alb*), hepatocyte nuclear factor 4 alpha (*Hnf4α*), coagulation factor VII (*F7*) and coagulation factor IX (*F9*) were determined by quantitative RT–PCR. All the data are normalized to mRNA expression level of beta-actin (*Actb*) and are expressed as the mean ± S.D. of triplicate experiments. HB-EGF and heparin-IC indicate heparin-binding EGF-like growth factor and heparin-immobilized poly(*N*-isopropylacrylamide-*co*-2-carboxyisopropylacrylamide) (poly(IPAAm-*co*-CIPAAm))-grafted tissue culture polystyrene (TCPS), respectively. HB-EGF$_X$/heparin-IC indicates heparin-IC surface with different amounts of affinity-bound HB-EGF. The amounts of affinity-bound HB-EGF on heparin-IC are 241.2 ± 36.1, 76.2 ± 3.8, 45.4 ± 7.7, and 10.3 ± 2.3 ng/cm^2. PIPAAm + HB-EGF$_Y$ indicates PIPAAm surfaces with different amounts of soluble HB-EGF in culture medium containing heparin (1.0 μg/cm^2). The total soluble HB-EGF amounts of PIPAAm$_Y$ + HB-EGF for a 4-day cultivation are 300, 150, 30 ng/cm^2 (as amounts of soluble HB-EGF per addition: 100, 50, 10 ng/cm^2, respectively).

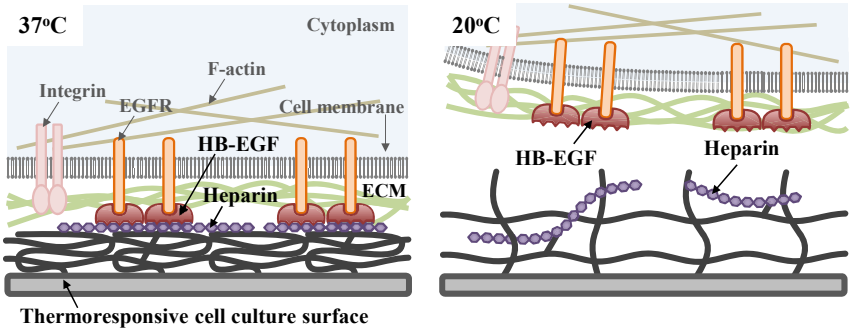

Fig. 9. Schematic illustration of temperature-dependent affinity regulation on a hepatocyte, HB-EGF, and heparin-immobilized thermoresponsive surface. Left, at 37 °C regular culture temperature (37 °C), adhesion and survival of cultured rat hepatocytes maintained on a surface that immobilizes HB-EGF through the affinity-binding of heparin with shrunken thermoresponsive polymer chains. Right, after lowering the temperature to 20 °C, the cultured hepatocytes detach due to an increase in the steric hindrance of the swollen grafted thermoresponsive polymer chains. HB-EGF is transferred from the surface to the detached hepatocytes because of its affinity-binding to EGF receptors.

functions of cultured hepatocytes; in particular, the amount of albumin secreted from hepatocytes on HB-EGF/heparin-IC surfaces was almost twice that observed with soluble growth factors. During a 4-day cultivation of hepatocytes, no additional HB-EGF supplementation of the medium was required on HB-EGF/heparin-IC surfaces, whereas the frequency of soluble HB-EGF dosing was three times that on PIPAAm surfaces. More importantly, the transplantation of hepatocyte sheets while hepatic functions were maintained was possible through use of HB-EGF/heparin-IC surfaces. Heparin-modified thermoresponsive surfaces bound with HB-EGF have the potential to provide a functional hepatocyte sheet-based culture system and effective hepatocyte-based tissue engineering for the treatment of liver diseases.

Conflict of interest

Teruo Okano, Ph.D. is a founder and a member of the board of CellSeed Inc., which has licenses for certain cell sheet-related technologies and patents from Tokyo Women's Medical University. Teruo Okano and Masayuki Yamato are stakeholders in CellSeed Inc.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.reth.2016.03.003.

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