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Maturation of the Extracellular Matrix and Cell Adhesion Molecules in Layered Co-cultures of HepG2 and Endothelial Cells

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We previously reported that using thermo-responsive culture surfaces, a layered co-culture was achieved by placing an endothelial cell sheet onto a layer of human hepatoma cell line HepG2 in order to develop a culture model that mimics hepatic lobules. In the layered co-culture cells, the expression levels of liver-specific genes gradually increased. A cross-sectional view of the layered co-culture cells showed that the thickness of the layer slowly increased after layering, as did extracellular matrix (ECM) deposition around HepG2 cells. In this study, we report that the molecular compositions of ECM and cell-adhesion molecules changed in the layered co-culture cells. Gene expression of integrin α4 and decorin gradually increased after layering, and the time-course pattern of these genes was correlated with that of liver-specific genes. Moreover, the layered co-culture system has the ability to assemble a branching network of fibronectin fibrils. These results suggest that a vastly different extracellular environment in layered co-culture cells may induce an increase in liver-specific functions.

Key words: cell-sheet technology, co-culture, ECM, gene expression, hepatocyte.

Abbreviations: BPAECs, bovine pulmonary artery endothelial cells; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hGAPDH, human glyceraldehyde-3-phosphate dehydrogenase; PIPAAm, poly(*N*-isopropylacrylamide); PVDF, polyvinylidene fluoride.

Previously, we reported the development of a culture surface produced using a thermo-responsive polymer, poly(*N*-isopropylacrylamide) (PIPAAm) (*1*). This culture surface permits the harvest of large cell sheets maintaining both intact cell–cell contacts and native cell–matrix association (*2*). Using this thermo-responsive surface, we constructed a layered co-culture system in order to develop a culture model that more closely mimics hepatic lobules in which layers of hepatocytes and endothelial cells are interconnected to form a continuous three-dimensional tissue lattice. A layered co-culture was achieved by placing an endothelial cell sheet onto the primary hepatocytes, which allowed for continuous expression of the differentiated functions of the hepatocytes (*3*). We also reported that in a layered co-culture system with the human hepatoma cell-line HepG2 and bovine pulmonary artery endothelial cells (BPAECs) forming a sheet, the expression levels of albumin and cytochrome P450 genes were gradually and strongly increased (*4*). Cross-sectional views of the layered co-culture cells by confocal laser microscopy showed that the thickness of the layer had slowly increased after layering, as did extracellular matrix (ECM) deposition around HepG2 cells. These structural changes were correlated with a gradual increase in expression of liver-specific genes. The relatively slow kinetics of these gene transcript increases may be due to the slow induction of ECM and the slow maturation of niche formation caused by homotypic and heterotypic cell–cell interaction.

The interactions between cells and the ECM together with cell–cell interactions can have profound effects on cell morphology and function *in vivo*. Several ECM components within the liver undergo modulation throughout various physiological processes (*5*), and ECM provides a critical scaffold for hepatocytes to regulate proliferation and differentiation (*6*, *7*). Previous studies have demonstrated that primary hepatocytes cultured as spheroids and on a single surface, then overlaid with a second layer of collagen or Matrigel sandwich configurations, demonstrate improved morphology and enhanced levels of albumin gene expression compared to conventional hepatocyte monolayer cultures (*8–10*). The importance of an ECM component in the maintenance of hepatocyte function and morphology is well known. However, the molecular constituents of customized ECMs and the precise mechanism of regulation of ECM assembly, which induce increases in liver-specific functions, are not sufficiently understood.

In the present study, we investigated the molecular composition of ECM and cell-adhesion molecules and ECM assembly in layered co-culture cells of HepG2 and

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BPAEC sheets. Expression levels of these genes changed compared to monolayer culture cells and several ECM and cell-adhesion genes gradually increased in a time-dependent manner after layering. The vastly different extracellular environment of layered co-culture cells may induce an increase in liver-specific function. Our layered co-culture system may contribute to understanding how the specificity of the extracellular environment regulates liver-specific function in hepatocytes.

MATERIALS AND METHODS

Cells and Cell Culture—M2 HepG2 cells (*11*) were cultured in Dulbecco's modified Eagle's medium (SIGMA, St Louis, MO) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2. HepG2 cells were plated on type I collagen-coated plastic dishes (35 mm in diameter) (Iwaki, Tokyo, Japan) at a cell density of 1.0×10^6 cells/dish.

BPAECs were purchased from Cell Applications, Inc. (San Diego, CA) and cultured in EBM medium supplemented with 5% fetal bovine serum, 12 μg/ml bovine brain extract, 1 μg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 50 μg/ml gentamycin and 50 ng/ml amphotericin B using the EGM-MV BulletKit (Cambrex Bio Science, Inc., Wakersville, MD) at 37°C under humidified atmosphere with 5% CO_2 and 95% air. BPAECs with four to five population-doubling levels were plated on PIPAAm-coated UpCell dishes (35 mm in diameter) (CellSheed, Tokyo, Japan) and type-I collagen-coated plastic dishes.

Layered Co-culture of HepG2 Cells and BPAEC Sheets—Construction of the layered co-cultured cells was as follows. HepG2 cells were cultured at 37°C until confluence and co-cultured with stratified BPAEC sheets. The three-dimensional manipulation of the BPAEC sheets and assembly of the layered co-cultured cells were performed as previously described (*4*, *12*, *13*). Briefly, BPAECs were plated on the UpCell dishes at a cell density of 1.0×10^6 cells/dish and cultured for 1 week at 37°C. After removing the culture medium, a sheet of polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA) was placed over the BPAEC monolayer. The dishes were then moved to a CO_2 incubator at 20°C for 5 min, during which the BPAEC sheets detached spontaneously from the dish surfaces. The overlaid PVDF membrane, together with the BPAEC sheet, was peeled off the dishes and transferred onto a monolayer of HepG2 cells. After incubation at 37°C for 2 h, the PVDF membrane was removed from the BPAEC sheet using tweezers. The stratified sheets were then cultured in a mixture of EBM medium and Dulbecco's modified Eagle's medium (1:1 v/v ratio) at 2 ml of medium/dish.

RNA Isolation and cDNA Synthesis—Total RNA from cells was extracted using RNAiso (Takara, Kyoto, Japan) according to the manufacture's protocol and quantified by measuring absorbance at 260 nm; purity was assessed from the 260 nm:280 nm absorbance ratio. cDNA for real-time PCR was synthesized by reverse transcription of 4 μg of total RNA with random hexamer primers and Super Script II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

PCR Array Analysis—For the PCR array experiments, an RT^2 profiler PCR Array (SuperArray Bioscience, Frederick, MD) was used to simultaneously examine the mRNA levels of 89 genes, including five housekeeping genes, in 96-well plates according to the manufacturer's protocol. Total RNA from BPAEC/HepG2 cells or HepG2 cells was isolated 20 days after overlaying of the BPAEC sheets. Real-time PCR was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA).

Real-Time PCR Using the SYBR Green I Method—Real-time PCR was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The standard reaction was performed in a 96-well plate and was composed of 10 μl of SYBR Premix Ex Taq™ II (Takara), 10 pmol each of the forward and reverse primers, 40 ng of HepG2 or BPAEC cDNA and distilled water to a final volume of 20 μl. To create a standard curve, a series of diluted standard plasmids, ranging from 1.8×10^7 to 1.8×10^3 copies/μl, was used instead of HepG2 or BPAEC cDNA and were amplified at the same time. The thermocycling conditions were 95°C for 10 s, following by 40 cycles of 95°C for 5 s and 60°C for 31 s.

Species-specific primers against a region of low-homology between human and bovine cDNA were designed using Primer Express Software (ver. 2.0, Applied Biosystems) (Table 1).

Immunofluorescence Staining for Fibronectin—Cultured cells surfaces were washed three times with PBS and the cells were then fixed in 4% paraformaldehyde in PBS for 20 min. After permeabilization with 0.5% Triton X-100 in PBS for 5 min, the cells were blocked with 3% bovine serum albumin in PBS for 10 min and

Table 1. **Sequences of oligonucleotide primers for real-time PCR.**

Gene name	Sequence
Human Integrin α4	Forward: 5′-GGGAGTGATCAAATT AGAAGGCAAT-3′
	Reverse: 5′-GTGGCAGGGACTTCATAT AAAGAAA-3′
Human GAPDH	Forward: 5′-GGAGTCCCTGCCACACTCAG-3′
	Reverse: 5′-GGCCCCTCCCCTCTTCA-3′
Bovine Decorin	Forward: 5′-GCTGAAGAGCTCAGGCATTGA-3′
	Reverse: 5′-ATGCGGATGTAGGAGAGCTTCT-3′
Bovine Fibronectin	Forward: 5′-GAAGGCAGCGGACGTATCAC-3′
	Reverse: 5′-TCCCCCGGTTGTCCTTCT-3′
Bovine GAPDH	Forward: 5′-CATCTCCGCCACACTGAGAA-3′
	Reverse: 5′-CCCTTCCTCAGGGCCTTAGA-3′

incubated with rabbit anti-human fibronectin monoclonal antibody (SIGMA) solution (1:50) for 1 h in a moist chamber. This antibody cross-reacts with bovine fibronectin. After three washes with 0.1% BSA–PBS, cells were incubated with goat anti-mouse secondary antibody laveled with Alexa 594 (Invitrogen) solution (1:200) for 1 h at 37°C.

The nuclei were stained with 0.01 mM Hoechst 333258 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in PBS for 10 min at room temperature. Confocal microscopy was performed using a Zeiss LSM 510 microscope (Carl Zeiss, Oberkochen, Germany).

Statistical Analysis—Data is presented as mean ± SD. Single-group comparisons were evaluated using the Wilcoxon rank sum test. $P < 0.05$ was regarded as significant.

RESULTS

Gene Expression of Various ECM and Adhesion Molecules Increased in Layered Co-cultured HepG2 Cells—In order to investigate the changes in gene expression between layered co-cultured and monolayer cultured HepG2 cells, we carried out PCR array assays (Table 2). We used a commercial RT^2 profiler PCR Array, which can detect changes in the expression of 84 genes encoding human ECM proteins, including basement membrane constituents, genes playing a role in ECM structure, molecules important to cell–cell and cell–matrix adhesion, transmembrane molecules and others. We previously reported that in the layered co-cultured cells, the gene-expression levels of albumin and cytochrome P450 were significantly increased and peaked at 20 days after the BPAEC sheets were overlaid (*4*), so PCR arrays were performed with cells cultured for 20 days after overlaying. Of the 84 genes contained in this array system, 37 genes in layered co-cultured cells were expressed at levels at least two times higher than in the monolayer cultured HepG2 cells (Table 2). Five transmembrane molecules, one cell–cell adhesion molecule, eleven cell–matrix adhesion molecules, six other adhesion molecules, three basement membrane constituents, one collagen and ECM structural constituent, seven ECM proteases, one ECM protease inhibitor, and the genes for two other ECM molecules showed a greater than 2-fold increase in their expression levels. Meanwhile, three genes were expressed at levels at least two times lower in the layered co-cultured HepG2 cells. These results indicate that in a layered co-culture system, gene-expression levels of various ECM and adhesion molecules increase, implying that the extracellular environment is vastly different from that of monolayer cultured HepG2 cells.

The Gene-Expression Level of Human Integrin α4 Gradually Increased in Layered Co-cultured HepG2 Cells—The results of the array analysis show that the expression of nine integrin subunits genes increased in layered co-cultured HepG2 cells. Notably, integrin α3 and integrin α4 genes were expressed at levels at least five times higher than in the monolayer cultured HepG2 cells (Table 2). To analyse the chronological changes in the expression of integrin α3 and integrin α4 genes, we performed quantitative real-time PCR analysis using primers specific for human genes on days 0, 5, 10, 15 and 20 after layering. The primers used were designed from regions of low homology between human and bovine cDNA to avoid cross-amplification of bovine integrin α4 (Table 1). Human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) from each cell was used as the internal control. Layered co-culturing induced human integrin α4 mRNA expression in a time-dependent manner (Fig. 1A). The gene expression of integrin α4 gradually increased from 5 days after layering and continued increasing until day 20. The gene expression of human integrin α3 did not change in the layered co-cultured and monolayer cultured HepG2 cells (data not shown).

The Gene Expression of Bovine Decorin Was Increased Until Day 15 in Layered Co-culture Cells—We used the PCR array system for human genes to analyse gene-expression changes in HepG2 cells. To investigate heterotypic cellular interactions in the layered co-culture system, we measured the gene-expression levels of ECM proteins in BPAEC. We performed real-time PCR analysis on days 0, 5, 10, 15 and 20 after layering using primers specific for bovine decorin and fibronectin genes. Bovine glyceraldehyde-3-phosphate dehydrogenase (bGAPDH) was used as the internal control. The expression of the bovine decorin gene gradually increased after layering and peaked at 15 days in layered co-culture cells and decreased thereafter (Fig. 1B). At 15 days, decorin gene expression increased greater than 6-fold in layered co-culture cells. The results of PCR array showed integrin α3 and laminin α2 genes induction were observed (Table 2). To confirm the induction of these genes, the mRNA induction levels of these genes were determined by real-time PCR. No induction mRNA expressions were found for the integrin α3 and laminin α2 genes (data not shown). These results suggest that the induction of integrin α3 and laminin α2 genes observed in the PCR array analysis were false-positive results.

An Organized Branching Network of Fibronectin Fibrils Was Observed in the Layered Co-culture Cells—Integrin has its own binding specificity and signalling properties, and activated integrin α4β1 has been reported to support the assembly of fibronectin fibrils (*14*, *15*). The results of the array analysis showed that the gene expression of human fibronectin in layered co-cultured HepG2 cells was equal to that in monolayer cultured cells (data not shown). Similarly, there was no significant time-dependent change the gene expression of bovine fibronectin between layered co-cultured and monolayer cultured cells (Fig. 1C). These results suggest that the gene expression of fibronectin is not induced by layered co-culturing. To analyse the fibronectin matrix assembly in a layered co-culture system, we performed immunofluorescence staining with an antibody that can detect fibronectin derived from both HepG2 cells and BPAEC (Fig. 2). Fibronectin and cellular nuclei of both HepG2 cells and BPAEC were double stained with Alexa594-labelled fibronectin antibody and Hoechst 33258 in both layered co-cultured and monolayer cells cultured at 20 days. Confocal laser-scanning microscopic analysis was performed for both the x–z optical cross-sectional

Table 2. **ECM and adhesion molecules gene-expression profile of layered co-culture of HepG2 cells and BPAEC sheets.**

Accession no.	Gene name	Fold induction
Transmembrane molecules		
NM_000615	Neural cell-adhesion molecule 1	2.7
NM_000442	Platelet/endothelial cell-adhesion molecule (CD31 antigen)	4.4
NM_000450	Selectin E (endothelial adhesion molecule 1)	11.2
NM_000655	Selectin L (lymphocyte adhesion molecule 1)	2.2
NM_003119	Spastic paraplegia 7, paraplegin (pure and complicated autosomal recessive)	2.0
Cell–cell adhesion		
NM_004360	Cadherin 1, type 1, E-cadherin (epithelial)	2.7
Cell-matrix adhesion		
NM_000610	CD44 molecule (Indian blood group)	8.3
NM_181501	Integrin, α1	2.9
NM_002203	Integrin, α2 (CD49B, α2 subunit of VLA-2 receptor)	2.3
NM_002204	Integrin, α3 (antigen CD49C, α3 subunit of VLA-3 receptor)	9.2
NM_000885	Integrin, α4 (antigen CD49D, α4 subunit of VLA-4 receptor)	5.3
NM_002205	Integrin, α5 (fibronectin receptor, α polypeptide)	2.7
NM_002206	Integrin, α7	3.9
NM_000632	Integrin, αM (complement component 3 receptor 3 subunit)	3.9
NM_002211	Integrin, β1 (fibronectin receptor, β polypeptide, antigen CD29 includes MDF2, MSK12)	4.0
NM_000212	Integrin, β3 (platelet glycoprotein IIIa, antigen CD61)	3.3
NM_003919	Sarcoglycan, epsilon	8.2
Other adhesion molecules		
NM_001904	Catenin (cadherin-associated protein), β1, 88kDa	3.1
NM_001332	Catenin (cadherin-associated protein), delta 2 (neural plakophilin-related arm-repeat protein)	−2.2
NM_003278	Tetranectin (plasminogen binding protein)	4.5
NM_002160	Tenascin C (hexabrachion)	4.8
NM_003247	Thrombospondin 2	14.6
NM_004385	Versican	2.4
NM_000638	Vitronectin	2.6
Basement membrane constituents		
NM_000426	Laminin, α2 (merosin, congenital muscular dystrophy)	13.7
NM_002291	Laminin, β1	2.2
NM_002293	Laminin, γ1 (formerly LAMB2)	3.4
NM_003118	Secreted protein, acidic, cysteine-rich (osteonectin)	−2.8
Collagens and ECM structural constituents		
NM_004370	Collagen, type XII, α1	−11.1
NM_001850	Collagen, type VIII, α1	3.5
ECM proteases		
NM_006988	ADAM metallopeptidase with thrombospondin type 1 motif, 1	9.7
NM_007037	ADAM metallopeptidase with thrombospondin type 1 motif, 8	2.6
NM_139028	ADAM metallopeptidase with thrombospondin type 1 motif, 13	5.2
NM_005940	Matrix metallopeptidase 11 (stromelysin 3)	2.7
NM_002427	Matrix metallopeptidase 13 (collagenase 3)	16.5
NM_005941	Matrix metallopeptidase 16 (membrane-inserted)	9.5
NM_002423	Matrix metallopeptidase 7 (matrilysin, uterine)	10.7
ECM proteases inhibitors		
NM_000362	TIMP metallopeptidase inhibitor 3 (Sorsby fundus dystrophy, pseudoinflammatory)	2.4
Other ECM molecules		
NM_001901	CConnective tissue growth factor	2.1
NM_000358	Transforming growth factor, β-induced, 68 kDa	4.7

Genes that changed greater than 2-fold by double-layered culture at 20 days after overlaying a bovine pulmonary artery endothelial cells (BPAEC) sheet onto hepatic HepG2 cells as determined by RT^2 *Profiler*^{TM} PCR Array: Human Extracellular Matrix and Adhesion Molecules.

view (Fig. 2a, d, g) and standard x–y sections (Fig. 2b–c, e–f, h–i). In the layered co-culture system, fibronectin was visualized as layers under both BPAEC and HepG2 cells (Fig. 2a, d). An organized branching network of fibronectin fibrils was observed around both BPAEC and HepG2 cells (Fig. 2e–f); however, at 5 days culture, fibronectin fibrils were observed as a single layer around BPAEC (Fig. 2b–c). These results suggest that a branching network of fibronectin fibrils around HepG2 cells was assembled during culturing in a layered

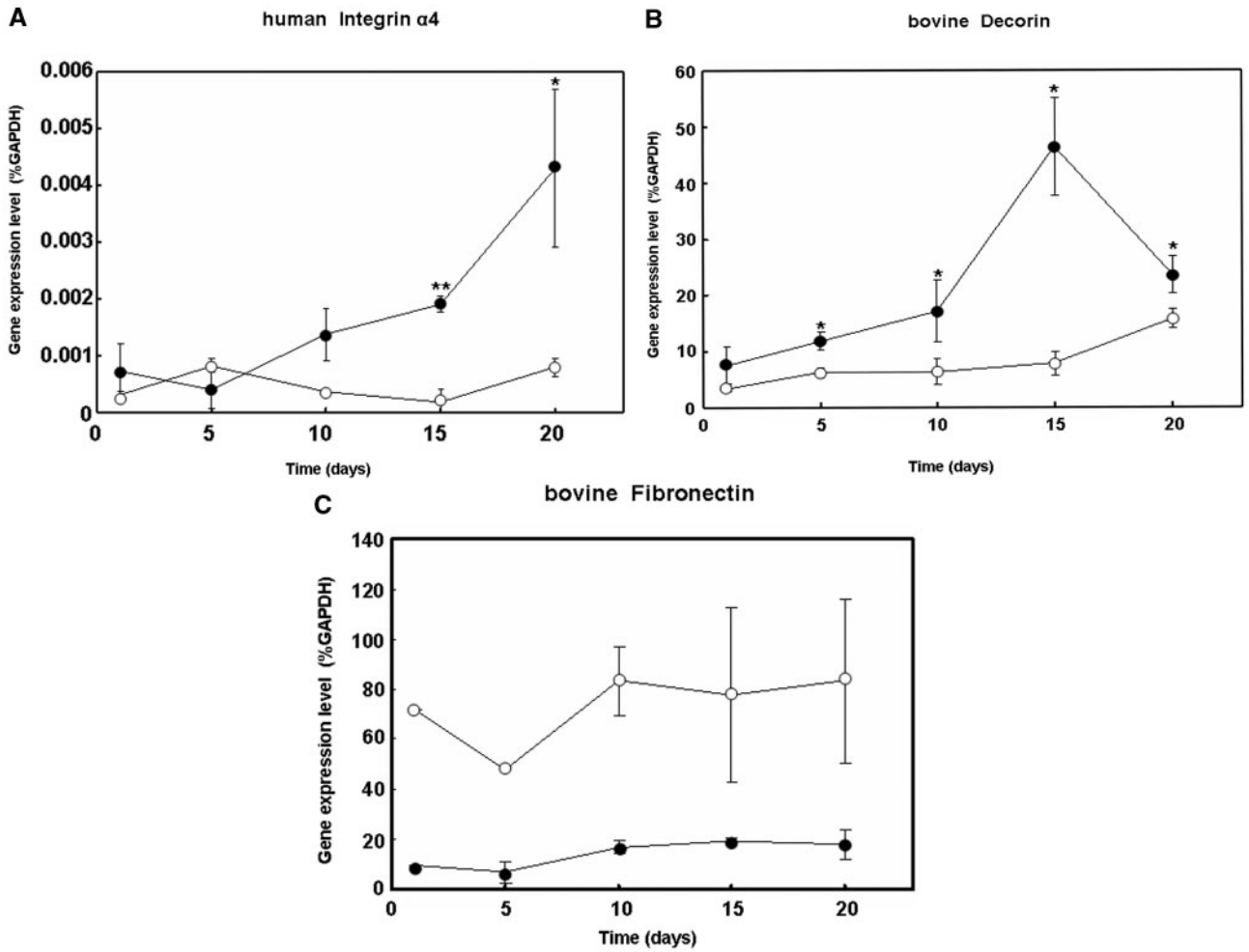

Fig. 1. **Comparison of (A) human integrin α4, (B) bovine decorin, and (C) bovine fibronectin gene-expression levels between layered co-culture cells (solid circle) and monolayer-cultured HepG2 cells (open circle) at different time points after overlaying the bovine pulmonary artery endothelial cell sheet onto HepG2 cells.** Total RNA was isolated from the cell sheets. After synthesizing cDNA from total RNA, gene-expression levels were analysed by real-time PCR. Data were normalized to corresponding human GAPDH or bovine GAPDH gene expression levels. Data represent the means ± standard deviation from four separate experiments. Error bars indicate the standard deviation of mean changes. $^{*}P < 0.05$, $^{**}P < 0.01$.

co-culture system. Fibronectin was also visible in monolayer-cultured cells; however, the fibrils were considerably shorter and rarely extended between HepG2 cells (Fig. 2h–i). These results suggest that a layered co-culture system provides the ability to assemble a fibronectin matrix to HepG2 cells.

DISCUSSION

In a layered co-culture system with HepG2 and endothelial cells forming a sheet using a thermo-responsive culture surface, the molecular composition of ECM and cell-adhesion molecules and the structure of ECM changed greatly in conjunction with an increase in the liver-specific function of HepG2 cells.

Decorin, a member of the small leucine-rich proteoglycan gene family, plays key roles in modulating collagen fibrillogenesis and cell proliferation (*16*, *17*). Decorin leads to protracted down-regulation of the epidermal growth factor receptor (EGFR) tyrosine kinase and other members of the ErbB family of receptor tyrosine kinases and causes protracted internalization and degradation of EGFRs primarily through a caveolar-mediated pathway (*18–20*). This leads to the protracted induction of endogenous $p21^{WAF1}$, a potent inhibitor of cycline-dependent kinases, and ultimate cell-cycle arrest in the G_1 phase (*21*, *22*). Moreover, decorin binds to collagen and may regulate fibrillogenesis and collagen phagocytosis (*23*, *24*). Cross-sectional views of confocal laser microscopy showed that proliferation of layered co-cultured HepG2 cells was reduced compared to monolayer culture, and HepG2 cells gradually attained a cuboidal shape (*4*). The gene expression of bovine decorin was gradually increased after layering and peaked at 15 days in layered co-culture cells and decreased thereafter (Fig. 1B). These results suggest that increases in the gene expression of decorin suppresses aberrant signalling events, thereby influencing

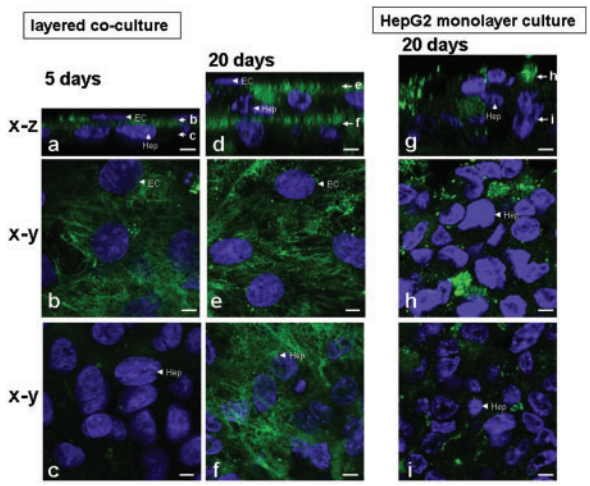

Fig. 2. **Expression and localization of fibronectin in layered co-culture cells after overlaying with bovine pulmonary artery endothelial cell sheet at 5 days after layering (a–c), at 20 days after layering (d–f), and monolayer cultured cells at 20 days (g–i) by immunofluorescence analysis.** Cells were fixed, permeabilized, and stained with anti-fibronectin monoclonal antibody and Alexa 594 anti-mouse IgG (green). Cellular nuclei were visualized with Hoechst 33258 (blue). Top panels are confocal laser micrographs of the *x–z* optical cross-sectional view in co-cultured (a, d) and monolayer cultured cells (g). Lower panels are standard confocal *x–y* sections in layered co-cultured (b–c, e–f) and monolayer cultured cells (h–i). EC: bovine pulmonary artery endothelial cell, Hep: HepG2 cells, Bars: 5 μm.

ECM assembly and inducing increased liver-specific functions in layered co-culture cells.

Integrins are the major cell-surface receptors connecting cells to the surrounding ECM. They not only support cell attachment, but also play important roles in ECM assembly and mediate adhesion to the ECM and transducer signals (*14*, *25–28*). In layered co-cultured HepG2 cells, the time-course pattern of the expression of human integrin α4 correlated with that of liver-specific genes (Fig. 1A) (*4*). This result suggests that signal transduction was gradually increased by integrin α4 receptors to the extracellular matrix, and it influenced the increased liver-specific function in layered co-cultured HepG2 cells. An RGD-independent mechanism of integrin α4β1-mediated fibril assembly can cause changes in fibronectin conformation and maintain connections as cells pull on fibrils (*29*, *30*). In the layered co-culture cells, the gene expression of fibronectin was not induced (Fig. 1C). However, a branching network of fibronectin fibrils around HepG2 cells was assembled during culturing in the layered co-culture system, but not in monolayer HepG2 cells (Fig. 2). A layered co-culture system apparently provides the ability to assemble a fibronectin matrix to HepG2 cells. Tumor cells frequently exhibit decreased adhesiveness due to a failure to deposit stromal fibronectin, permitting more rapid proliferation, migration, invasion and metastasis (*31*, *32*). Liu *et al*. reported that down-regulation of fibronectin significantly enhances MAGE A3 expression. MAGE A3, as a downstream target of fibronectin, accelerates cell-cycle progression through p21 reduction, an effect facilitated by diminished p53 levels (*33*). In a layered co-culture system, an organized branching network of fibronectin fibrils may act as an inhibitor of HepG2 cell proliferation and metastasis.

The gene expression of the basement membrane constituents increased in layered co-culture cells (Table 2). The expression of human laminin α5 genes was gradually increased in a time-dependent manner (data not shown). The basement membrane can regulate cellular adhesion, proliferation and differentiation (*34*, *35*). Matrigel, which contains several basement membrane proteins, has been used to maintain the liver-specific functions of hepatocytes (*9*, *10*). The molecular composition of the ECM in our layered co-culture cells was similar to that of the basement membrane, which can influence cellular functions.

In HepG2 spheroids, strong homophilic interaction of E-cadherin has been reported as a major factor for morphological transition (*36*). In our layered co-culture cells, the gene expression of human E-cadherin was 2.7-fold higher than in monolayer cultured HepG2 cells (Table 2); however, the gene expression of many kinds of ECM and cell-adhesion molecules was increased in the layered co-culture cells. In our layered co-culture cells, heterotypic interactions through heterotypic secreted ECM play a fundamental role and can induce increased liver-specific functions.

Our analysis may aid in understanding how the specificity of the extracellular environment can regulate liver-specific functioning in hepatocytes. The layered co-culture system may be a valuable model to study the role of heterotypic cell–cell and cell–matrix interactions in hepatocyte functional maintenance. A greater understanding of how cell–cell and cell–matrix interactions modulate tissue functions will generate ideas for manipulating liver tissue functions *in vitro* for therapeutic and drug-screening applications.

CONFLICT OF INTEREST

None declared.

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