Theme: Chemistry for Health
Symposium: The Chemistry-Biology Interface: Drug Targets and Diagnostics

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The overlap between chemistry and biology is increasing as many scientists focus on this rapidly developing interface in order to achieve a better balance between research and real-world applications. One of the most exciting, promising and innovative areas at this chemistry-biology interface is the area of Drug Targets and Diagnostics, encompassing subjects such as cell signalling, proteomics/genomics, drug delivery, tissue engineering, biomarkers and diagnostics.

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Directing hepatic differentiation of embryonic stem cells with protein microarray-based co-cultures

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Embryonic stem cells hold considerable promise in tissue engineering and regenerative medicine as a source of tissue-specific cells. However, realizing this promise requires novel methods for guiding lineage-specific differentiation of stem cells. In this study, we developed a micropatterned co-culture platform for stimulating hepatic differentiation of mouse embryonic stem cells (mESCs). Studies of mESC and hepatic cell adhesion preferences revealed that mESCs required fibronectin for attachment, while hepatic cells (HepG2) preferred collagen (I) substrate and did not adhere to fibronectin. Printing columns of collagen (I) and fibronectin spots (300 μm diameter), followed by sequential seeding of the two cell types, allowed the positioning of clusters of mESCs adjacent to groups of hepatic cells within the same microarray. These micropatterned co-cultures were maintained for up to two weeks in hepatic differentiation media supplemented. To examine the differentiation, mESCs were selectively extracted from the co-culture using laser microdissection and analyzed using real-time reverse transcriptase (RT)-polymerase chain reaction (PCR). These analyses revealed that mESCs co-cultured with HepG2 cells showed a decrease in pluripotency gene expression concomitant with up-regulation of endodermal genes. In addition, the co-culture format induced a significant increase in the expression of liver genes compared to mESCs cultured alone. In conclusion, micropatterned co-cultures of mESCs and hepatic cells showed a significant promise in driving stem cell differentiation towards hepatic phenotype. In the future, this cell culture platform will be further enhanced to enable efficient conversion of mouse and human ESCs to hepatocytes.

Introduction

Given the shortage of donor livers, the concept of liver-related cell therapies has emerged as an alternative strategy. However, the scarcity of human hepatocytes remains a serious roadblock in the development of cell-based therapies. Embryonic stem cells (ESCs) are capable of unlimited self-renewal and can acquire any cell phenotype, thereby offering an ideal source of hepatocytes. However, the efficiency of stem cell conversion to hepatocytes, as well as other terminally differentiated cells, remains low, confounding the use of ESCs as a source of mature tissue-specific cells.

To promote the differentiation of ESCs towards a hepatic fate in vitro, it is important to define a microenvironment or niche conducive to liver-specific commitment of stem cells. The microenvironment encompasses a diverse set of cues including mature hepatocytes, non-parenchymal liver

Insight, innovation, integration

This paper describes novel micropatterned co-cultures of mouse embryonic stem cells and hepatocytes created on matrix protein arrays. Our method allowed placing stem cells and hepatocytes on the same surface separated by a distance of tens of micrometers. Spatial segregation of cell types afforded by micropatterning allowed the selective retrieval of stem cells from the co-cultures using laser-capturating (a variant of laser capture microdissection) for downstream gene expression studies. This analysis revealed enhancement of hepatic gene expression in embryonic stem cells co-cultured with hepatocytes compared to stem cells cultured alone. Overall, micropatterned co-cultures described here provide a general strategy for investigating the effects of heterotypic interactions on tissue-specific lineage selection of stem cells.
cells, recruited inflammatory cells, as well as secreted
growth-modulating molecules and the extracellular matrix
(ECM). Therefore, research efforts for directing mouse
or human ESCs towards hepatic lineage have sought to
recapitulate aspects of the liver microenvironment through
the incorporation of growth and differentiation factors, ECM
coatings, constitutive expression of hepatic transcription
factors, as well as co-cultivation with other liver paranchymal
or non-paranchymal cells.¹

The co-culture system has been widely used to maintain the
function of hepatocytes in vitro and is thought to better mimic
the intercellular contacts and endocrine signaling observed
in vivo.² Co-cultures have been employed for long-term
phenotype maintenance in primary hepatocytes,³,⁴ for
constructing in vitro liver models for hepatic fibrosis or
bioartificial livers,⁵,⁶ increasing liver-specific functions of
fetal hepatocytes,⁷ as well as differentiating stem cells
into specific lineages.³,⁸,⁹ Co-cultivation of two cell types
in a random configuration limits the ability to control
the extent of cell–cell interactions. To remedy this, micro-
patterning approaches including photoresist photolithography,⁶
polymer microwells,⁹,¹⁰ polymer microwells,¹¹ electrochemically
switchable surfaces¹²,¹³ and robotically printed microarrays¹⁴,¹⁵
have been used to create micropatterned co-cultures of
adult cells.

A small number of reports have investigated co-cultures of
liver cells with ESCs,¹⁶–¹⁸ mesenchymal stem cells,¹⁹ or
hematopoietic stem cells²⁰ in order to bias stem cells towards
hepatic lineage selection. The goals of the present study were
(1) to develop a novel strategy for placing small groups of
hepatocytes next to clusters of stem cells and (2) to
analyze stem cell gene expression without the loss of the local
microenvironment context. To achieve the first goal, printed
arrays of ECM proteins were employed to identify the
adhesion preferences of mouse embryonic stem cells
(mESCs) and hepatic cells. Printing of cell type-specific
ECM components as alternating columns of spots (300 μm
diameter) followed by sequential seeding of the two cell types
allowed the positional control of small groups of
hepatocytes next to clusters of stem cells and (2) to
analyze stem cell gene expression without the loss of the local
microenvironment context. To achieve the first goal, printed
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adhesion preferences of mouse embryonic stem cells
(mESCs) and hepatic cells. Printing of cell type-specific
ECM components as alternating columns of spots (300 μm
diameter) followed by sequential seeding of the two cell types
allowed the positioning of small groups of mESCs next to
islands of hepatic cells (see Fig. 1). Importantly, spatial
segregation of cell types allowed the selective retrieval of stem
cells using laser catapulting for downstream gene expression
studies by RT-PCR. The laser catapulting/RT-PCR tandem
permitted the analysis of stem cell gene expression in the
context of local microenvironment and pointed to enhancement
of hepatic gene expression in stem cell–hepatocyte co-cultures.

In addition, this location-specific analysis revealed phenotypic
differences in gene expression between clusters of mESCs next to
cultures of mature hepatocytes, with stronger expression of
hepatic-related genes in the interface and weaker in the center of the stem cell cluster. In summary,
this paper describes a novel method for creating stem cell
co-cultures based on cell attachment to protein microarrays
and proposes a novel method for analyzing stem cell
function without losing local microenvironment context. Beyond mESC–hepatic co-cultures, protein microarrays
may offer a general strategy for placing stem cells next to
differentiated cells in order to investigate the effects of
eheterotypic interactions on tissue-specific lineage selection of
stem cells.

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**Materials and methods**

**Chemicals and materials**

Glass slides (75 × 25 mm) were obtained from VWR international (West Chester, PA). 3-Acryloyxpropyl trichlorosilane
was purchased from Gelest, Inc (Morrisville, PA). Sulfuric acid, hydrogen peroxide, ethanol, collagen from rat tail (type I),
collagen from Engelbreth–Holm–Swarm murine sarcoma basement membrane (type IV), laminin, epidermal growth
factor, bovine serum albumin, dexamethasone and Tween 20
were obtained from Sigma-Aldrich (St. Louis, MO). Phosphate-buffered saline (PBS) 10x was purchased from Cambrex (Charles City, IA). Dulbecco’s modified Eagles’ medium (DMEM), minimal essential medium (MEM), Iscove’s Modified Dulbecco’s Medium (IMDM), sodium pyruvate,
non-essential amino acids, l-glutamine, ES-qualified fetal
bovine serum (FBS), certified FBS, 2-mercaptoethanol,
CellTracker™ Green CMFDA and Red CMTPX probes
for long-term tracing of living cells, and 4′,6-diamidino-2-
phenylindole, diacetate (DAPI) were purchased from Invitrogen
Life Technologies (Carlsbad, CA). QuantiTect Reverse
Transcription Kit was purchased from Qiagen (Valencia, CA). FastStart Universal SYBR Master Mix was purchased from Roche (Indianapolis, IN). Glucagon and insulin were
obtained from Eli-Lilly (Indianapolis, IN). ESGRO (leukemia
inhibitory factor: LIF), primary mouse embryo fibroblasts,
fibronectin, and ES cell characterization kit were obtained from Millipore (Temecula, CA). Monoclonal anti-human/mouse α-fetoprotein antibody was purchased from R&D Systems (Minneapolis, MN). Anti-mouse IgG FITC conjugated was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Mouse embryonic stem cells (D3) and hepatic cells (HepG2) were purchased from ATCC (Manassas, VA).

Surface modification

Glass slides were cleaned by immersion in piranha solution consisting of a 3 : 1 mixture of concentrated sulfuric acid and 35% w/v of hydrogen peroxide for 10 min. The glass slides were thoroughly rinsed with deionized (DI) water and dried under nitrogen. For silane modification, the glass slides were treated in an oxygen plasma chamber (YES-R3, San Jose, CA) at 300 W for 5 min and then placed in a solution containing 3-acryloyloxytrimethoxysilane in anhydrous toluene (20 μL per 40 mL) for 10 min. The reaction was performed in a glove box filled with nitrogen to eliminate atmospheric moisture. The slides were rinsed with fresh toluene, dried under nitrogen and cured at 100 °C for 2 h. The silane quality was assessed using LSE Stokes ellipsometer (Gaertner Scientific, Chicago, IL) and contact angle goniometer (Rame-Hart, Netcong, NJ). The silane-modified glass slides were stored in a desiccator before use.

Protein microarraying

All ECM proteins were dissolved in 1 X PBS at a 0.2 mg mL⁻¹ concentration, with the addition of 0.005% (v/v) Tween 20. Protein microarrays were contact-printed under ambient conditions on silane-modified glass slides using a MicroCaster hand-held microarrayer system (Schleicher & Schuell) or GMS 417 robotic arrayer (Genetic Micro Systems, Inc.). Collagen (I) and fibronectin were arrayed in alternating columns to create a six-by-twelve array of spots. In the case of MicroCaster, the spot size was around 500 μm in diameter. The center-to-center distance between the spots was 1250 μm. With a GMS 417 arrayer, spots of 300 μm diameter were arrayed with 375 μm pitch. The glass slides with the protein microarrays were stored in a refrigerator for at least one month without detriment to arrays.

Construction of micropatterned co-cultures of mESCs and hepatic cells

Mouse ESCs (D3 cells) were maintained with growth-arrested murine embryonic fibroblast (MEF) feeder cells on gelatin-coated tissue culture plates at 37 °C in a humidified 5% CO₂ atmosphere. The culture medium consisted of DMEM supplemented with 15% ES-qualified FBS, 200 U mL⁻¹ penicillin, 200 μg mL⁻¹ streptomycin, 2 mM L-glutamine, 1 mM nonessential amino acids, 100 nM 2-mercaptoethanol, and 1000 U mL⁻¹ LIF. For the cell seeding experiments, the glass slides containing protein microarray were cut and placed into wells of a conventional six-well plate. The samples were sterilized with 70% ethanol, and washed twice with 1 X PBS. Cellular micropatterning was carried out by exposing glass slides to D3 cell suspension in culture medium at a concentration of 1 X 10⁶ cells mL⁻¹. After 1 h of incubation at 37 °C, the medium containing unattached cells was removed and the surfaces were washed twice with 1 X PBS. The cell patterns formed on the glass slide were imaged by a brightfield microscope (Carl Zeiss Inc., Thornwood, NJ).

Hepatoma cells (HepG2) were employed as model hepatocytes in our studies. HepG2 cells were maintained in MEM supplemented with 10% FBS, 200 U mL⁻¹ penicillin, 200 μg mL⁻¹ streptomycin, 1 mM sodium pyruvate, and 1 mM nonessential amino acids at 37 °C in a humidified 5% CO₂ atmosphere. To introduce HepG2 cells, glass slides containing surface-bound arrays of mESCs were exposed to HepG2 cell suspension at a concentration of 1 X 10⁶ cells mL⁻¹. After 1 h of incubation at 37 °C, unbound cells were removed by washing with 1 X PBS twice and the culture was maintained in differentiation medium consisting of IMDM supplemented with 20% FBS, 200 U/mL penicillin, 200 μg mL⁻¹ streptomycin, 1 mM nonessential amino acids, 0.5 U mL⁻¹ insulin, 14 ng mL⁻¹ glucagon, and 100 nM dexamethasone.

Alkaline phosphatase staining and intracellular immunostaining

Alkaline phosphatase activity of ES cells was assessed using an ES characterization kit according to the manufacturer’s instructions. For the AFP immunostaining, cells were fixed with 4% paraformaldehyde in PBS for 20 min and then permeabilized with 0.2% Triton X-100 for 5 min. The cells were then incubated in blocking solution (1% BSA in 1 X PBS) for 1 h at room temperature and exposed to 1:100 diluted α-fetoprotein (AFP) antibody overnight at 4 °C. The following day, the cells were incubated in 1:50 diluted secondary antibodies conjugated with FITC and then they were counterstained with DAPI. The cells were washed between each step with 1 X PBS containing 0.005% Tween 20 three times for 5 min. All incubations were performed at room temperature if not specified. The stained cells were visualized and imaged using a confocal microscope (Zeiss LSM Pascal).

Laser catapulting of stem cells and RT-PCR analysis of gene expression

Prior to laser catapulting, cells on glass slides were fixed with ice-cold 70% ethanol, and dried under nitrogen. Fixed cells were stored in an airtight container at −80 °C and were catapulted within 2 w. ES cells were retrieved from micropatterned surfaces by the PALM LMPC system (PALM Microlaser Technologies). Extracted cells were stabilized in 200 μL of 1 X Applied Biosystems (AB) lysis buffer and stored at −20 °C. Total RNA was extracted from the cell lysates using a 6100 Nucleic Acid PrepStation (Applied Biosystems) according to the manufacturer’s instructions. The RNA from extracted cells was precipitated and resuspended in DI water. cDNA was synthesized using Quantitect Reverse Transcription Kit according to the manufacturer’s instructions. Briefly, 4 μL of wipeout buffer was added to the RNA sample (20 μL) and the sample was incubated at 42 °C for 2 min. Then, 16 μL of Reverse Transcription Mix was added and the sample was incubated at 42 °C for 40 min. The reaction was terminated by heating the sample for 3 min at 95 °C.

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The oligonucleotide sequences for genes of pluripotency, Oct4 and Nanog, were adapted from the literature.24 The oligonucleotide sequences for genes of three germ layers, β-fetoprotein (Afp) and Sox17 (endoderm), Acta (mesoderm) and β-tubulin 3 (Tubb3, ectoderm), and β-actin (housekeeping gene) were adapted from the Real-Time PCR Primer and Probe Database (http://medgen.ugent.be/rtprimerdb). The oligonucleotide sequences for liver-associated genes, β-glutamyl transferase (Ggt), albumin, transthyretine, glucose-6-phosphatase, γ-glutamyl transferase, have been reported previously. SYBR real-time PCR was performed using FastStart Universal SYBR Master (Rox) Mix. Primer (Sigma Genosys) concentrations were optimized before use. SYBR Green Master Mix was used with the appropriate concentrations of forward and reverse primers (Table 1), and cDNA in a total volume of 12 μL. All PCR reactions were done in duplicate. PCR amplification was performed as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 10 s and 68 °C for 1 min on Mastercycler Realplex (Eppendorf). The relative expression level of each gene was calculated using the comparative threshold cycle (Ct) method using β-actin as a housekeeping gene and an internal standard. Median Ct values of duplicate samples were used to calculate ΔCt of the housekeeping gene for the same sample. A denaturing curve for each gene was used to confirm homogeneity of the PCR product. We found it challenging to average out PCR results from several experiments as ΔCt values varied from one experiment to the next. Therefore, a representative PCR result (out of n = 3) is shown in this paper.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 5’ to 3’</th>
<th>Conc./μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>F: ACGGCCAGGTGCATCTACATTG</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>R: ATACCCCAAGAGGAGGCTGGA</td>
<td>0.5</td>
</tr>
<tr>
<td>Nanog</td>
<td>F: CCTAAGGCTCCACAGATGC</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>R: CGGCTTGGATCCTACCCCCCTG</td>
<td>1</td>
</tr>
<tr>
<td>Oct-4</td>
<td>F: GAAGAGAAGAGATTGACCTTG</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>R: TCTTTAGGCTGAGCTGCAAG</td>
<td>1</td>
</tr>
<tr>
<td>Sox17</td>
<td>F: CGAGCCCAGGAGGCTGTC</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>R: TGCCAAGTCCAGGCTCCT</td>
<td>1</td>
</tr>
<tr>
<td>β-Tubulin 3 (Tubb3)</td>
<td>F: CCTAAGGCTCCACAGATGC</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>R: AGGAGGCTGACAGACACATC</td>
<td>1</td>
</tr>
<tr>
<td>α-Actin, cardiac (Acta)</td>
<td>F: TTCCTGATCTCAGTAGGTA</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>R: TTGCGCTCATTGGAAGTGA</td>
<td>1</td>
</tr>
<tr>
<td>α-fetoprotein (Afp)</td>
<td>F: CGAGCAGAAGGACTGCTT</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>R: TACAGCTGACTGCAAG</td>
<td>1</td>
</tr>
<tr>
<td>Transthyretine (Ttr)</td>
<td>F: AGGACATTTGGATTCCAGCT</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>R: GGGCTTCTGGATCTGTGCA</td>
<td>1</td>
</tr>
<tr>
<td>Albumin (Alb)</td>
<td>F: GCCAGGCTGCTGCAAGA</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>R: GGCGTCTTTGCACTATGCA</td>
<td>1</td>
</tr>
<tr>
<td>Glucose-6-phosphatase (G6p)</td>
<td>F: TGCTGCTCCATTCGCCCTC</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>R: GGCTTACAGAGATCAAGAGATGC</td>
<td>1</td>
</tr>
<tr>
<td>γ-Glutamyl transferase (Ggt)</td>
<td>F: CGTTTGCTCATGACAGAGAG</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>R: AAATCCACCCACACTGACC</td>
<td>1</td>
</tr>
</tbody>
</table>

The oligonucleotide sequences for Oct4 and Nanog, were adapted from the literature.24 The oligonucleotide sequences for genes of three germ layers, α-fetoprotein (Afp) and Sox17 (endoderm), Acta (mesoderm) and β-tubulin 3 (Tubb3, ectoderm), and β-actin (housekeeping gene) were adapted from the Real-Time PCR Primer and Probe Database (http://medgen.ugent.be/rtprimerdb). The oligonucleotide sequences for liver-associated genes, β-glutamyl transferase (Ggt), albumin, transthyretine, glucose-6-phosphatase, γ-glutamyl transferase, have been reported previously. Quantitative real-time PCR was performed using FastStart Universal SYBR Master (Rox) Mix. Primer (Sigma Genosys) concentrations were optimized before use. SYBR Green Master Mix was used with the appropriate concentrations of forward and reverse primers (Table 1), and cDNA in a total volume of 12 μL. All PCR reactions were done in duplicate. PCR amplification was performed as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 10 s and 68 °C for 1 min on Mastercycler Realplex (Eppendorf). The relative expression level of each gene was calculated using the comparative threshold cycle (Ct) method using β-actin as a housekeeping gene and an internal standard. Median Ct values of duplicate samples were used to calculate ΔCt of the housekeeping gene for the same sample. A denaturing curve for each gene was used to confirm homogeneity of the PCR product. We found it challenging to average out PCR results from several experiments as ΔCt values varied from one experiment to the next. Therefore, a representative PCR result (out of n = 3) is shown in this paper.

### Results and discussion

In this study, ECM microarrays were employed to assemble micropatterned co-cultures of mESCs and hepatic cells. Preferential adhesion of mESCs and hepatic cells on fibronectin and collagen (I), respectively, allowed the placement of these cells on the adjacent protein islands (300 or 500 μm diameter) within the same cell microarray. Analysis of gene expression revealed enhanced endodermal and liver-specific differentiation of mESCs co-cultivated with hepatic cells compared to stem cell monocultures.

**Micropatterned stem cell co-cultures formed on protein arrays**

Both mESCs and hepatocytes require surfaces coated with ECM proteins for attachment and cultivation. Gelatin-coated plastic substrates are typically used for maintenance of mESCs, while hepatocytes attach and function well on substrates coated with collagen (I, IV) or laminin.26 The adhesion preferences of mESCs were tested by printing arrays of ECM proteins including collagen (I) and (IV), laminin, and fibronectin, followed by cell seeding. These cell adhesion studies (results not shown) revealed that after 1 h incubation, mESCs preferentially attached on fibronectin spots, with minimal adhesion observed on other matrix proteins. Conversely, when seeded on the ECM proteins discussed above, HepG2 cells did not adhere on fibronectin, whereas attachment could be observed on other ligands. To take advantage of the cell attachment preferences, columns of fibronectin and collagen (I) spots were printed onto silane-modified glass slides and the two cell types were sequentially seeded onto the same surface (see Fig. 1 for an overview of the process). We previously showed that glass surfaces modified with acylated silane resist attachment of hepatocytes, since these epithelial cells do not produce appreciable quantities of endogenous ECM proteins and therefore require substrates to be pre-coated with adhesive ligands.27 The silane-modified surfaces do become fouled by the protein adsorption from solution over time; however, incubation of hepatocytes or mESCs on these substrates for a short period of time (~ 1 h) allowed the confinement of these cells to the printed protein domains, with limited attachment observed on the silane-modified glass regions. Due to partial fouling of the silane-modified surfaces, cells were able to
expand and grow outside of the original attachment sites over time (days) in culture, as shown in Fig. 2.

Fig. 2A shows a close-up view of the protein spots after seeding mESCs. As can be seen from this image, stem cells are confined to specific locations on the surface (fibronectin domains), while collagen (I) spots, fluorescently stained for visualization, remain free of cells. The mESCs were allowed to proliferate on the spots for 24 hours to ensure formation of a confluent monolayer prior to seeding of hepatic cells. Minimal to no attachment of hepatic cells on top of the stem cells was observed during co-culture construction. To highlight the distinct spatial arrangement of stem cells and hepatic cells, the co-cultures were stained with alkaline phosphatase—a commonly used marker of stem cells. As shown in Fig. 2B, an intense alkaline phosphatase signal was observed from mESC clusters (right column), while a minimal signal was seen from hepatic cell clusters (left column of spots). The co-cultures arrangement of alternating columns of hepatic and stem cell clusters occurred on a large scale as shown in Fig. 2C. After adding hepatic cells, co-cultures were maintained in a differentiation media containing FBS, insulin, and dexamethasone for up to 10 days. As a control, mono-cultured ESCs were also maintained in the same media.

Fig. 2A shows a close-up view of the protein spots after seeding mESCs. As can be seen from this image, stem cells are confined to specific locations on the surface (fibronectin domains), while collagen (I) spots, fluorescently stained for visualization, remain free of cells. The mESCs were allowed to proliferate on the spots for 24 hours to ensure formation of a confluent monolayer prior to seeding of hepatic cells. Minimal to no attachment of hepatic cells on top of the stem cells was observed during co-culture construction. To highlight the distinct spatial arrangement of stem cells and hepatic cells, the co-cultures were stained with alkaline phosphatase—a commonly used marker of stem cells. As shown in Fig. 2B, an intense alkaline phosphatase signal was observed from mESC clusters (right column), while a minimal signal was seen from hepatic cell clusters (left column of spots). The co-cultures arrangement of alternating columns of hepatic and stem cell clusters occurred on a large scale as shown in Fig. 2C. After adding hepatic cells, co-cultures were maintained in a differentiation medium containing FBS, insulin, and dexamethasone for up to 10 days. As a control, mono-cultured ES cells were also maintained in the same medium.

After initial seeding, mESCs and hepatic cells have been attached to 300 μm diameter spots with 375 μm edge-to-edge spacing (Fig. 2A and B); however, as discussed above, silane-modification of the surfaces allowed for expansion of the cells out of their original locations. As shown in Fig. 2C–E, this expansion occurred in both mESC mono-cultures and co-cultures. In the case of co-cultures, cell proliferation and spatial expansion led to the creation of a hepatic–stem cell interface, where physical contact between the cell types was possible. Based on our microscopy observations, the boundary between mESCs and hepatic cells remained distinct and minimal re-sorting of the two cell types was observed.

Effects of mESC–hepatic cell co-cultures on pluripotency and germ layer selection

In order to assess the effects of hepatic cells on differentiation and maturation, mESCs gene expression indicative of pluripotency, germ layer selection and hepatic phenotype were analyzed. In a typical experiment, cells were enzymatically removed from a surface and then were subjected to gene expression analysis using RT-PCR. However, this method of nucleic acid collection does not allow the connection of gene expression to a local microenvironment experienced by cells during cultivation. The ability to capture cell function without the loss of the local microenvironment context is particularly appealing for micropatterned cultures where cells may be experiencing different signals at different locations of the same surface. Previously, we showed that laser microdissection-mediated cell retrieval from micropatterned co-cultures followed by RT-PCR analysis could be used for connecting cell phenotype to a location on a micropatterned surface. In the present study, the same approach was employed for analysis of mESC differentiation in the micropatterned co-cultures by removing stem cells at different cultivation time-points. Fig. 3A shows an example where the stem cell cluster has been removed (right side), but the hepatic cell

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As shown in Fig. 3B, the expression level of *Nanog* relative to a housekeeping gene, *β-actin*, at day 1 was similar to that of undifferentiated ESCs, which suggested that mESCs were largely pluripotent after 1 d of co-culture in differentiation media. (*Note:* all of the days in this paper mean ‘days after co-culture’. Therefore, day 1 indicates the second day of culture for ESCs.) Over the time in culture, the pluripotency gene expression of mESCs decreased gradually in both mono- and co-cultures; however, this decrease was more pronounced in the case of co-cultures. After 8 d of co-culture, *Nanog* was still observed, although the levels decreased to 35 and 17% in a mono-culture and co-culture respectively, compared to mESC maintained under conditions promoting pluripotency. The results presented in Fig. 3B imply that a certain population of stem cells remained undifferentiated and coexisted with more differentiated mESCs. Similar trends in the down-regulation of gene expression were observed with another pluripotency gene *Oct4* (data not shown).

We next examined the expression of the genes associated with three germ layers: endoderm (marker gene-*Sox17*), ectoderm (marker gene Class III *β*-tubulin (*Tubb3*)), and mesoderm (marker gene-cardiac *α*-actin (*Actac*)). *Sox17* is a Sry-related HMG-box transcription factor developmentally expressed in both the definitive endoderm and extraembryonic endoderm. The liver develops from a definitive endoderm; therefore, the expression of genes associated with this germ layer is an important prerequisite of hepatic differentiation of ESCs. *Tubb3* is specific for a neuronal differentiation and *Actac* is initially expressed in the endomesoderm, but subsequently in the mesoderm. Fig. 3C shows the expression level of each gene at day 1 and day 8. Interestingly, mESCs co-cultivated with hepatic cells showed a three-fold increase in *Sox17* gene expression compared to mESCs cultured alone in a differentiation medium. This up-regulation in endodermal gene expression was concomitant with decreased levels of mesoderm marker gene expression (*Actac*) and constant levels of ectoderm (*Tubb3*) gene.

The results shown in Fig. 3C suggest that co-cultivation with hepatic cells induced endodermal differentiation of mESCs—an important prerequisite of hepatic differentiation. It should be noted that some endodermal differentiation was observed in mESCs mono-cultures adherent on fibronectin microarray spots and cultivated in hepatic differentiation medium; however, this was considerably lower compared to co-cultures (Fig. 3C).

**Early and mature hepatic gene expression in mESCs co-cultures**

During liver development, hepatocytes originate from a definitive endoderm and the first evidence of hepatic specification within the endodermal cells is the up-regulation of transcripts of the genes encoding *α*-fetoprotein and albumin. *Afp* is widely used as a marker gene of definitive endoderm as well as early hepatic differentiation. *Alb* is a key marker for functional hepatocytes and is the most abundant protein synthesized by mature hepatocytes. In addition, another liver-specific serum protein, transthyretin (*Ttr*) (also known as pre-albumin), was used in our studies as an early liver marker. We compared the definitive endoderm and

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**Fig. 3 RT-PCR analysis of pluripotency and germ layer gene expression in stem cell micropatterns.** Stem cells were cultured in hepatic differentiation media either alone or together with hepatic cells (HepG2). (A) Mouse ESCs were selectively retrieved from the co-culture using laser catapulting and were immediately used in RT-PCR studies. (B) Expression of pluripotency gene *Nanog* decreased over time for both mono- and co-cultivated mESCs; however, the co-culture induced a more pronounced and more rapid decrease in *Nanog* expression. (C) Expression levels of germ layer genes at day 1 and day 8. The endodermal marker, *Sox17*, was up-regulated, while other germ layer markers remained unchanged or decreased.
early liver gene expression in mESCs cultured alone and in co-cultures with HepG2 cells. Liver gene expression in mouse a hepatoma cell line (Hepa 1-6) served as a benchmark for comparison in our stem cell differentiation studies.

As shown in Fig. 4A the early liver genes were detected from day 3 onwards in the mESC–hepatic cell co-cultures, but appeared only after day 5, and at a much lower level, in the mESC monocultures. After becoming detectable at day 3, the level of Afp transcripts at day 8 in co-culture was 33.5% of Hepa 1-6 cells, suggesting that many ESCs still remained at early stages of hepatic lineage. In the case of Ttr, the transcripts rapidly increased to the maximum level (11.8% of Hepa 1-6), and remained at this level throughout the culture period (Fig. 4A).

Serum albumin (Alb) is the most abundant protein synthesized by hepatocytes. Its production starts in the early stages of liver development and reaches the maximum level in the adult liver. In our experiments, Alb expression increased with time and by day 8 reached levels equivalent to 20.45% of that observed in Hepa 1-6. Significantly, 5–10-fold higher levels of Alb gene expression were observed in mESCs co-cultured with hepatic cells compared to mESC monocultures. This is an important indicator of enhanced hepatic differentiation of mESCs in the micropatterned co-cultures.

RT-PCR analysis of late liver gene expression in micropatterned mESC co-cultures vs. mono-cultures (see Fig. 5) showed that mature liver genes were not observed in mono-cultures after 8 d of cultivation. By contrast, the presence of hepatic cells (co-cultures) induced expression of G6p and Ggt genes in mESCs, pointing to the appearance of maturing hepatoblast-like cell populations. In the case of G6p, gene expression became detectable in co-cultures from day 3 (data not shown) and reached levels equivalent to 32.76% of
that observed in Hepa 1-6. Ggt showed a similar trend to G6p and the level at day 8 was 24.49% of that observed in mouse hepatic cells. Importantly, neither G6p nor Ggt gene expression was observed in mESC mono-cultures cultivated in hepatic differentiation medium.

An important goal of this paper was to develop an analytical technique that would complement micropatterned stem cell co-cultures and would allow the characterization of stem cell phenotype expression in a location-specific manner. Laser catapulting offered the possibility of collecting desired cells from the micropatterned substrate and performing downstream RT-PCR analysis without losing the local microenvironment. In our experiments, mESCs were originally confined to fibronectin spots but then grew both upward as well as outward in the direction of hepatic cell islands (Fig. 2D). We sought to investigate the expression of mature hepatic genes in stem cells at the center of the embryoid body-like cluster vs. the stem cells residing on the periphery of the island, in close proximity to hepatic cells. As shown in Fig. 5A, to investigate the location-specific difference in gene expression, stem cells were laser catapulted from the center of the 300 μm diameter stem cells cluster (far from hepatic cells) as well as from the edge of the stem cell cluster (near hepatic cells). This location-specific analysis revealed heterogeneity in stem cell gene expression (Fig. 5B) with 10–16-fold higher expression of mature liver genes observed at the edge of the stem cell cluster, next to the hepatocytes, compared to the center of the cluster, further away from hepatocytes. These preliminary results point to the importance of heterotypic paracrine–juxtacrine interactions for the induction of mature hepatic phenotype in ESCs. Importantly, stem cells and hepatic cells were morphologically different and could be easily identified using brightfield microscopy prior to catapulting. Furthermore, we verified the species selectivity of the PCR assays for mouse G6p and Ggt genes.

Conclusions

This study employed protein arrays to create micropatterned co-cultures of mESCs with hepatic cells. When compared with mono-cultures, micropatterned co-cultures showed enhanced endodermal and hepatic gene expression concomitant with a more rapid decrease in pluripotency. Interestingly, among genes representative of the three germ layers, only endodermal gene expression was up-regulated in the mESC–hepatic cell co-cultures, while other germ layer genes were either unaffected or down-regulated. The presence of hepatic cells in micropatterned co-cultures also induced the expression of early and mature liver genes in mESCs. In terms of early liver genes, ESCs in a co-culture at day 3 started producing detectable amount of transcripts, while those in a monoculture started at day 5. Although some early liver genes were detected in ESC monocultures exposed to differentiation medium, no late liver genes were observed. This suggests that the communication between ESCs and hepatic cells was particularly important for induction of mature hepatic phenotype in stem cells. In addition, location-specific analysis of stem cells in the micropatterned co-cultures showed heterogeneity in gene expression as a function of distance between adjacent heterotypic cell clusters. Taken together, our study outlines new methods of cultivating and analyzing stem cells and highlights the possibility of using hepatocytes as the source of “instructive” signals for guiding liver-specific differentiation of mESCs. In the future, the protein microarray-based co-culture platform described here may be used for differentiation of human ESCs towards hepatic lineage or may be adapted to deriving other tissue-specific cells from ESCs.

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References