

# Microenvironmental Regulation of the Sinusoidal Endothelial Cell Phenotype *In Vitro*

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Liver sinusoidal endothelial cells (LSECs) differ, both structurally and functionally, from endothelial cells (ECs) lining blood vessels of other tissues. For example, in contrast to other ECs, LSECs possess fenestrations, have low detectable levels of platelet endothelial cell adhesion molecule 1 expression, and in rat tissue, they distinctively express a cell surface marker recognized by the SE-1 antibody. These unique phenotypic characteristics seen in hepatic tissue are lost over time upon culture *in vitro*; therefore, this study sought to systematically examine the effects of microenvironmental stimuli—namely, extracellular matrix and neighboring cells, on the LSEC phenotype *in vitro*. In probing the role of the underlying extracellular matrix, we identified collagen I and collagen III as well as mixtures of collagen I/collagen IV/fibronectin as having a positive effect on LSEC survival. Furthermore, using a stable hepatocellular model (hepatocyte–fibroblast) we were able to prolong the expression of both SE-1 and phenotypic functions of LSEC such as factor VIII activity and AcLOL uptake in cocultured LSECs through the production of short-range paracrine signals. In the course of these experiments, we identified the antigen recognized by SE-1 as CD32b. **Conclusion:** Collectively, this study has identified several microenvironmental regulators of liver sinusoidal endothelial cells that prolong their phenotypic functions for up to 2 weeks in culture, enabling the development of better *in vitro* models of liver physiology and disease. (HEPATOLOGY 2009;50:920-928.)

Liver sinusoidal endothelial cells (LSECs) are specialized endothelial cells that play important roles in liver physiology and disease.<sup>1</sup> LSEC have several features that distinguish them from other endothelial cells (EC). Morphologically, they are characterized by the presence of open fenestrae arranged in sieve plates, and the lack of an organized basement membrane.<sup>2</sup> Function-

ally, LSEC also can act as scavengers, eliminating soluble waste macromolecules from portal venous blood or immune complexes<sup>3</sup>; are critical for inducing CD8+T cell tolerance<sup>4</sup>; and synthesize and release Factor VIII, a critical co-factor in the intrinsic coagulation pathway.<sup>5,6</sup> Thus, it is evident that a distinct endothelial phenotype is required for the specialized functions of the liver *in vivo*. Upon isolation from the hepatic microarchitecture, LSEC lose many of their phenotypic functions. We and others have previously demonstrated that the modulation of the hepatocellular phenotypes *in vitro* can lead to the development of robust *in vitro* models of some aspects of liver biology.<sup>7-9</sup> Here, we investigate the role of the cellular microenvironment on the LSECs phenotype *in vitro* with the goal of elucidating fundamental aspects of LSEC biology as well as improving the fidelity of *in vitro* models of liver tissue.

One of the major challenges in LSEC biology has been the lack of a gold standard biochemical marker for the normal, differentiated LSEC phenotype. The morphological gold standard is the visualization of fenestrations by scanning electron microscopy, a method fraught with technical limitations. The closest biomarker for LSEC phenotype is the antigen recognized by the SE-1 antibody.<sup>10</sup> This antibody, unlike those that recognize the

Abbreviations: cDNA, complementary DNA; EC, endothelial cell; ECM, extracellular matrix; HEK, human embryonic kidney; LDL, low-density lipoprotein; LSEC, liver sinusoidal endothelial cell; PECAM-1, platelet endothelial cell adhesion molecule 1; RECA, rat endothelial cell antigen.

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ubiquitous endothelial marker CD31, is specific to LSECs in rat tissue, and expression of the SE-1 antigen has been shown to positively correlate with the presence of fenestrations. However, the unknown identity of this antigen has made it difficult to translate findings in the rat liver to other species such as mouse and human or to perturb it with molecular tools such as RNA interference. Over the course of our studies, we sought to identify the antigen for SE-1 to address these challenges and aid in the mapping of our findings to a medical framework.

When cultured alone after isolation, rat LSECs lose expression of SE-1, increase expression of CD31, and undergo apoptosis after 2 days.<sup>11,12</sup> Studies on the role of the microenvironment have shown that perturbations of the underlying matrix can prolong the maintenance of fenestrations.<sup>13</sup> Cocultivation with primary hepatocytes can also prolong the loss of phenotype for several days as measured by continued expression of the SE-1 antigen,<sup>14</sup> suppression of CD31 expression,<sup>11</sup> and stimulation of proliferation.<sup>15</sup> This effect is thought to be due to paracrine signals such as vascular endothelial growth factor but is limited in duration, due in part to the concomitant loss of hepatocyte phenotype over several days in culture. In the present study, we exploit two microtechnology platforms previously developed in our group to systematically examine the effect of cell–matrix and cell–cell interactions on LSEC survival and phenotype.<sup>16,17</sup> These platforms allow the investigation of LSEC responses to individual matrix species collagen I, collagen III, collagen IV, fibronectin, and laminin and their combinations, as well as enable analysis of the relative impact of contact versus diffusible paracrine signals in coculture with hepatocytes. In the course of these studies, we also identify the antigen recognized by the SE-1 antibody and map our findings to both mouse and human LSECs.

## Materials and Methods

See Supporting Information for details on the isolation and culture of hepatocytes, LSEC, and fibroblasts.

**Coculture of LSECs with Neighboring Cells.** Cellular cocultures were performed using a recently developed micromechanical reconfigurable culture method to enable tracking of individual cell types, separation into pure populations for analysis, and deconvolution of contact-mediated versus soluble signals.<sup>17</sup> (See Supporting Information for details.)

**Protein Purification and Identification Using Mass Spectrometry.** Isolated LSECs were cultured for 1 day, and lysed in 1 mL of RIPA buffer (Upstate Biotechnology, Waltham, MA) with protease inhibitors cocktail (Roche, Indianapolis, IN). The monoclonal antibody SE-1 was covalently coupled to CNBr-activated Sepharose 4B (Amer-

sham, Uppsala, Sweden) and used to affinity-purify the unknown cell surface antigen from LSEC lysates as described.<sup>18</sup> The isolated protein was identified by peptide mass fingerprinting using an LTQ linear ion-trap mass spectrometer (ThermoFisher, San Jose, CA) (Supporting Information).

**Rat CD32b Cloning and Transfection.** CD32b complementary DNA (cDNA) was synthesized (GENE-ART, Regensburg, Germany) and cloned using the pcDNA3.1 vector. Human embryonic kidney (HEK) cells were transfected with rat CD32b or an empty vector using Lipofectamine. Analysis was performed using fluorescence-activated cell sorting and western blotting.

**Factor VIII Assay.** Factor VIII activity was assayed as described using the Coatest C/4 kit (Diapharm, Franklin, OH).<sup>19</sup>

**Extracellular Matrix Array.** Extracellular matrix (ECM) arrays containing combinatorial mixtures of ECM molecules were fabricated as described.<sup>16</sup>

**Statistical Analysis.** Experiments were repeated three times with duplicate or triplicate samples for each condition. For functional assays, one representative outcome is presented. All data are expressed as the mean  $\pm$  standard deviation. Statistical significance was determined using the Student *t* test (Microsoft Excel).

**Uptake of Acetylated Low-Density Lipoprotein.** Cultured cells were incubated with a medium containing 5  $\mu$ g/mL of acetylated low-density lipoprotein (LDL)-Alexa 488 conjugated (Molecular Probes, Eugene, OR) for 4 hours at 37°C and examined by fluorescence microscopy.

## Results

**Expression of Endothelial Markers in Hepatic Tissue and in Cultured LSECs.** To characterize the phenotype of ECs in intact hepatic tissue, and after their isolation and culture, we used several previously described markers for ECs present in distinct hepatic vascular beds. These included SE-1, platelet endothelial cell adhesion molecule 1 (PECAM-1), and rat endothelial cell antigen (RECA). As seen in Fig. 1A (top panel), immunohistochemistry with the endothelial marker SE-1 on rat liver sections demonstrated a strong staining of the sinusoidal endothelial cells. No reactivity was observed in central or portal tract endothelium. In contrast, PECAM-1 was highly expressed in central and portal tract endothelium, and very low expression was detected in LSECs. The RECA antibody stained the ECs of both the sinusoids and large vessels. Next, we assessed the expression of the endothelial markers SE-1, PECAM-1, and RECA, in cultured LSECs at 1 and 3 days after isolation. At day 1, the

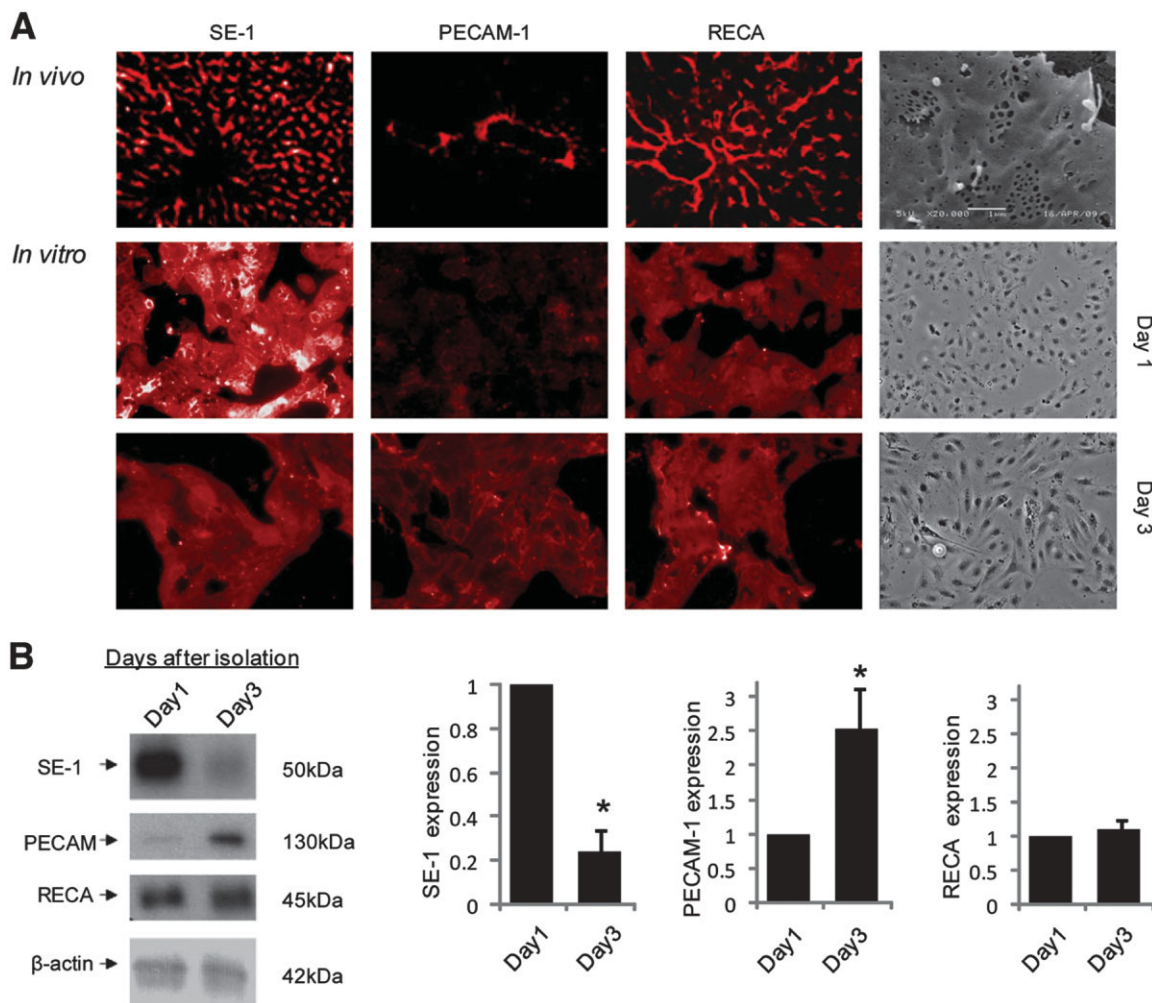


Fig. 1. Characterization of LSECs *in vivo* and *in vitro*. (A) Liver sections of normal rat or LSECs isolated from rat liver and cultured for 1 or 3 days were stained with the SE-1, anti-PECAM-1, or RECA antibodies. The far right panel shows scanning electron microscopy images of LSECs cultured at day 1 and phase contrast images of LSECs cultured at day 1 or 3. Fluorescence and phase contrast images were acquired at an original magnification of 20 $\times$ . (B) Western blotting analyses of total protein from LSECs cultured for 1 or 3 days using the SE-1, anti-PECAM-1, or RECA antibodies. Quantification and normalization are shown in the right panel ( $n = 3 \pm$  standard deviation). \* $P < 0.05$ .

*in vitro* expression of SE-1, PECAM-1, and RECA, correlate with the *in vivo* expression of these markers. Nevertheless, we documented a strong decrease in the expression of SE-1 and an increase in the expression of PECAM-1 3 days after isolation. The expression of RECA did not display significant changes (Fig. 1A,B). Scanning electron microscopy verified that the cell membrane of isolated LSECs at day 1 contained sieve plates with grouped fenestrae (Fig. 1A and Supporting Fig. 1).

**Identification of the Antigen Recognized by the SE-1 Antibody.** To begin linking the expression of the LSEC marker SE-1 to cellular function, we performed affinity purification of the protein recognized by the SE-1 antibody from LSEC lysates, followed by mass spectrometry analysis. These experiments revealed the protein to be CD32b (Fig. 2A). To confirm the identity of the recog-

nized protein, LSEC lysates were immunoprecipitated with an SE-1 antibody, and the immunoprecipitated material was detected by way of western blotting using two different anti-CD32 antibodies. In both cases, the SE-1 antibody and the anti-CD32 recognized the same antigen (Fig. 2B). Furthermore, the CD32b cDNA was transiently transfected in HEK cells and probed with the antibody against SE-1. In these experiments, CD32b expressed in HEK cells was recognized by monoclonal antibody SE-1 using both flow cytometry and western blot analysis (Fig. 2C). Immunohistochemical analysis revealed that SE-1 and polyclonal CD32b antibody staining colocalized in rat liver sections (Fig. 2D). We also demonstrated LSECs as the main source of CD32b (Supporting Fig. 2). Taken together, these data demonstrate that the SE-1 antibody recognizes the cell surface receptor CD32b.

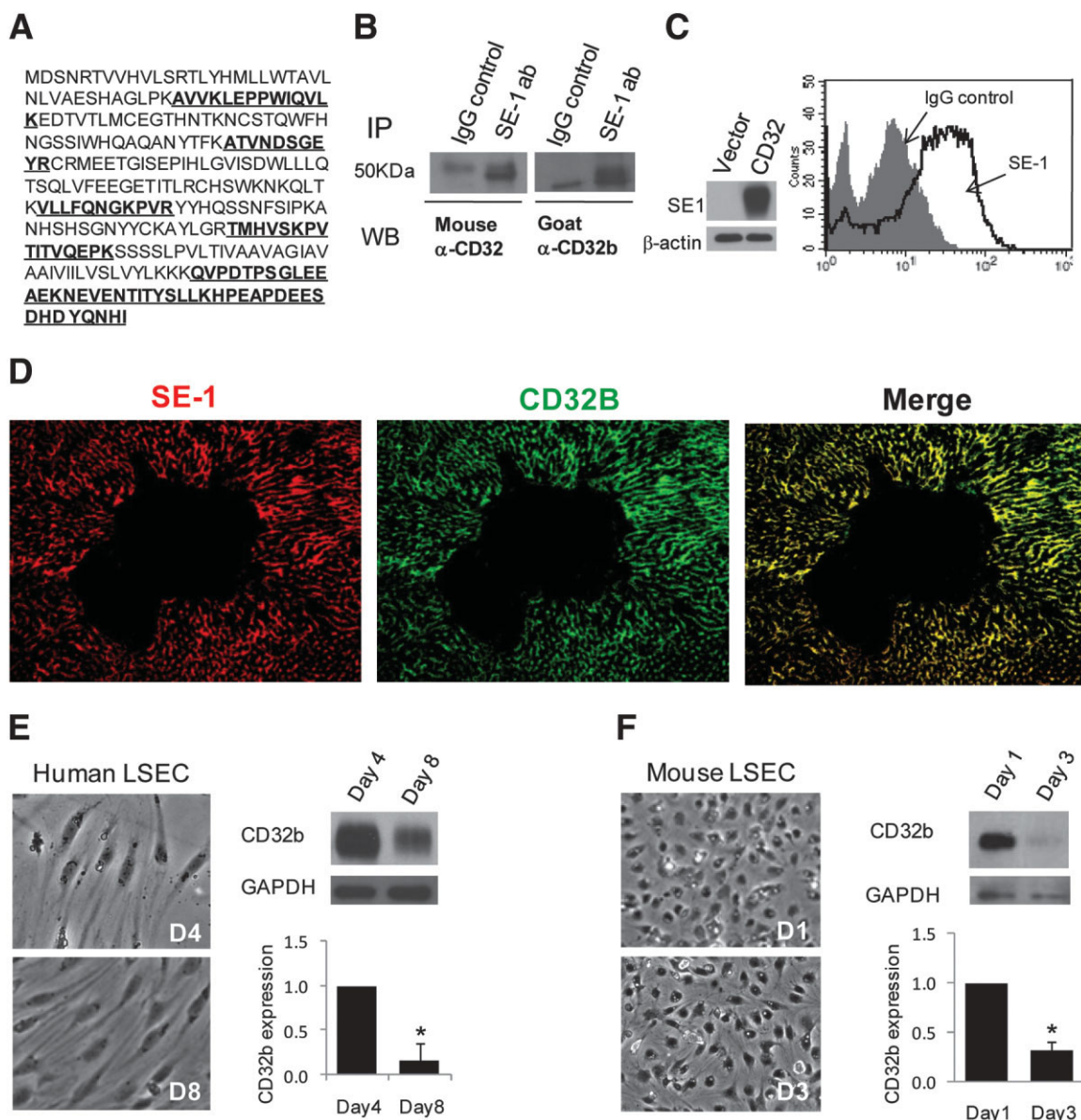


Fig. 2. Identification and characterization of the antigen recognized by the SE-1 antibody. (A) Amino acid sequences of the rat CD32b molecule identified by mass spectrometry. Boldface indicates amino acids predicted by mass spectrometry. (B) LSEC lysates were immunoprecipitated with the SE-1 antibody, and the immunoprecipitated material was detected by way of western blotting either using mouse anti-CD32 or goat anti-CD32b antibodies. (C) HEK cells transfected with rat CD32b cDNA or with the empty vector and analyzed by way of western blotting using the SE-1 antibody (right). HEK cells were transfected with CD32b cDNA and stained with the SE-1 antibody or an immunoglobulin G control and analyzed using fluorescence-activated cell sorting. (D) Double immunostaining was performed in liver rat sections using the monoclonal SE-1 antibody and the goat anti-CD32b polyclonal antibody, followed by fluorescently labeled secondary antibodies (original magnification 20 $\times$ ). Total protein lysates of (E) human or (F) mouse LSECs cultured for 4 or 8 days or 1 or 3 days were analyzed by way of western blotting using the CD32b antibody.

The identification of the antigen recognized by SE-1, the CD32b molecule, allowed us to extend our studies to mouse and human LSECs, because the SE-1 antibody does not cross-react with these species. We assessed the expression of CD32b in human and mouse LSECs by way of western blotting. In both cases, we showed a down-regulation of CD32b over time (Fig. 2E,F), demonstrating that this pattern is conserved across several species. Interestingly, *in vivo* expression of CD32b in mouse liver

also correlated with patterns of SE-1 staining in rat liver (Supporting Fig. 3C).

**Effect of ECM on the Survival of Liver Sinusoidal ECs in Culture.** To assess the effects of different components of extracellular matrix on the fate of primary rat LSECs, we patterned cells atop combinatorial matrix mixtures of interstitial matrix molecules (fibronectin, collagen I, collagen III) and basement membrane components (laminin, collagen IV) using a microarray platform we

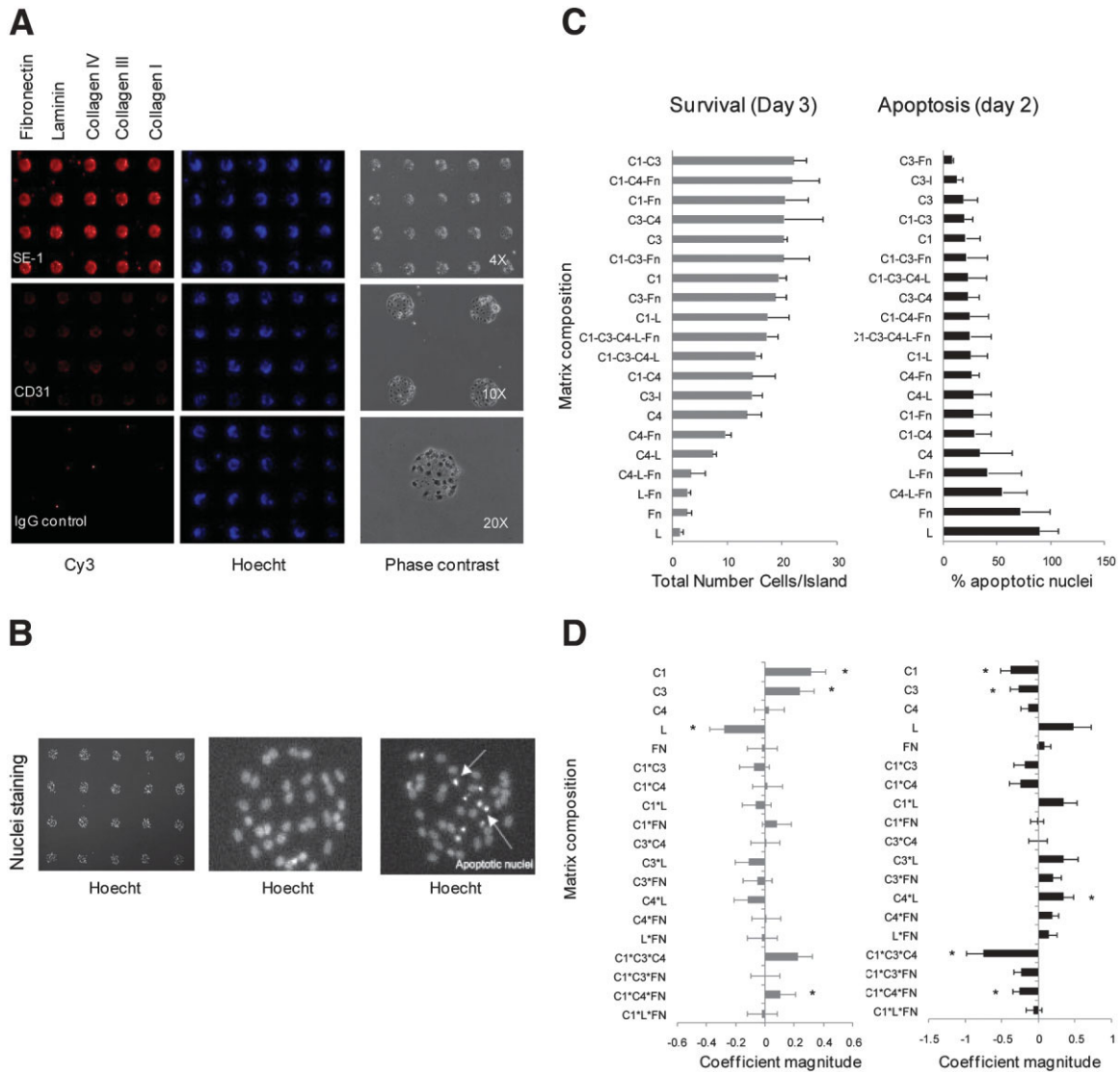


Fig. 3. Effect of the ECM components on the survival of cultured LSECs. (A) An example of cells patterned atop an ECM microarray at day 1. The left panel shows staining with the SE-1, anti-PECAM-1, and immunoglobulin G control antibodies, respectively, followed by incubation with Cy-3-conjugated secondary antibody, the corresponding nuclei are visualized by Hoechst staining (middle panel), and phase contrast images are seen in the right panel. (B) Representative images of nuclei seen by Hoechst staining with apoptotic nuclei highlighted with arrows. (C) Average and factorial analysis of the percentage of apoptotic nuclei at day 2 observed atop varying matrix compositions. (D) Average and factorial analysis of the total number per cells at day 3 observed atop varying matrix compositions.

have described.<sup>16</sup> At day 1 after seeding, LSECs expressed high and similar levels of SE-1 (CD32b) and low levels of PECAM-1 across all conditions. All islands showed similar numbers of ECs and absence of apoptotic nuclei on day 1. The presence of attached ECs on each island on day 2 and 3 as detected by microscopy was used to score survival over time. A representative part of the array is shown in Fig. 3A. In contrast to the first day after seeding, notable differences in the number of surviving cells and the percentage of apoptotic nuclei per island emerged over the next 48 hours. The differences in survival and apoptosis were dependent on the initial underlying matrix composition (Fig. 3B). Islands containing collagen I or III

exhibited the lowest percentage of apoptotic nuclei per island and the highest number of cells per island. In contrast, fibronectin and laminin negatively affected the survival of LSECs, correlating with the highest percentage of apoptotic nuclei per island and the lowest number of cells per island. Factorial analysis revealed that collagen I and collagen III have significant positive effects on cell survival, and laminin has a strong negative effect. We also identified the positive effect of the mixture of collagen I/collagen IV/fibronectin in reducing apoptotic nuclei and increasing cell number. Interestingly, collagen IV alone did not modulate cell survival; however, in the context of collagen I and III, it had positive effects (Fig. 3C,D).

**Role of Supportive Cells in Maintaining LSEC Phenotype.** Our laboratory and others have previously shown that coculture of hepatocytes with 3T3 fibroblasts can rescue the loss of the hepatocellular phenotype in culture,<sup>8</sup> and that both cell contact and short-range paracrine signals were necessary for rescue.<sup>17</sup> We used a similar approach to assess the role of supportive cells in maintaining the LSEC phenotype in culture. Cocultures were performed using a reconfigurable cell culture substrate.<sup>17</sup> The system can be configured in contact mode, where contact-mediated signaling can occur between the two populations, or in gap mode, where the populations are separated by an 80- $\mu\text{m}$  gap and can only communicate by diffusible soluble signals (Fig. 4A).

Using this platform, LSECs were cultured together with three different combinations of neighboring cells, including hepatocytes, hepatocytes mixed with 3T3 fibroblasts as a stable liver tissue model, and 3T3 fibroblasts (Fig. 4B). In each case, cocultures were performed in both gap (80- $\mu\text{m}$  separation) and in contact configuration.

Immunocytochemical and western blot analysis showed that the expression of SE-1 (CD32b) was optimally maintained in cultures combining LSECs with hepatocytes and 3T3 fibroblasts (Fig. 4C,E). We also measured factor VIII activity as a biochemical marker of LSEC phenotype and Ac-LDL uptake as a measure of the endocytic capability of LSECs. As with SE-1, cocultures of LSECs with hepatocytes and fibroblasts maintained the highest uptake of Ac-LDL (Fig. 4G) and produced the highest levels of factor VIII (Fig. 4F). No significant factor VIII activity was detected in hepatocyte or hepatocyte/fibroblast cocultures, confirming that factor VIII is produced solely by LSECs (data not shown). As expected, albumin secretion—a measure of hepatocellular function—was highest in the cultures containing hepatocyte/fibroblast cocultures (Fig. 4D). Thus, tricultures of LSECs with hepatocytes and fibroblasts atop collagen I provided the best cellular microenvironment of the conditions tested for retention of the LSEC phenotype. We also measured LSEC proliferation, and found that whereas hepatocyte and hepatocyte/fibroblast cocultures enhanced LSEC proliferation, the increase in labeling index of approximately 20% of nuclei was unlikely to be sufficient to account for the two- to three-fold increase in phenotypic functions. In contrast, we did not observe any effect of fibroblasts alone on LSECs (Supporting Fig. 4).

We sought to determine whether contact between the cell populations was required for rescue of the LSEC phenotype. Therefore, we compared LSECs cultured in gap and contact modes. Western blots of SE-1 (CD32b) demonstrated that the degree of rescue obtained from short-range soluble signals in gap mode was equivalent to that

obtained in contact mode (Fig. 4H). Thus, our findings indicate that hepatocyte/fibroblast coculture stabilizes the LSEC phenotype through diffusible factors. In order to identify these soluble factors, we first examined the expression levels of several cytokines in the conditioned media of the different culture combinations by using a rat cytokine microarray system containing antibodies against 19 cytokines and other proteins. Using this approach, we did not detect any significant changes between this cytokine panel in the well-mixed media after 24 hours of conditioning; however, differences that occur over shorter time scales would not be detectable in this platform (Supporting Fig. 5).

Next, we performed transcriptome measurements in order to identify soluble factors secreted by hepatocytes upon coculture with fibroblast. Data from these experiments identified 17 distinct genes up-regulated in hepatocytes and with predicted effects on the endothelium (Supporting Table 1). Therefore, in order to assess whether labile diffusible factors could propagate signals between cell populations over a distance of more than 80  $\mu\text{m}$ , we performed transwell experiments in which LSECs shared their media with hepatocyte/fibroblast cocultures but were separated by 1 mm. Neither the transwell nor conditioned media models were able to rescue the expression of SE-1 (CD32b), as was seen in the short-range gap and contact cocultures (Fig. 4I and data not shown).

Finally, we evaluated the longevity of SE-1 expression under different conditions. At day 14, SE-1 (CD32b) expression was only detectable in the triculture configuration. Notably, over this second week of culture, expression of SE-1 was increased in contact cultures compared with the 80- $\mu\text{m}$  gap culture. Furthermore, in the contact mode at day 14, we observed a migration of LSECs toward the hepatocyte/fibroblast domain, and these cells express the highest levels of SE-1. These data suggest that reciprocal signaling or contact-mediated signals may play a role over the second week of culture (Fig. 4J).

## Discussion

The current study was designed to examine the effect of distinct microenvironmental stimuli—in particular ECM components and paracrine effects of supportive cell types—on the LSEC phenotype. Using two platforms, we demonstrated that specific matrix molecules and short-range soluble signals emanating from a relatively stable hepatocellular model (hepatocyte/fibroblast cocultures) are able to prolong survival and the expression of LSEC phenotypic markers (SE-1 expression AcLOL uptake and factor VIII activity) for up to 2 weeks. Notably, the antigen recognized by the antibody SE-1, was identi-

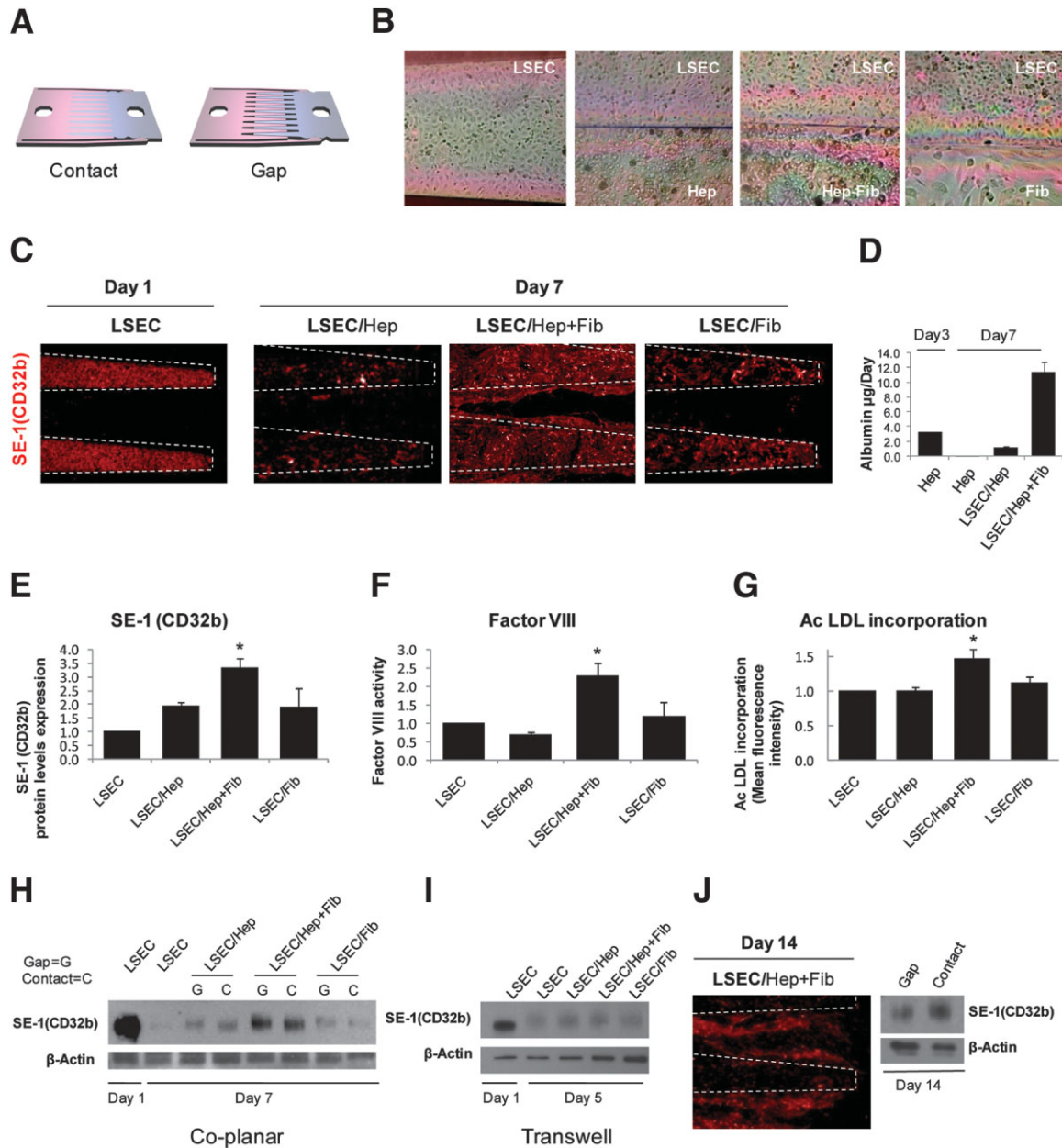


Fig. 4. Role of neighboring cells in maintaining the LSEC functional phenotype. (A) Schematic representation of the micromechanical platform used for coculture experiments. (B) Phase contrast images of LSECs cocultured with three different combinations of neighboring cells (C) LSECs cocultured with three different combinations of neighboring cells were stained with the monoclonal antibody SE-1, followed by conjugated secondary antibody. (D) Albumin secretion by hepatocytes alone or in coculture with LSECs or LSECs/fibroblasts. (E) SE-1(CD32b) quantification of three independent experiments between day 6 and 7. (F) Representative experiment of factor VIII activity in the supernatants of the different cocultures. (G) Uptake of Ac-LDL by the cultured LSECs, a representative experiment of three individual experiments. (H) LSEC cocultures with three different combinations of neighboring cells were performed in two modes (gap or contact) for 7 days. Protein lysates of LSECs were analyzed by way of western blotting using the antibody SE-1(CD32b). (I) SE-1(CD32b) expression on LSECs alone or with three different combinations of neighboring cells using the transwell system. (J) Immunostaining and western blotting at day 14 using the antibody SE-1 ( $n = 3 \pm$  standard deviation). \* $P < 0.05$ .

fied as CD32b. This particular finding will allow us to better understand the putative role of CD32b (SE-1) on liver sinusoidal endothelial cells, extend this type of study to other species (such as mouse and human), and perturb this antigen with molecular tools such as RNA interference.

In our model, the expression of endothelial CD32b was maintained for up to 14 days in an optimal configuration involving cocultivation of LSECs with both hepatocytes and fibroblasts. CD32b is a low-affinity Fc $\gamma$  receptor constitutively expressed by human adult liver sinusoidal ECs that has the same expression pattern as

that described for SE-1 in rats, where only the sinusoidal cells and not the ECs of the large vessels express the antigen.<sup>20</sup> Here, we demonstrated that during the process of LSEC differentiation in culture, there is a loss of CD32b expression in rat, mouse, and human LSECs, which shares the same dynamics with the loss of the gold standard phenotypic marker, cytoplasmic fenestrations.<sup>21,22</sup> CD32b plays a major role in the endocytosis of immune complexes in the rat and has been found to be dysregulated in disease, potentially linking the LSEC phenotype with the accumulation of immune complexes observed in some human liver diseases.<sup>20,23</sup>

Previous studies have shown that hepatocytes cocultured with fibroblasts can retain a differentiated hepatocyte phenotype over several weeks in culture.<sup>24,25</sup> In this study, we observed that the cocultivation of LSECs with hepatocyte/fibroblast cocultures are better able to preserve the hepatocyte phenotype (as determined by the expression of SE-1, uptake of Ac-LDL, and factor VIII activity) than hepatocyte/LSEC cultures under the conditions tested, demonstrating that the triculture model has the potential to promote the stabilization of both ECs and hepatocytes for up to 2 weeks. Recent evidence points to the importance of LSECs as a source of factor VIII, a critical cofactor in the intrinsic coagulation pathway.<sup>6</sup> Our findings on the effect of neighboring cells on LSECs are consistent with observations in the literature. For example, a small population of SE-1–positive cells was observed over 13 days in a three-dimensional perfused coculture of hepatocytes and LSECs,<sup>13</sup> where hepatocytes in the three-dimensional perfused environment are more phenotypically stable than in monolayer culture.<sup>26</sup> In short-term experiments (up to day 4), hepatocytes (that are concurrently losing their phenotype) have been shown to prevent CD31 expression and stimulate proliferation in ECs.<sup>11,15</sup> Thus, it is reasonable to expect that a monolayer tissue model that combines a relatively stable hepatocyte phenotype (hepatocytes/fibroblasts) with LSECs would support the LSEC phenotype over a relatively long time scale. Previous studies have shown that LSECs can positively impact hepatocyte function in culture at early time points (24 hours).<sup>27</sup> In comparison, hepatocyte functions in the 3T3 coculture model reported here stabilize over approximately 5 to 7 days. Thus, the generation of tricultures to stabilize LSEC function may also have beneficial effects on hepatocyte function early in culture.

Our studies indicate that diffusible factors emanating over short distances,  $L$  ( $80 \mu\text{m} < L < 1 \text{mm}$ ), were responsible for the rescue of the endothelial phenotype over the first week in culture. *In vivo*, it is feasible that hepatocytes and ECs communicate via diffusible signals

over short-length scales such as those in the space of Disse. In an attempt to identify diffusible factors that may play a role in this process, we conducted a comparative analysis of 19 cytokines in the media to determine whether a single factor could be identified to explain these findings; however, no significant differences were identified between supportive (hepatocyte/fibroblast) and nonsupportive (fibroblasts or hepatocytes alone) cultures. We also performed a gene expression profiling study comparing hepatocytes and hepatocytes in coculture with fibroblasts aiming to reveal potential soluble factors that could contribute to the maintenance of the LSEC phenotype. The candidates revealed by these experiments include pleiotrophin, insulin-like growth factor I, and angiotensin, the potential action of which in this context correlate with previously published data (Supporting Table 1).

We also examined the independent role of ECM coatings on the LSEC phenotype. In liver fibrosis, there is a shift in the type of ECM in the space of Disse from the normal low-density basement membrane-like matrix (collagen IV, collagen V, and laminin) to interstitial type matrix containing fibril-forming collagen (mainly collagen I and III).<sup>28</sup> In our array, these different matrices and their mixtures were included to simulate the *in vivo* microenvironment and to capture the transformation of matrix composition observed in liver fibrosis. Our experiments identified the interstitial matrix collagen I and III and mixtures of collagen I/collagen IV/fibronectin as having a positive effect on LSEC survival. These findings are consistent with prior reports that combinations of ECM can modulate LSEC fenestrations<sup>13</sup>; however, the positive role of fibrillar matrix on LSECs in culture was not expected. Alteration of integrin expression or signaling could explain these findings, and *in vivo* it has been shown that LSEC integrin expression is altered during the process of capillarization in liver cirrhosis.<sup>29</sup>

Taken together, these studies reveal several factors that are critical for the maintenance of the functional phenotype of LSECs in culture. This experimental framework should contribute to our understanding of the complex circuitry necessary to maintain cellular function *in vivo* and *in vitro* and may provide an *in vitro* model that captures the phenotype of multiple hepatic cell populations over many days in culture. In the future, incorporation of additional nonparenchymal cells such as cholangiocytes and stellate cells will allow even higher fidelity liver models to be assembled and investigated.

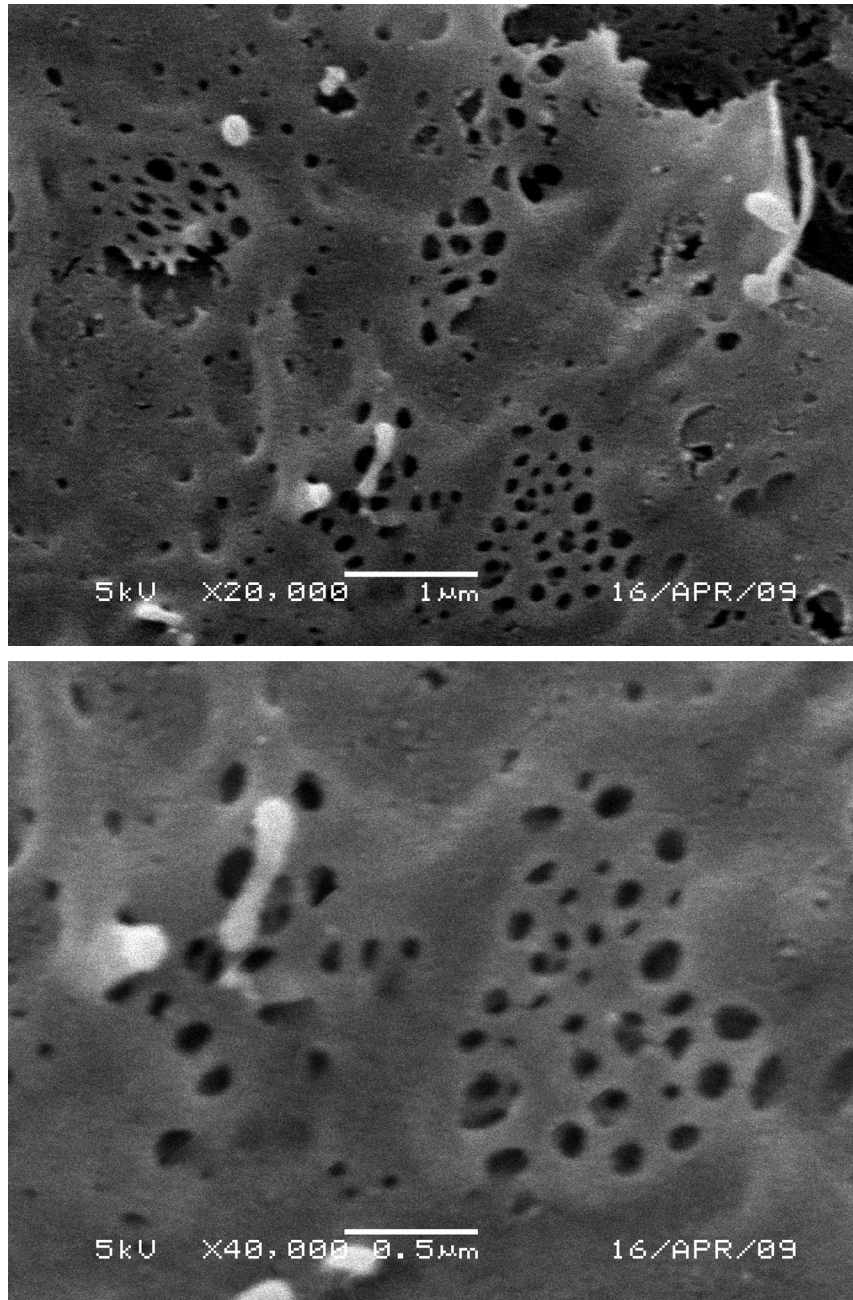
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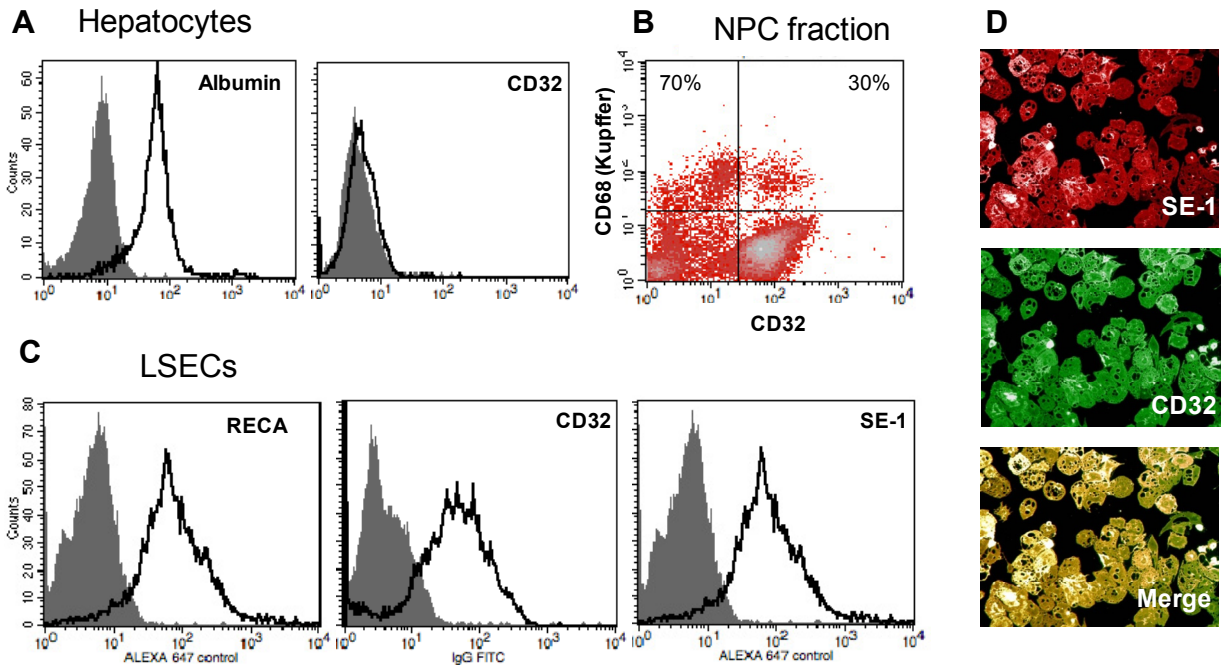
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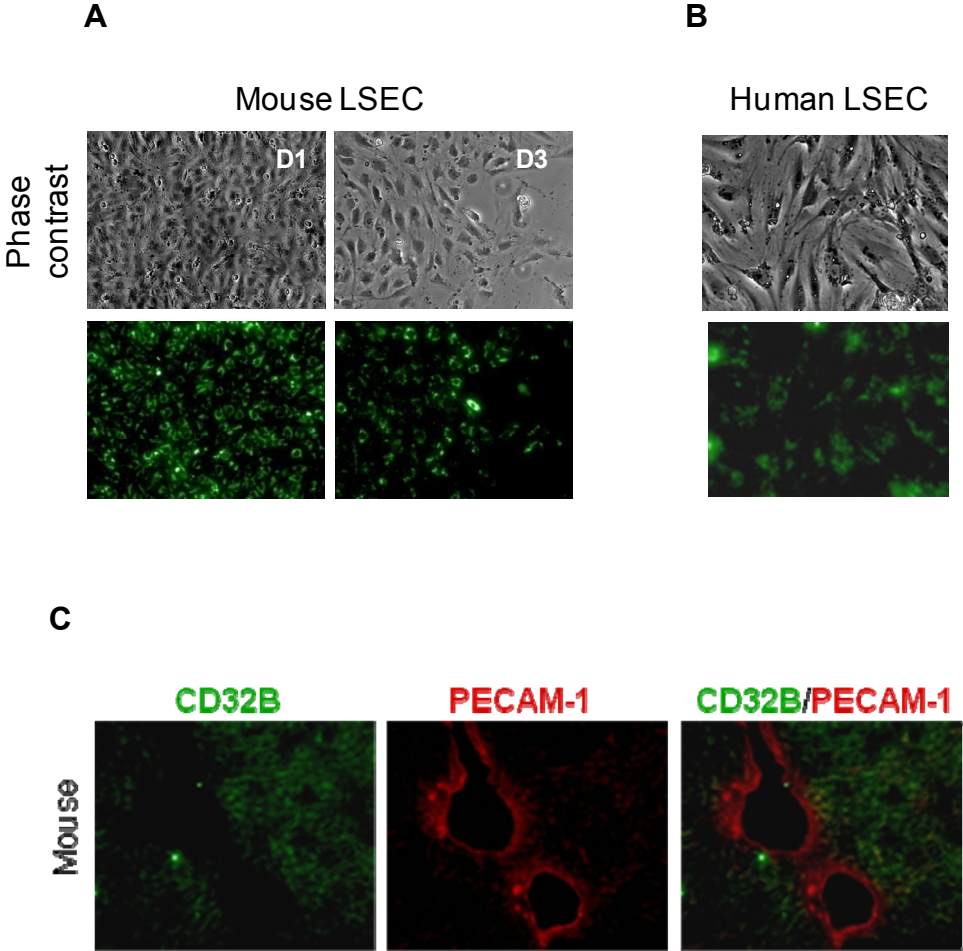
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Supplemental figure 1

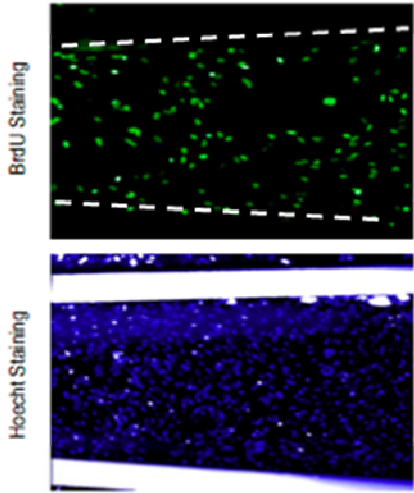


Supplemental figure 2

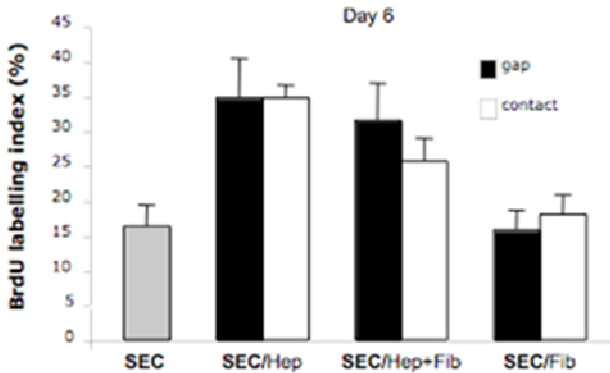


Supplemental figure 3

**A**

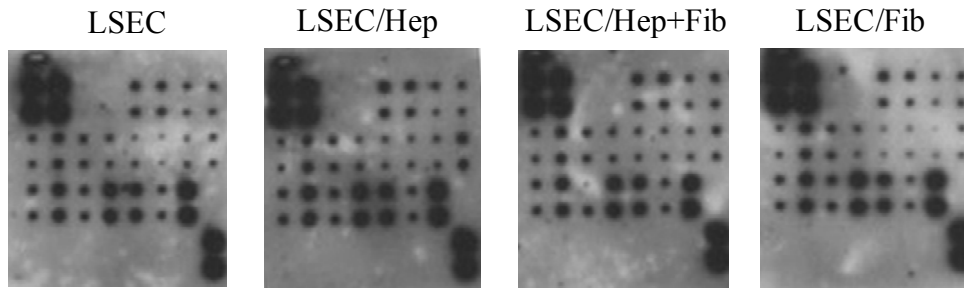


**B**



Supplemental figure 4

**A**



**B**

CINC-2, CINC-3, CNTF, Fractakline, GM-CSF, IFN-gamma, IL-1 alpha, IL-1 beta, IL-4, IL-6, IL-10, LIX, Leptin, MCP-1, MIP-3 alpha, NGF-beta, TIMP-1, TNF-alpha, VEGF

<u>Accession Number</u>	<u>Gene description</u>	<u>fold change</u>
	<b>Coagulation cascade</b>	
NM_053491	<i>RPlg: plasminogen</i>	105.33
NM_022924	<i>F2: coagulation factor II</i>	81.57
M84000	<i>alpha-1-macroglobulin</i>	75.19
NM_017143	<i>F10: coagulation factor X (1)</i>	41.41
NM_024382	<i>Serpind1: leuserpin-2</i>	33.35
NM_012803	<i>Proc: protein C</i>	14.21
AA875097	<i>Fibrinogen, A alpha polypeptide</i>	10.83
	<b>Vasomotor properties</b>	
NM_134432	<i>Agt: angiotensinogen (2)</i>	32.21
	<b>Antioxidants related</b>	
NM_013048	<i>Ttpa: tocopherol transfer protein alpha (3)</i>	40.15
	<b>Growth factors and mitogen</b>	
M15481	<i>Insulin-like growth factor I (IGF-I) (4)</i>	10.32
NM_130752	<i>Fgf21; fibroblast growth factor 21</i>	6.14
NM_017066	<i>Ptn: pleiotrophin (Heparine binding factor) (5)</i>	3.50
	<b>Hemoglobulin related</b>	
NM_012582	<i>Hp: haptoglobin</i>	57.62
NM_053318	<i>Hpx: hemopexin</i>	30.63
AA945178	<i>Transferrin</i>	2.98
	<b>Others</b>	
NM_134326	<i>Alb: Albumin (positive control)</i>	30.56
NM_019373	<i>Apom: apolipoprotein M</i>	35.64

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# Supplemental Material

## Material and methods

### Cell Culture

**Hepatocyte Isolation.** Rat hepatocytes were isolated by in situ collagenase perfusion through the portal vein according to the method of Seglen, with minor modifications (1). Detailed procedures for hepatocyte isolation and purification have been previously described (2). Routinely, 200-300 million cells were isolated with 85%-95% viability, as judged by trypan-blue exclusion. Hepatocyte culture medium consisted of Dulbecco's Modified Eagle Medium with high glucose, 10% (v/v) fetal bovine serum, 0.5 U/ml insulin, 7 ng/ml glucagon, 7.5 µg/ml hydrocortisone, and 1% (v/v) penicillin-streptomycin.

**Liver Sinusoidal Endothelial Cells Isolation.** Rat and mouse LSECs were isolated as previously described with minor modifications (3). Briefly, supernatants containing nonparenchymal cells (obtained as described above) were centrifuged at 300 X g for 20 minutes. The resulting pellet was resuspended in Dulbecco's phosphate-buffered saline (PBS) and then centrifuged at 900 X g for 25 minutes through a 25%/50% Percoll gradient. The interface of the gradient containing Kupffer cells and sinusoidal endothelial cells (Non parenchymal fraction) was plated in 6-well plates and incubated at 37°C for 10 minutes. The nonadherent cells are the sinusoidal endothelial cell fraction, which were cultured in the presence of 40ng/ml of rat vascular endothelial cells growth factor (R&D, Minneapolis, MN). Human non-parenchymal fraction containing LSEC were obtained from (CellzDirect, Durham, NC) and (LONZA, Walkersville, MD). Isolation of LSEC was performed as above with minor modifications.



***Fibroblast culture.*** Swiss 3T3 fibroblasts were purchased from ATCC (Manassas, VA). These fibroblasts were cultured in Dulbecco's Modified Eagle Medium with high glucose, 10% (v/v) fetal bovine serum, and 1% (v/v) penicillin-streptomycin.

### **Co-culture of LSEC with neighboring cells**

Cellular co-cultures were performed using a recently developed micromechanical reconfigurable culture method to enable tracking of individual cell types, separation into pure populations for analysis, and deconvolution of contact-mediated versus soluble signals. Each micromechanical substrate has two complementary parts. Each cell population was plated on individual parts, and then the two parts were assembled to enable 'contact' or 'gap' culture (80 $\mu$ m separation). Detailed description of the device and procedures for cells seeding are available elsewhere (4). Briefly, the micromechanical substrates were coated in collagen solution (50  $\mu$ g/ml in water) at 37°C for at least 45 min.  $1.5 \times 10^6$  LSEC were seeded onto one half of each device, while supportive cell types (fibroblasts, hepatocytes, or a mix of both) were seeded onto the other half. After 6 h, the complementary parts were assembled into their initial configuration for a particular experiment.

### **Immunocytochemistry**

Isolated LSEC or liver tissue sections were fixed with 4% paraformaldehyde and incubated for 30 minutes at room temperature with blocking buffer (PBS with 2% BSA) (Sigma, St.Louis, MO). After blocking, tissue sections and cells were stained with primary and secondary antibodies for

1 hour at room temperature. Washes with PBS were performed between incubations. Finally, nuclei were visualized by DNA staining with Hoescht reagent ( $10^{-3}$ mg/ml). Samples were mounted using Fluoromount-G (Southern Biotechnology, Birmingham, AL) and fluorescence images were acquired. The following primary antibodies were used: mouse anti-Rat Hepatic Sinusoidal Endothelial Cells (SE-1)(1:100; IBL-America, Minneapolis, MN), goat anti CD32-B(1:200, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-PECAM-1 (1:100; BD Pharmingen, San Jose, CA) and mouse RECA (1:100; Serotec, Oxford, UK). The secondary antibodies used were Cy3-conjugated anti-mouse IgG (1:500; Jackson Immuno Research, West Grove, PA) and Alexa 488 conjugated anti-goat IgG (1:500; Invitrogen, Eugene, OR)

### **Western Blot Analysis**

Samples from culture LSEC were lysed in RIPA buffer (Upstate Biotechnology, Waltham, MA) with protease inhibitors cocktail (Roche, Indianapolis, IN). Lysates were separated using polyacrylamide gel electrophoresis in nonreduced conditions and transferred to a polyvinylidene fluoride membrane. The blots were subsequently blocked with Tris-buffered saline containing 0.05% Tween 20 and 5% nonfat dry milk, and incubated with the following primary antibodies: mouse anti-Rat Hepatic Sinusoidal Endothelial Cells (SE-1)(1:300; IBL-America, Minneapolis, MN), goat anti CD32-B(1:200, Santa Cruz Biotechnology, Santa Cruz, CA), Mouse anti CD32 (1:200; BD Pharmingen, San Jose, CA), mouse anti-PECAM (1:200; BD Pharmingen, San Jose, CA), mouse RECA (1:200; Serotec, Oxford, UK) overnight at 4C, followed by an incubation with rabbit anti-mouse or anti-goat horseradish peroxidase-conjugated secondary antibodies (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room temperature. After 30

minutes, immunodetection was performed using the ECL blotting detection system (Pierce, Rockford, IL).

### **Immunoprecipitation**

LSEC cells contained in a 35-mm tissue culture dish were washed twice with PBS and were lysed in RIPA buffer (Upstate Biotechnology, Waltham, MA) with protease inhibitors cocktail (Roche, Indianapolis, IN). Immunoprecipitation were performed with 5 $\mu$ g of mAb SE-1 as previously described (3).

### **Preparation for scanning electron microscopy**

Endothelial cells were cultivated in 24-multiwell plates on collagen-coated Thermanox cover slips for scanning electron microscopy. Coverslips with liver sinusoidal endothelial cells were rinsed twice with PBS and fixed with 2% glutaraldehyde in 0.1 mol/l Na-cacodylate buffer (with 0.1 mol/l sucrose) at pH 7.4 for 2 h. Samples were dehydrated in a graded ethanol series (70%, 80%, 90%, 95%, 100%, 100%), critical point dried, and sputter coated with gold. The samples were examined with a scanning electron microscopy.

### **ECM Array**

ECM arrays containing combinatorial mixtures of ECM molecules were fabricated as previously described (5). Each array slide contained 8 10x10 arrays consisting of 20 select combinations of

5 ECM molecules (rat collagen I, human collagen III, mouse laminin, mouse collagen IV, human fibronectin) with 5 replicates per mixture. LSEC were seeded onto the arrays at  $1 \times 10^6$  cells/mL and allowed to attach overnight. Arrays were cultured in LSEC media for a period of 2 or 3 days prior to fixation, DNA labeling, and immunostaining for SE-1 and PECAM-1, which were performed as described above. Cell numbers and the percentage of cells exhibiting apoptotic nuclei were assessed using Metamorph image analysis software. For each matrix condition, the 5 replicate measurements were used to calculate the average and standard error for the mixture. Three experiments utilizing independent LSEC isolations were quantified in this manner. Main- and interaction effects as well as statistical significance were evaluated using factorial analysis with Minitab statistical software (Minitab, State College, PA)

### **Protein Purification, and Identification by Mass Spectrometry**

Isolated LSEC were cultured for one day, and lysed in 1 ml of RIPA buffer (Upstate Biotechnology, Waltham, MA) with protease inhibitors cocktail (Roche, Indianapolis, IN). MAb against SE-1 was covalently coupled to CNBr-activated Sepharose 4B (Amersham, Uppsala, Sweden) and used to affinity-purify the unknown cell surface antigen from LSEC lysates as previously described (REF). Protein was eluted with 0.1% trifluoroacetic acid (Fluka, Steinheim, Germany). Eluates were vacuum-dried to remove trifluoroacetic acid and resuspended in Laemmli buffer (Bio-Rad laboratories, Hercules, CA). The affinity chromatography-purified protein was resolved on SDS-polyacrylamide gel electrophoresis and stained with Silver (Invitrogen, Carlsbad, CA). The protein band was excised from the gel and further processed for analysis by the Taplin Biological Mass Spectrometry Facility at Harvard Medical School. The

protein was identified by peptide mass fingerprinting using LTQ linear ion-trap mass spectrometer (ThermoFisher, San Jose, CA). Peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Peptide sequences (and hence protein identity) were determined by matching protein databases with the acquired fragmentation pattern by the software program, Sequest (ThermoFisher, San Jose, CA). Spectral matches were manually examined and multiple identified peptides per protein were required.

### **FACS analysis and protein array**

For FACS analysis, hepatocytes, LSEC and NPC were used. The following primary antibodies were used: FITC mouse anti CD32 (1:20; pharmingen), Rabbit anti-albumin (1:100; MP Biomedicals, OH), Alexa 647 mouse anti CD68 and mouse RECA (1:20 and 1:100; Serotec) and mouse SE-1 (1:100; IBL-America). The secondary antibodies used were alexa 635-conjugated anti-mouse IgG and Alexa 488 conjugated anti-rabbit IgG (1:400; Invitrogen, Eugene, OR). In each sample 15,000 cells were acquired and analyzed using a FACSCalibur apparatus (BD Biosciences) and the CellQuest Pro software.

### **Cytokine array**

The array membranes (Raybiotech, Inc., Technology Parkway, Norcross, GA) were blocked with 5% BSA/Tris buffered saline (TBS) (0.01 M Tris-HCl pH 7.6/0.15 M NaCl) for 30 min and then incubated with 1 mL of two fold-diluted conditioned media for 2 h. After extensive washing with TBS/0.1% Tween 20 to remove unbound proteins, the membranes were incubated with

biotin-conjugated anti-cytokine antibodies. Following extensive washing, signals were detected by incubation with HRP-conjugated streptavidin (2.5 pg/mL) coupled with an ECL system (Applied Biosystems).

### **Gene expression profiling**

Hepatocytes or hepatocytes in co-culture with fibroblast were cultured for 9 days. At day 9, RNA was extracted from the hepatocytes from both groups, using TRIzol-LS (Gibco, Gaithersburg, MD). Each RNA sample was labeled and hybridized to an Affymetrix microarray. Scanning and data analysis were performed as described before (6).

### **Figures Legends**

***Supplemental figure 1: Scanning electron microscopy image of cultured LSEC documenting the presence of fenestrations.*** Rat LSEC were isolated from normal livers, cultured for 24 h, and processed for SEM. Pictures were taken at 20,000X and 40,000X.

***Supplemental figure 2: CD32 expression in Hepatocytes, LSEC, and Kupffer cells.*** Primary hepatic cells were isolated from normal rat livers. Expression of CD32 was evaluated in (A) Hepatocytes (Albumin<sup>+</sup>), (B) Kupffer cells (CD68<sup>+</sup>) and (C) LSEC (RECA<sup>+</sup>) by flow cytometry. (D) Double immunostaining of rat LSEC using the goat anti-CD32B and the antibody SE-1,

followed by an anti-goat 488-conjugated and an anti-mouse Cy3-conjugated secondary antibodies.

**Supplemental figure 3: Characterization of mouse and human LSEC.** (A) Alexa 488 Ac-LDL incorporation by mouse LSEC. (B) Alexa 488 Ac-LDL incorporation by human LSEC. (C) Double immunostaining of mouse liver section using the goat anti-CD32B and the rat anti-PECAM-1, followed by an anti-goat 546-conjugated and an anti-rat 488 secondary antibodies.

**Supplemental figure 4: Assessment of LSEC proliferation.** (A) representative image of BrdU-labeled nuclei of LSEC in co-culture with supportive cells in the gap configuration. (B) BrdU positive nuclei quantification.

**Supplemental figure 5: Protein analysis of supernatants derived from LSEC/Hepatocytes/Fibroblasts co-cultures.** (A) Using a protein array, the expression of 19 cytokines and growth factors was measured from supernatants of the 3 different co-culture conditions indicated. (B) List of cytokines/growth factors present in the array.

**Supplemental Table 1: List of differentially expressed genes in hepatocytes co-cultured with fibroblasts compared to hepatocytes alone.**

Genes included in this table meet the following criteria: first, they are  $\geq 2X$  higher expressed in hepatocytes in co-cultured compared with hepatocytes alone; second, their protein products are predicted to be secreted.

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5. Flaim CJ, Chien S, Bhatia SN. An extracellular matrix microarray for probing cellular differentiation. *Nat Methods* 2005;2:119-125.
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