Nuclear Magnetic Resonance Metabolomic Footprinting of Human Hepatic Stem Cells and Hepatoblasts Cultured in Hyaluronan-Matrix Hydrogels

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Key Words. Nuclear magnetic resonance • Metabolomics • Footprinting • Human • Hepatic stem cells • Hepatoblasts • Extracellular matrix • Hyaluronans • Collagens

ABSTRACT
Human hepatoblasts (hHBs) and human hepatic stem cells (hHpSCs) were maintained in serum-free Kubota’s medium, a defined medium tailored for hepatic progenitors, and on culture plastic versus hyaluronan hydrogels mixed with specific combinations of extracellular matrix components (e.g., type I collagen and laminin). Nuclear magnetic resonance spectroscopy was used to define metabolomic profiles for each substratum tested. The hHpSCs on culture plastic survived throughout the culture study, whereas hHBs on plastic died within 7–10 days. Both survived and expanded in all hydrogel-matrix combinations tested for more than 4 weeks. Profiles of hundreds of metabolites were narrowed to a detailed analysis of eight, such as glucose, lactate, and glutamine, shown to be significant components of cellular pathways, including the Krebs and urea cycles. The metabolomic profiles indicated that hHpSCs on plastic remained as stem cells expressing low levels of albumin but no α-fetoprotein (AFP); those in hydrogels were primarily hHBs, expressing AFP, albumin, and urea. Both hHpSCs and hHBs used energy provided by anaerobic metabolism. Variations in hyaluronan-matrix chemistry resulted in distinct profiles correlating with growth or with differentiative responses. Metabolomic footprinting offers noninvasive and nondestructive assessment of physiological states of stem/progenitor cells ex vivo. STEM CELLS 2008;26:1547–1555

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION
Nuclear magnetic resonance (NMR) spectroscopy is used to analyze small molecules (<2,000 Da), the structures of macromolecules up to 60 kDa [1, 2], and metabolomic profiling with respect to biochemical pathways such as glycolysis, Krebs cycle, urea synthesis, and transamination [3]. NMR can detect diverse metabolites noninvasively and nondestructively without extensive sample preparation [2]. The metabolome may offer a more relevant view of in vivo metabolism compared with the proteome, to define a phenotype on the basis of amplification of sequential reactions within the cell [4].

Human hepatic stem cells (hHpSCs) and human hepatoblasts (hHBs) are small cells (both less than 12 μm) with overlapping but distinct antigenic profiles; they are located in vivo within or next to the ductal plates of fetal livers and the canals of Hering in adult livers [5, 6]. The methods for identification, isolation, and culture of cells in monolayer cultures or in purified hyaluronan hydrogels have been described in prior reports [5, 7–12] Cells were cultured in Kubota’s medium (KM), a serum-free medium designed for hepatic progenitors, and either on culture plastic or in hyaluronan hydrogels mixed with specific extracellular matrix components. Conditioned medium from cultures was collected daily and subjected to metabolomic profiling. Cell biological characterizations of cells in these conditions are described elsewhere (Turner et al., manuscript in preparation). This study focuses upon use of NMR to evaluate the cellular metabolome of the cells under these conditions.

MATERIALS AND METHODS

Most of the methods are described in the supplemental online data. Here are given only those that uniquely apply to the NMR studies.

Hyaluronan-Matrix Complexes

The complexes comprised two forms of hyaluronan (HA) [13]. Hyaluronan modified with a 3,3′-dithiobis(propanoic dihydrazide)
thiol group (HA-DTPH) is a chemically modified HA possessing multiple thiols for cross-linking with disulfide bridges [14] or with a bivalent electrophile [15]. Carbaryl-S (CMHA-S) is a carboxy-methylated HA derivative further modified with multiple thiols for cross-linking [16]. The cross-linking was achieved by treatment with polyethylene glycol diacrylate. We prepared complexes of HA-DTPH or CMHA-S with type I collagen (Sagma-Aldrich, St. Louis, http://www.sigmaaldrich.com) and/or laminin (Sigma-Aldrich). Seven variations of substratum were tested: (a) culture plastic (PL), (b) HA-DTPH + collagen in a gelatin form (H + G), (c) HA-DTPH + gelatin + laminin (H + G + L), (d) CMHA-S (C), (e) CMHA-S + laminin (C + L), (f) CMHA-S + gelatin (G), and (g) CMHA-S + gelatin + laminin (C + G + L). Additional details on the preparation of the hydrogels are given in the supplemental online data.

Hyaluronan Matrices as Substrata

The mixtures of extracellular matrix components (sECMs) were placed into six-well polystyrene culture dishes (Falcon; Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com) just after addition of the cross-linker and prior to gelation and incubation for 16 hours incubation at 37°C in a CO2 incubator (Forma Scientific, Thermo Scientific, Waltham, MA, http://www.thermo.com) to allow gelation to occur. One milliliter of KM was added to each hydrogel followed by HhPSCs and HhBs suspended in KM at seeding densities of 1 million cells per hydrogel (volume, 0.5 ml). If embedded into the hydrogels, cells were seeded prior to cross-linking. Cells embedded in the hydrogels remained as aggregates of cells throughout the hydrogel. Cells were cultured for 4 weeks, with media changes every day.

NMR Spectroscopy

Sample Collection. Samples were taken in triplicate from each experimental condition and each control on days 2, 8, and 12. Samples were stored at −80°C immediately following collection.

Sample Preparation. After thawing at room temperature, an aliquot of 540 μl of each sample was added to 5-mm magnetic resonance tubes (Wilmad Lab-Glass Inc., Buena, NJ, http://www.wilmad-labglass.com) along with 60 μl of a D2O (Cambridge Isotope Laboratories, Andover, MA, http://www.isotope.com) solution containing 81.84 mM formate (Alfa Aesar, Ward Hill, MA, http://www.alfa.com) for a chemical shift reference, and 0.2% NaN3 (Sigma-Aldrich) to inhibit bacterial growth.

1H NMR Spectroscopy. All 1H NMR spectra were obtained using a Varian 400 MHz NMR spectrometer (Varian Inc., Palo Alto, CA, http://www.varianinc.com). A standard one-pulse sequence with 60° pulse angle and a 1.5-second H2O presaturation was used, with an interpulse delay of 6 seconds, an acquisition time of 2,558 seconds, a sweep width of 4,650.08 Hz, and the transmitter offset centered on the H2O signal. The spectra were digitized with 24,000 points.

Spectral Processing. Processing of all NMR spectra was done in ACD/1D NMR Manager, version 8.0 (Advanced Chemistry Development, Inc., Toronto, ON, Canada, http://www.acdlabs.com). Exponential line broadening of 0.1 Hz and zero filling to 32,000 points were applied to each spectrum. After Fourier transformation, spectra were autophased and baseline corrected using uniform settings applied to all spectra. All spectra were referenced to the TSP peak at 0.00 ppm. Regions of the spectra associated with TSP (0.25 ppm and upfield), formate (8.23–8.51 ppm), and water (4.55–5.03 ppm) were used for integrations applied to the PCA-identified regions of interest. Those variations were managed in the statistical analyses as a random effect. Five hundred sixty pairwise comparisons were made between scaffolds all falling in the classes of C versus PL, C versus L, C versus G, and H versus L. Fixed time points were used for assessing substrata effects, so that data for different matrices were compared at the same time point. Major variables that were impossible to control in the samples were the distinctions in the human livers from one donor to another. Those variations were managed in the statistical analyses as a random effect. For each metabolite, a mixed model was fit with metabolite as the response. Times of 2, 8, and 12 days were used, and the following scaffolds were used: PL, C, L, G, C + L, H, and C + G + L. Fixed time points were used for assessing substrata effects, so that data for different matrices were compared at the same time point. Major variables that were impossible to control in the samples were the distinctions in the human livers from one donor to another. Those variations were managed in the statistical analyses as a random effect. Five hundred sixty pairwise comparisons were made between scaffolds all falling in the classes of C versus PL, C versus L, C versus G, and H versus L. Fixed time points were used for assessing substrata effects, so that data for different matrices were compared at the same time point. Major variables that were impossible to control in the samples were the distinctions in the human livers from one donor to another. Those variations were managed in the statistical analyses as a random effect. Fifty comparisons were made at each time point, a Bonferroni correction was done to account for multiple comparisons, and consequently only those p values less than .05/10 = .005 were considered statistically significant.

Model

The statistical model can be expressed as the equation $Y_{ijl} = \alpha + \beta_i + \gamma_j + \lambda_l + \Delta_{ij} + \epsilon_{ijl}$, where $\alpha$ is the overall mean response; $\beta$ is a fixed substrate effect; $\gamma$ is the effect of substrate and day and consequently has the subscripts $i$ and $j$; $\lambda$ is the random effect of liver, which is interpreted as the assumption that all livers in the study are considered to be random samples of all possible livers; $\Delta$ is a fixed effect of liver which is interpreted as the assumption that data for different matrices were compared at the same time point. Major variables that were impossible to control in the samples were the distinctions in the human livers from one donor to another. Those variations were managed in the statistical analyses as a random effect. Fifty comparisons were made at each time point, a Bonferroni correction was done to account for multiple comparisons, and consequently only those p values less than .05/10 = .005 were considered statistically significant.

RESULTS

Identification and Quantification of Metabolites in Cultured Cells

Areas of the spectra (Fig. 1) were not considered for PCA, because they contained exogenous signals not emanating from cell metabolism. These areas are represented as “dark” regions of the spectra, and appear near the vicinities of water, the controls, or spectral areas beyond the range of the signal. These
dark regions specifically fall into three distinct ranges. The first is 0.2 ppm or less, which contains only the internal standard TSP. The second dark region falls between 4.06 and 5.02 ppm, a region that includes the water peak, which is the strongest signal in the sample. The last dark region falls between 8.24 and 8.48 ppm and includes the known peak for formate, used as a standard to quantify the concentration [17] of metabolites present as a percentage of area under their respective spectral curve. Many of the significant metabolites, determined by PCA, fall within the region of 0.84 and 4.46 ppm, and this area is expanded for better visualization.

PCA is the primary pattern recognition algorithm used to simplify the enormous amount of biochemical information contained in 1H NMR spectra. PCA reduces the 1H NMR spectra to a set of eigenvalues, typically three or four values, called principal components, which can be plotted against one another as a measure of the metabolome; those clustering together have a similar metabolome and differ from those far away from the cluster. These graphs are called the score plots [4]. PCA was used to determine whether NMR spectra change systematically with time of incubation. Indeed, the time of incubation was one of the most significant sources of variability among the samples.

The time-dependent variability was captured most prominently by the first two principal components (PCs), PC1 and PC2, which are shown in Figure 2. The score plot in Figure 2A was generated by plotting the value (score) of each sample along PC1 versus its value for PC2. The samples are color-coded according to the day on which they were obtained, revealing substantial clustering of samples taken on different days in different regions of the plot. From day 2 to day 8 and then to day 12, samples tend to shift to the upper left in the plot. Still, there is a major overlap among the three day-defined clusters. Some overlap was due to systematic differences between liver 3 and livers 1 and 2 used in this study. To show these differences of spectral variability, samples obtained from livers 1 and 2 are plotted in Figure 2B, and samples obtained from liver 3 are plotted separately in Figure 2C. These two score plots clearly show that for each liver the day 2 samples are prominently displaced relative to the day 8 samples, which in turn are shifted relative to day 2 samples.

Figure 1. 1H nuclear magnetic resonance spectra. Spectra and peak identification of culture media obtained at 9.4 T. Metabolite assignments are shown in the upper left corner and correspond to the numbered spectra peaks. Chemical shift (ppm) is shown at the bottom from 0 to 9 (left to right). Scaled bins are immediately above and parallel to the horizontal chemical shift axis, except in dark regions. Dark regions fall in regions of <0.02, 4.06–5.02, and 8.24–8.48 ppm, eliminating peaks for 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt, water, and formate respectively. Many of the significant metabolites fall within the boxed region from 0.84 to 4.46 ppm, and this area is expanded for better visualization.

Figure 2. Score plots from principal component analysis of all 187 samples of 1H nuclear magnetic resonance spectra. Each point represents an individual supernatant sample, color-coded according to its incubation age. (A): Shown are all 187 samples. (B): Shown are 124 samples originated from livers 1 and 2. (C): Shown are 63 samples originated from liver 3. Note that samples in (C) are less dispersed than those in (B), and they are also shifted overall relative to samples in (B). Despite these differences, (B) and (C) show the same trend of day 12 samples being partially shifted relative to day 8 samples, which in turn are shifted relative to day 2 samples.
treatment are connected, to make clear the paths traveled in the score plot over the incubation period. Similar trajectories can be equated with similar metabolic changes in culture. This plot fails to identify significant differences in temporal trajectories that might be due to different treatments.

To determine which metabolites were primarily responsible for temporal changes in NMR spectra, the contributions (loadings) of each spectral bin to PC1 and PC2 are plotted in Figure 4. In the loading plot, each bin is shown as a point. The loading plot relates the relative contribution of the individual bins to the overall value of the eigenvalues, PC1, PC2, PC3, etc. Each bin contains one or more peaks representing a molecule in the medium sample [4]. The bins that have little or no impact on PC1 and PC2 are placed at or near the plot origin. The more significant the contribution of a bin to one or both principal components, the farther away from the origin is its position in the plot. The bins responsible for the temporal shift of the spectra in the PC1 versus PC2 score plots (i.e., the left-upward \([\sim 135^\circ]\) shift of points; Figs. 2, 3) are the bins that in the Figure 4 loading plots are shifted in the same \((\sim 135^\circ)\) or opposite \((\sim 215^\circ)\) direction from the plot origin. In other words, the spectral bins sensitive to incubation time are located in the top left and bottom right corners of the loading plot. Among these, bins were found containing the following metabolites: glucose, alanine, lactate, arginine, glutamine, and glutamate. These bins are labeled accordingly in the Figure 4 plot. Thus, PCA suggests that concentrations of the metabolites in the supernatant change systematically from day 2 to day 12.

The concentrations derived from the spectral data are expressed as a percentage area compared with that of formate, the concentration standard added to the samples. Representative samples are exhibited in Figure 5. In each case the substrate is labeled as the x-axis in the graph where the seven different scaffolds for the cells are compared: C, C + L, C + G, C + G + L, H + G, H + G + L, and PL. The last single column represents the endogenous amount of that metabolite found within the KM. The sample days represented as 2, 8, and 12 are color-coded and sequenced, thus giving a triplet of 2 (red), 8 (tan), 12 (blue) with day 2 on the left and day 12 on the right. The height of the histogram is demarcated by the y-axis, representing the percentage formate peak. By setting the area under the formate peak in all spectra to be 100% of the concentration, comparisons can be made among the spectra in regard to concentration of metabolite present.

Figure 5A, representing glucose, shows that all cultures of matrix hydrogels are consumers of available glucose within the system. Of the bins recognized as important in the PCA, three are representative of glucose. When peaks plotted for the three different glucose representative areas are compared, they show the same histogram (data not shown for all three graphs). The percentage translates into a net amount for each known metabolite and is recorded as a net loss or production within the mass balance of the system. Medium glucose is highest in the KM solution (the controls, no cells), whereas on other scaffolds, the net use of the glucose decreases from day 2 to day 12. Therefore, the cells are consuming glucose, not generating it, as would occur if they were more differentiated and capable of gluconeogenesis.

Figure 5B is the histogram of alanine. Again the x-axis represents the substratum, which is the type of matrix-hyaluronan scaffold, and the y-axis is the percentage formate region under the curve of the spectra. Alanine is produced in high quantity at day 2 of the culturing process and subsequently declines in production by day 12. There is a shift between the first two graphed sECMs, CMHA-S and CMHA-S-laminin, in which production falls and consumption begins by day 12.

Figure 5C represents the histogram for lactate. The level of lactate within KM is at a low level \((0.0157\%\) formate peak). However, cells by day 2 show a minimum of an eightfold increase in lactate. Tissue culture plastic is the substratum with the highest induction of lactate metabolites in the cells. Over the course of the experiment, lactate production subsequently falls to close to the amount found in KM. This suggests that the cells are recovering from the processing procedures that have injured them, relying less on anaerobic glycolysis for energy, and have subsequently settled down to a more normal metabolism indicated by the restoration of lactate to low levels.

Figure 5D represents arginine. The amino acid metabolite is present in KM at low levels compared with that found by day 2. Cells cultured on all forms of scaffolds/substrata demonstrate a high level of arginine, which subsequently falls in concentration quickly. By day 12, arginine levels almost reached a concentration equivalent to the starting amount introduced in culture media.

Figure 5E represents glutamine, which has an overall consumption rate on all substrates, as recognized between the media and the cultures on days 2, 8, and 12. However, net consumption of the glutamine goes down over the course of time and appears to stabilize within a narrow range by day 8. A common set of
metabolites for this experiment is listed in supplemental online Table 1, giving a basis of reference for phenotyping the footprint of the human hepatic progenitor metabolites.

The statistical model used (described in Materials and Methods) contains the predicted trends for each treatment over time with consideration of substratum. The model is constructed by using the actual values to predict what occurs after all factors are taken into account. As with any of the metabolites, the model is considered individualized. The overall p values for this model are significant for time but not for substratum or the day/substratum interactions, which consider all the factors at once. The statistical model shows that the overall mean response of any metabolite is the combination of a fixed substratum effect, a fixed time effect, a random liver effect, and the interaction of the substratum and time variables. These effects are documented in pairwise comparisons for significance, found in Table 1.

Metabolomic Profiles
The metabolites identified by PCA in Figure 1 (also described in supplemental online Table 1) can be mapped to cellular metabolism. Figure 6A illustrates the metabolic connection of many of the significantly identified metabolites, including alanine, glucose, lactate, glutamine, and arginine. In brief, it links glucose consumption and lactate production to glycolysis, glutamine consumption to the Krebs cycle, and alanine and arginine accumulation to the urea cycle. Those metabolites can further be used to describe the metabolic pathways in which the cells produce proteins identified by enzyme-linked immunosorbent assay. For example, during glycolysis, glucose is transformed through a chain of chemical modifications into pyruvate, and it then enters the Krebs cycle as acetyl-CoA, located within the mitochondria.

α-Ketoglutarate in the Krebs cycle can be affected by transamination reactions with alanine and aspartate to yield glutamate, which in turn is an intermediate toward the production of glutamine and/or albumin. Oxaloacetate from the tricarboxylic acid cycle is the byproduct of the interaction of the α-ketoglutarate with the aspartate, as well as the conversion from malate, and in similar fashion, the combination of oxaloacetate and glutamate forms aspartate and α-ketoglutarate. Aspartate is funneled into the urea cycle, leading to the production of ornithine and citrulline.

<table>
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<tr>
<th>Metabolite</th>
<th>Overall effect</th>
<th>Day 2</th>
<th>Day 8</th>
<th>Day 12</th>
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<tbody>
<tr>
<td>Arginine</td>
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<tr>
<td>Choline</td>
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<td>Ethanol</td>
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<tr>
<td>Lactate/threonine</td>
<td>XX</td>
<td>XX</td>
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<td>Alanine</td>
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<td>Lactate</td>
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<tr>
<td>Glucose-α</td>
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<td>Glutamate-2</td>
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<td>Glutamate-3</td>
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<td>Glucose-2</td>
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<td>Glutamine</td>
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<td>X &lt; .05</td>
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Figure 5. Metabolites identified by PCR. (A–F): Formate-normalized metabolites of glucose, alanine, lactate, arginine, glutamine, and glutamate are shown as they vary with time with respect to each substratum. The substrata are listed along the horizontal axis. Normal amounts of metabolites found within fresh Kubota’s medium are reported as baseline (controls). Days are demarcated as individual colors, with every three bar sets forming the pattern of day 2 on the left, day 8 in the middle, and day 12 on the right. The y-axis is representative of the percentage of metabolite normalized to the formate control. Abbreviations: C, carbylan-S; G, gelatin; H, hyaluronan modified with a 3,3’-dithiobis(propanoic dihydrazide) thiol group; L, laminin; PL, plastic.

Table 1. Cultures in hyaluronan hydrogels (hyaluronans with disulfide cross-linking) versus on plastic
of arginine and, subsequently, urea. Pyruvate can be metabo-
lized into alanine or lactate, with reversal of the process being
plausible. Measured end products, urea and albumin, demonstrate
that the cells are parenchyma, as only these cells synthesize these
compounds. The relationship between arginine and urea produc-
tion is indicated by the data in Figure 6B. The x-axis represents
the normalized arginine concentration compared with the net
urea analysis, where the y-axis represents the day. Across all
substrata tested, the trend from day 2 to day 12 is a decrease in
production of both arginine and urea. The base amount of
arginine in KM is 200 mg/l. From day 2 through day 12, the
amount of arginine production fell from 178.7516 to 30.74449
mg/dl arginine. The paths of the arginine (Fig. 6B, dotted line)
and the urea (Fig. 6B, unbroken line) are similar, because they
are directly tied to a coupled reaction in which arginine is used
for urea cycle and affect the urea cycle, tying arginine consumption to urea production. (B): Urea production compared with arginine. The data normalized to formate for the concentration of arginine in the system have been averaged over the substrates. Kubota’s medium (KM) contains a base concentration of arginine of 200 mg/l, which correlates to an extracted value of 0.0066 concentration. From day 2 (0.058988% formate per 178.7516 mg/dl concentration) through day 12 (0.010146% formate per 30.74449 mg/dl concentration), there is production of arginine in the system. However, the net time effect in the system is a loss of production of arginine. (C): Albumin production is represented in the graph along with glutamine reduction. As diagrammed in (A), both glutamine and glutamate can affect the production of the albumin. Glutamine is consumed by the culture system, and the cells on day 2 consume almost all of the glutamine in the KM. By days 8 and 12, the consumption has subsided to just over half of the available glutamine in the medium. Glutamate production still occurs throughout the lifespan of the culture, but it decreases as the cultures age. Albumin production also drops with time. Abbreviation: TCA, tricarboxylic acid.

Figure 6. Metabolic pathways identified by spectral analysis and immunoassays. (A): Metabolic pathways represented schematically within a normal cell. The block diagram denotes principal component-identified metabolites. Glucose consumption produces pyruvate, which in turn produces alanine and lactate, as well as acetyl-CoA. Acetyl-CoA is shunted into the TCA cycle, which is tied to key metabolites of α-ketoglutarate and oxaloacetate. α-Ketoglutarate is tied to production of glutamine and albumin through the common pathway divergent at glutamate. Metabolites of the TCA cycle act on aspartate and affect the urea cycle, tying arginine consumption to urea production. (B): Urea production compared with arginine. The data normalized to formate for the concentration of arginine in the system have been averaged over the substrates. Kubota’s medium (KM) contains a base concentration of arginine of 200 mg/l, which correlates to an extracted value of 0.0066 concentration. From day 2 (0.058988% formate per 178.7516 mg/dl concentration) through day 12 (0.010146% formate per 30.74449 mg/dl concentration), there is production of arginine in the system. However, the net time effect in the system is a loss of production of arginine. (C): Albumin production is represented in the graph along with glutamine reduction. As diagrammed in (A), both glutamine and glutamate can affect the production of the albumin. Glutamine is consumed by the culture system, and the cells on day 2 consume almost all of the glutamine in the KM. By days 8 and 12, the consumption has subsided to just over half of the available glutamine in the medium. Glutamate production still occurs throughout the lifespan of the culture, but it decreases as the cultures age. Albumin production also drops with time. Abbreviation: TCA, tricarboxylic acid.

hHpSCs and hHBs will survive and expand for weeks in three-
dimensional (3D) cultures in hyaluronan-matrix hydrogels and
with metabolomic profiles confirming that the cells lineage
restricted to or remained throughout as hHBs. Our past studies
demonstrated that hHpSCs, but not hepatoblasts, survived on
culture plastic; on plastic, the hepatoblasts disappeared by
―7–10 days [9]. Daily metabolomic phenotyping by NMR
assays of the hHBs cultured in the hydrogels provided a global
survey of metabolic pathways in the cells.
This is the first report, to our knowledge, of metabolomic phenotyping of a stem/progenitor cell population via unbiased high-throughput analytical methods using computational mathematics to interpret the metabolic footprint. The novelty of the approach is furthered indicated by the fact that there have been relatively few studies on metabolomics, even of mature hepatocytes, but none at all of hepatic progenitors. Most of the published reports are with respect to drug metabolism [2, 18, 19] or on a specific metabolic pathway, such as carbohydrates [20] or lipids [21]. Those studies characterize transcription factors or signaling pathways but do not analyze the complement of small molecules produced by the cells. It is possible that metabolomics were ignored because of the need for improved methods for doing metabolomic analyses on cells in culture. In this study, we carefully modified the extracellular matrix composition of the hydrogels to identify their influence on metabolomic differences indicative of a stem cell versus differentiated phenotype.

The extracellular matrix in combination with soluble signals (autocrine, paracrine, and endocrine) synergistically regulates all aspects of cells [22, 23]. Matrix effects on mature hepatocytes have long been known to regulate specific gene expression and, by inference, metabolic profiles [18, 23–27]. Changes in the chemistry of the matrix and soluble signals parallel the maturational stages of the cells [28–31]. The matrix chemistry near or in the liver’s stem cell niches is composed of hyaluronans and their receptors, CD44H, laminins and certain integrins (α4β1, α6β4), type III and IV collagen, and forms of chondroitin sulfate proteoglycans (PGs) with few sulfate residues [5, 11, 12, 29, 30]. The matrix chemistry transitions to that associated with fully mature cells and composed of collagen type I, some collagen type III, fibronectin, and forms of highly sulfated proteoglycans (e.g. heparin-PGs) but not laminin, hyaluronans, or type IV collagen [3–8, 23, 29, 30, 32, 33]. Because of the microenvironment [11, 12], the hHpSCs and hHBs are less likely to come into contact with the shear stresses from blood flow or with the hormonal factors contained within the blood of the sinusoids than their progeny adjacent to fenestrated endothelial cells of the sinusoids [34]. The fenestrated endothelia offer cellular boundaries in some regions and only matrix chemistry at the sites of the fenestrae. The knowledge of the matrix chemistry was used to define the variations in hyaluronan-hydrogel matrix tested.

Applying metabolomic analytical methods, extensive multivariate statistical analyses were performed on the NMR data; these analyses further illustrated that the changes in the matrix chemistry can give rise to significant changes in the production of certain metabolites, including consumption of specific compounds. Implications of the changes in the matrix chemistry and comparison with what occurs with the control cultures (plastic) are indicative of what is going on within the cells on matrix scaffolds, especially those in 3D cultures. Table 1 is indicative of the comparison of cells on plastic and matrix-hyaluronan mixes. Of the 560 paired comparisons, those between the CMHA-S and plastic show the most significance in metabolic divergence. This is not to be confused as an identification of the best culturing conditions. The choice of culturing conditions is dictated, in part, by the desired biological responses of the cells. However, using this method of analysis, CMHA-S does exhibit properties that make it the preferred system, of those assayed, for the maintenance of hepatoblasts with minimal differentiation.

The relevance of being three-dimensional is also strongly implicated by our findings. The hHpSCs and hHBs in serum-free KM in monolayer cultures showed a strong selection for hHpSCs within 1 or 2 weeks [7, 9, 11, 12]. In the hyaluronan hydrogels, the selection proved true for hepatoblasts under all variations of hyaluronan and matrix components (e.g., HA-DTPH alone, HA-DTPH with laminin, and HA-DTPH with collagen). The variations in matrix components added to the hyaluronans affected expansion rates or differentiated functions in subtle ways, all the conditions assayed were equivalent in the ability to sustain human hepatic progenitors at the hepatoblast lineage stage. Three-dimensionality of cells when embedded in type I collagen resulted in more rapid differentiation, whereas in hyaluronans, it resulted in more rapid expansion. Therefore, the response of the cells is defined by the chemistry of the matrix components and by the medium conditions, as well as by being in a three-dimensional state. Alternatively, it may be a factor of whether a matrix component(s) is presented on one or multiple cell surfaces.

Serum was avoided, given that it is not defined, results in differentiation of mesenchymal and parenchymal progenitors, and results in selective overgrowth of mesenchymal cell populations [35]. The serum-free conditions contributed to greater stability and uniformity of gene expression, corroborating similar findings in prior studies [18].

The form of cross-linking of the hydrogels proved important in their influence on the cells. Aldehyde-cross-linked hydrogels were found to be supportive, albeit resulting in slow growth and with technical difficulties in retrieval of the cells [36]. The hydrogels used here are cross-linked by disulfide bridges and were found to be permissive for rapid growth and for ease of cell recovery. This permissiveness is assumed to be due to the cells’ ability to enzymatically modify the scaffolding, a requirement for cells to go through cell division.

Metabolomic profiles (Fig. 5; Table 1; also described in supplemental online data) readily indicate cells in aerobic versus anaerobic metabolism. Our data indicate that the hHpSCs on culture plastic and the hHBs in the hydrogels rely primarily on aerobic glycolysis, especially initially in the incubation. Alanine and lactate are indicative of anaerobic glycolysis [37]. Others have reported that low oxygen concentrations in mammalian embryonic tissues are normal. Thus, maintenance of cultures at low oxygen tensions should be selective conditions for stem cells and early progenitors [38–40]. Cells engaged primarily in anaerobic, not aerobic, metabolism are highly indicative of stem cells [41]. This correlates with the known oxygen conditions in vivo, in which the site of the liver’s stem cell niche has significantly lower levels of oxygen than that next to the more mature parenchymal cells [40].

Also, hHpSCs and hHBs are remarkably tolerant of ischemia, surviving for hours in the livers of asystolic donors whereas mature parenchymal cells typically die within an hour following cardiac arrest [9, 42]. Mammalian cells act in response to a hypoxic environment by increasing their carbohydrate consumption and switching to anaerobic respiration, a pattern also found in stem cells [40, 43]. Glycolytic enzymes are present in the cytosol and do not require mitochondria. Glycolytic metabolic pathways, the “Warburg effect,” are known to be associated with cancer cells, now recognized to be transformed stem cells. Lactate is an end product of anaerobic glycolysis and is indicative of low oxygen availability, a relatively undifferentiated state [37], or consumption of it by specific cell types. Anaerobic use of glucose with low production of lactate has been reported within the hepatic culturing system. Specific stem cell populations, such as hemopoietic progenitors, are able to survive in this state by using glycolytic pathways [40, 44]. Grayson et al. [40] and Cipolleschi et al. [44] report that human mesenchymal stem cells have increased glucose consumption and lactate production under such conditions. Certain lineage stages of cells, especially stem cells, are able to survive under hypoxic conditions over extended periods of time [40], tolerance for ischemia [42], and relatively undifferentiated state.
The data here show that glucose consumption is high and that lactate production is high (Fig. 4). Each of the metabolites is reduced over time, which can be explained as an initial inflammatory process and acute injury responses when the cells are first plated and then adaptation to culture with a gradual shift to lineage stages optimally supported by the culture conditions used. Transamination of pyruvate, which leads to increased amounts of alanine, occurs to help decrease systems of lactate.

Conversion from anaerobic to aerobic metabolic pathways is indicative of differentiation [41]. Differentiation is also indicated by metabolomic profiles implicating gluconeogenesis and the urea cycle pathway. Gluconeogenic amino acids are alanine, threonine, tyrosine, and phenylalanine [2]. Profiles for these can be constructed using the NMR phenotyping, where reduction could be indicative of that metabolic pathway. The urea cycle pathway includes biomarkers of arginine, glutamine, and urea [45]. Arginine is taken up by the cells and is used to produce urea [2]. As a direct player in production (Fig. 6), the log-log graph of arginine compared with production of urea follows the same path. Furthermore, the impact of this pathway and the albumin production pathway is complicated where the production of arginine, glutamine, and urea could be indicative of that metabolic pathway. The urea cycle is constructed using the NMR phenotyping, where reduction in the matrix components added to the hyaluronans resulted in metabolomics of human hepatic stem/progenitor cell populations indicate that the cells remain stably at the maturation stage of hepatic stem cells if on culture plastic or at the next lineage stage, hepatoblasts, if cultured in hyaluronans mixed with various extracellular matrix components. Variations in the matrix components added to the hyaluronans resulted in changes in the metabolomic profiles indicative of changes in growth or differentiative properties of the cells.

This application is directly applicable to cells in 3-D cultures or in bioreactors in which one can better mimic the multifaceted complexity of in vivo situations (2, 11, 41, 42). The database being established should prove ideal for rapid daily assessment of cell populations to see whether they are surviving, expanding, undergoing lineage restriction and/or differentiating to specific adult fates.

Bioreactors are being created with useful features (e.g., dissolution boundaries, see through portals) and are complemented by platforms of analyses to overcome the limitations of access to cells within the devices. Metabolomic profiles offer non-invasive and non-destructive assays to assess the cells in bioreactors or other forms of 3-D culture conditions.

### CONCLUSION

Metabolomic profiles of human hepatic stem/progenitor cell populations indicate that the cells remain stably at the maturation stage of hepatic stem cells if on culture plastic or at the next lineage stage, hepatoblasts, if cultured in hyaluronans mixed with various extracellular matrix components. Variations in the matrix components added to the hyaluronans resulted in changes in the metabolomic profiles indicative of changes in growth or differentiative properties of the cells.

The authors indicate no potential conflicts of interest.

### REFERENCES

22. Lauffenburger D, Griffith L. Who’s got pull around here? Cell organi-
27. Kiatt M, Confort-Gouny S, Vion-Dury J et al. Quantitation of metabo-
31. Lauffenburger D, Griffith L. Who’s got pull around here? Cell organi-
34. Zheng Shu X, Liu Y, Palumbo F et al. In situ crosslinkable glycos-
36. Kiatt M, Confort-Gouny S, Vion-Dury J et al. Quantitation of metabo-
40. Lauffenburger D, Griffith L. Who’s got pull around here? Cell organi-
44. Zheng Shu X, Liu Y, Palumbo F et al. In situ crosslinkable glycos-


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Stem Cells 2008;26;1547-1555; originally published online Mar 6, 2008;
DOI: 10.1634/stemcells.2007-0863

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