

Oxygen-mediated enhancement of primary hepatocyte metabolism, functional polarization, gene expression, and drug clearance

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The liver is a major site for the metabolism of xenobiotic compounds due to its abundant level of phase I/II metabolic enzymes. With the cost of drug development escalating to over \$400 million/drug there is an urgent need for the development of rigorous models of hepatic metabolism for preclinical screening of drug clearance and hepatotoxicity. Here, we present a microenvironment in which primary human and rat hepatocytes maintain a high level of metabolic competence without a long adaptation period. We demonstrate that co-cultures of hepatocytes and endothelial cells in serum-free media seeded under 95% oxygen maintain functional apical and basal polarity, high levels of cytochrome P450 activity, and gene expression profiles on par with freshly isolated hepatocytes. These oxygenated co-cultures demonstrate a remarkable ability to predict in vivo drug clearance rates of both rapid and slow clearing drugs with an R^2 of 0.92. Moreover, as the metabolic function of oxygenated co-cultures stabilizes overnight, preclinical testing can be carried out days or even weeks before other culture methods, significantly reducing associated labor and cost. These results are readily extendable to other culture configurations including three-dimensional culture, bioreactor studies, as well as microfabricated co-cultures.

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The liver is the largest internal organ and the hub of carbohydrate, lipid, and protein metabolism. Liver metabolism plays a central role in the clearance, modification, and incidental toxicity of most nutrients and xenobiotics. Consequently drug-induced liver toxicity and unpredicted drug metabolism are major causes of postmarket drug withdrawal (1, 2). With cost of drug development escalating to \$400 million/drug there is an urgent need for the development of rigorous models of liver metabolism in the context of ADME/Tox (absorption, distribution, metabolism, excretion, and toxicity) screening. This need is exacerbated by the failure of animal studies to predict drug clearance and toxicity, as well as the disastrous clinical and financial consequences of postmarket drug withdrawal (2, 3).

One model found to be useful in the prediction of drug metabolism is the culture of primary human hepatocytes (4, 5). In current practice, isolated hepatocytes are cultured in suspension, a configuration shown to maintain high levels of cytochrome P450 (CYP450) activity up to 6 h in vitro. This technique allows for the characterization of rapidly clearing drugs (4, 5). However, the inherent difficulties in evaluating the metabolism of slow-clearing drugs under such short time periods bars many promising compounds from clinical validation (4). An alternative approach developed by several groups, including ours, is to support the long-term function of primary hepatocytes using specialized tissue culture configurations (6–8).

Previously, Dunn et al. demonstrated long-term synthetic and metabolic activity in primary hepatocytes entrapped between two layers of collagen (9, 10), while others demonstrated similar enhancement of function in hepatocytes following their aggregation into spheroids (11). An alternative strategy is the cocul-

ture of hepatocytes with non-parenchymal cells such as 3T3-J2 fibroblasts or endothelial cells (8, 12, 13). Recently, micropatterns of hepatocytes and 3T3-J2 were shown to acquire high levels of CYP450 gene transcription and metabolic activity following 11 days of culture (14). While these culture configurations offer significant metabolic competence, they do so only after a long adaptation period of between 7 to 10 days of culture during which the primary cells slowly adapt their metabolic activity to the in vitro microenvironment (6, 14).

One strategy to eliminate this long adaptation period and attain a high level of metabolic activity from the onset of culture is to minimize the stress associated with the transition between the in vivo to the in vitro microenvironment. A critical aspect of the microenvironment which is dramatically different between in vivo and in vitro is oxygen supply (15). In vivo a mixture of arterial and venous blood continuously supply over 2,000 nmol/mL of oxygen to hepatocytes, while in vitro oxygen's low solubility in culture media offers less than 200 nmol/mL to the cells (7, 16). While this has traditionally limited hepatocytes to subconfluent cultures (15), oxygen supply becomes an even greater concern during the initial phase of cell attachment when oxygen uptake rates are 300% greater than normal (17, 18). It is not surprising therefore that reducing oxygen concentration negatively affects hepatocyte metabolism (19–21). However, it is surprising that the culture of primary hepatocytes under high partial pressures of oxygen is not reported to improve their metabolic activity (19, 21, 22).

Our recent development of an oxygen-carrying matrix allowed us to identify a negative effect of serum on oxygen-enhanced metabolism (16). As serum has been previously shown to cause loss of hepatocyte polarity and gene expression during the onset of culture (23, 24), it suggests that a serum-free, oxygen-rich microenvironment would minimize adaptation stress and allow for high levels of metabolic activity from the onset of culture. If this hypothesis holds, it suggests a simple approach to enhance the metabolic activity of other highly metabolic cells such as cardiomyocytes, β -cells, or neurons, and could potentially enhance our ability to induce these phenotypes during embryonic stem cell differentiation.

In support of this hypothesis we demonstrate that serum has a predominantly negative effect on the metabolic function of primary rat and human hepatocytes. In the absence of serum, the effects of an oxygen-rich seeding environment are pronounced,

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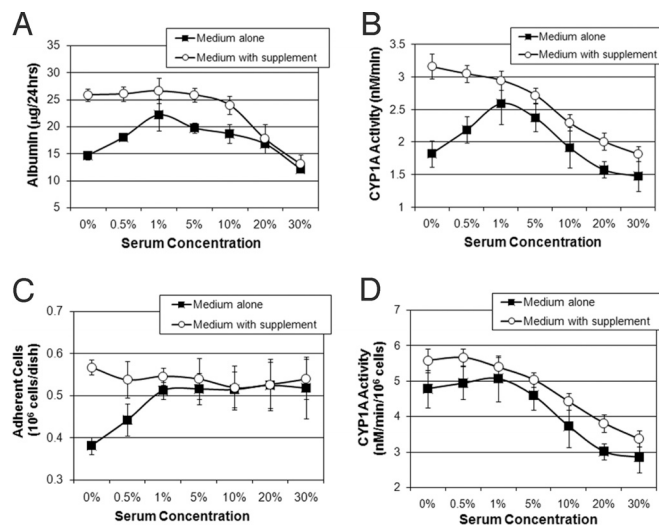


Fig. 2. Serum has a predominantly negative effect on the function of primary rat hepatocytes. (A) Rates of albumin synthesis and (B) Cyp1A1/2 activity following overnight seeding in serum-free media containing increasing concentrations of HI-FBS. (C) Total protein analysis demonstrates increased cell attachment with serum up to 1% concentration. In the presence of defined attachment supplement (with supplement) cellular attachment is decoupled from serum. (D) Cyp1A1/2 activity normalized to number of adhered cells, hepatocyte metabolic function is seen to be inversely correlated with serum content in the seeding media.

Serum Has a Detrimental Effect on Short-Term Hepatocyte Function. Primary cells are thought to require significant adaptation to serum (23). To evaluate the effect of serum on the function of primary hepatocytes, primary rat hepatocytes were seeded at a density of 100,000 cells/cm² on collagen-coated plates in serum-free medium supplemented with increasing concentrations of heat-inactivated FBS. Fig. 2*A* shows albumin production during the first 24 h of culture, while Fig. 2*B* shows Cyp1A activity following 24 h of culture. Both albumin secretion and Cyp1A activity significantly increased with serum up to 1% concentration by $52 \pm 8\%$ ($P = 0.001$, $n = 3$) and $30 \pm 10\%$ ($P = 0.021$, $n = 3$), respectively. However, higher concentration of serum led to a significant decrease in albumin secretion and Cyp1A activity by $45 \pm 4\%$ ($P = 0.017$, $n = 3$) and $43 \pm 1\%$ ($P = 0.001$, $n = 3$), respectively. As cellular attachment is thought to be mediated by serum, we quantified cell attachment by measuring total protein as a surrogate measure of cell number which is linearly correlated with DNA content and cell counting in primary hepatocyte cultures (Fig. S1). Fig. 2*C* shows that cell adhesion increased in the presence of serum up to 1% concentration corresponding to the increased function. To compensate for this loss in cell adhesion, we supplemented the serum-free media with H μ REL defined attachment supplement. Fig. 2*C* shows that in the presence of the attachment supplement, serum had little effect on cellular adhesion. More importantly, under these conditions Cyp1A activity increased by $74 \pm 4\%$ ($P = 0.020$, $n = 3$), Fig. 2*B*. Normalizing albumin production and Cyp1A activity to adherent cell number demonstrates that serum has a predominantly negative effect on hepatocyte function during the first 24 h of culture (Fig. 2*D*).

Oxygenated Co-Cultures Support High Levels of Cyp1A1/2 and Cyp2B1/2 Activity. Oxygen is an important component of the hepatic microenvironment (15). While passive diffusion of oxygen is not thought to be limiting during normal culture (15), it fails to supply the oxygen requirements of primary rat and pig hepatocytes during the first 24 h of culture when oxygen

Table 1. Cyp1A activity in rat hepatocytes after 24 h of culture (nanomolar per minute per 10⁶ cells)

	Hepatocytes		Hepatocyte - Endothelial	
	Serum free	10% Serum	Serum free	10% Serum
Normal oxygen	1.48 ± 0.12	0.70 ± 0.50	1.45 ± 0.35	1.20 ± 0.60
High oxygen	2.44 ± 0.20	0.89 ± 0.30	3.45 ± 0.21	1.29 ± 0.13

demands are three to four times higher than their basal levels (17, 18). Therefore, seeding primary hepatocytes under a partial pressure of 95% oxygen can potentially reduce the stress associated with the transition from in vivo to in vitro culture, supporting superior survival and metabolic function. Table 1 shows Cyp1A activity in monocultures and co-cultures of primary rat hepatocytes seeded overnight in 21% or 95% oxygen, corresponding to 7 or 32 mg/L oxygen at the air-liquid interface respectively. Both cultures were stabilized to atmospheric levels of oxygen for 30 min prior to the EROD assay to ensure basal levels of activity. Cyp1A activity was markedly elevated following culture in 95% oxygen. Monocultures activity was increased by 64% ± 8% while activity in co-cultures increased by 138 ± 6%. Interestingly, this increase in function could not be detected when the cells were cultured in the presence of 10% serum. Similar results were observed in monocultures and co-cultures of human hepatocytes (Table S1). Hepatocyte viability in co-cultures at 24 h was also significantly increased from 84 ± 4% at normal oxygen tension to 96 ± 2% at high oxygen tension ($P = 0.005$, $n = 3$) (Fig. S2). To further characterize the metabolic competence of co-cultures seeded in 95% oxygen, we quantified the activities of CYP1A1/2 and CYP2B1/2 enzymes in both rat and human cells. Fig. 3A compares Cyp1A1/2 and Cyp2B1/2 activity in primary rat hepatocytes co-cultured with endothelial

cells in 95% oxygen for 24 h (day 1) to the activity of rat hepatocytes from the same isolation in suspension (day 0). Similar results are reproduced in Fig. S3 for cryopreserved human hepatocytes. Our data demonstrates that both human and rat oxygenated co-cultures support high levels of CYP450 activity, comparable to that of hepatocytes from the same isolation cultured in suspension.

Long-Term Maintenance of Hepatic Metabolic Competence in Oxygenated Co-Cultures. To further characterize the potential of oxygenated co-cultures we quantified albumin synthesis, urea production, and Cyp1A activity over 7 days in culture. Co-cultures of hepatocytes with endothelial cells were seeded in serum-free culture media under normal oxygen tension (co-cultures) or 95% oxygen [co-cultures (oxygen)]. As a second control representing widely used culture configuration we co-cultured hepatocytes with endothelial cells in serum-containing culture medium [co-cultures (serum)]. Fig. 3C demonstrates that albumin secretion during the first day of culture in 95% oxygen was 3-fold higher compared to serum-cultured cells ($P = 0.016$, $n = 3$), and 2-fold higher compared to serum-free cultured cells ($P = 0.049$, $n = 3$). No significant differences were detected by day 7. Interestingly, while urea production (Fig. 3D) was not statistically different at the onset of culture, it increased by day 7 in co-cultures seeded in 95% oxygen to be 4-fold higher than serum-cultured cells ($P = 0.001$, $n = 3$), but was not statistically different from the serum-free condition ($P = 0.057$, $n = 3$). To compare long-term Cyp1A activity to current model systems, we carried out two suspension measurements, one with freshly isolated hepatocytes and another with isolated hepatocytes following 2 h of incubation at 37 °C to account for the rapid loss of function in suspension. As shown above Fig. 3B demonstrates that Cyp1A activity in co-cultures seeded under high oxygen tension was comparable to that of hepatocytes in suspension. During the first day of culture, the activity was 59% higher than that of serum-cultured cells ($P = 0.001$, $n = 3$), and 70% higher than serum-free cultured cells ($P = 0.001$, $n = 3$). However, at day 7 of culture, there was no significant difference between the co-cultures suggesting similar levels of metabolic competence. Long-term metabolic and synthetic activity was further supported by microscopy. Fig. 4B demonstrates that oxygenated co-cultures of primary human hepatocytes retain their distinct polygonal morphology through day 9 of culture.

Gene Expression and Functional Polarization in Oxygenated Co-Cultures.

To expand the characterization of oxygenated co-cultures we carried out gene expression analysis of phase I/II enzymes as well as drug transporters in both rat and human hepatocytes. As gene transcription of human hepatocytes is more clinically relevant it is presented in Fig. 4A, while rat data are shown in Fig. S4. Gene transcription of human hepatocytes following 24 h of culture was compared to mRNA isolated from the cryopreserved hepatocytes stock. Fig. 4A demonstrates that oxygenated co-cultures maintain a remarkable level of gene transcription following the first day of culture, comparable to in vivo levels of transcription. This result stands in contrast to the gene transcription levels of hepatocytes in serum-containing co-cultures which show dramatically lower levels of gene expression. To evaluate the functional activity of drug transporters in co-cultures seeded in 95% oxygen we stained human hepatocyte in oxygenated co-cultures with CDFDA, a compound which is metabolized into a fluorescent marker, and transported by polarized cells via MRP2 into bile canaliculi (25). Fig. 4C demonstrates that human hepatocytes seeded in 95% oxygen form functional bile canaliculi at the onset of culture. Another aspect of hepatocyte polarity is the presence of 3-O-sulfated heparan sulfate (HS4C3) on the basal surfaces of the cells (26). Heparan sulfate plays a critical role in the clearance of lipoproteins which are thought to

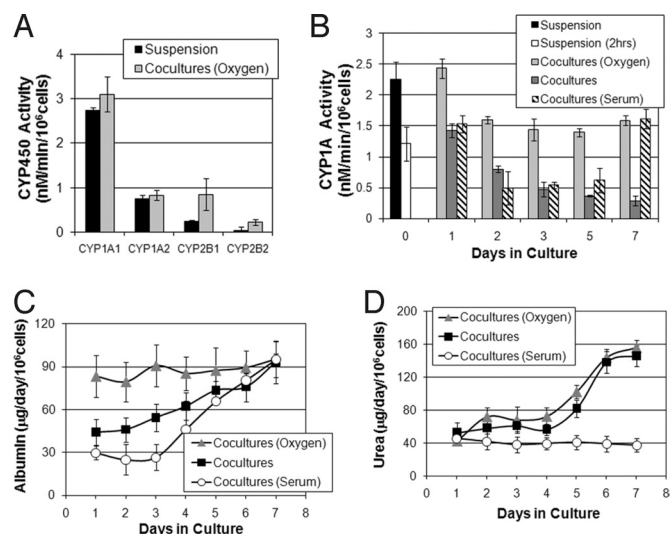


Fig. 3. Long-term function of serum-free oxygenated rat hepatocyte-endothelial co-cultures. (A) Activity of Cyp1A1/2 and Cyp2B1/2 in oxygenated co-cultures on the first day of culture (day 1) compared to hepatocytes from the same isolation cultured in suspension (day 0). (B) Long-term maintenance of Cyp1A1/2 activity in all three co-cultures compared to that of freshly isolated hepatocytes in suspension (Suspension). To account for the rapid loss of function in suspension, a second measurement was carried out on freshly isolated cells following 2 h of culture at 37 °C. (C) Rates of albumin synthesis and (D) urea production in serum-free hepatocyte-endothelial co-cultures seeded under normal oxygen tension (Cocultures) or 95% oxygen [Cocultures (Oxygen)]. Rates are compared to hepatocytes-endothelial co-cultures maintained in serum-containing culture medium [Cocultures (Serum)]. Synthetic activity in oxygenated co-cultures is maintained from the onset of culture.

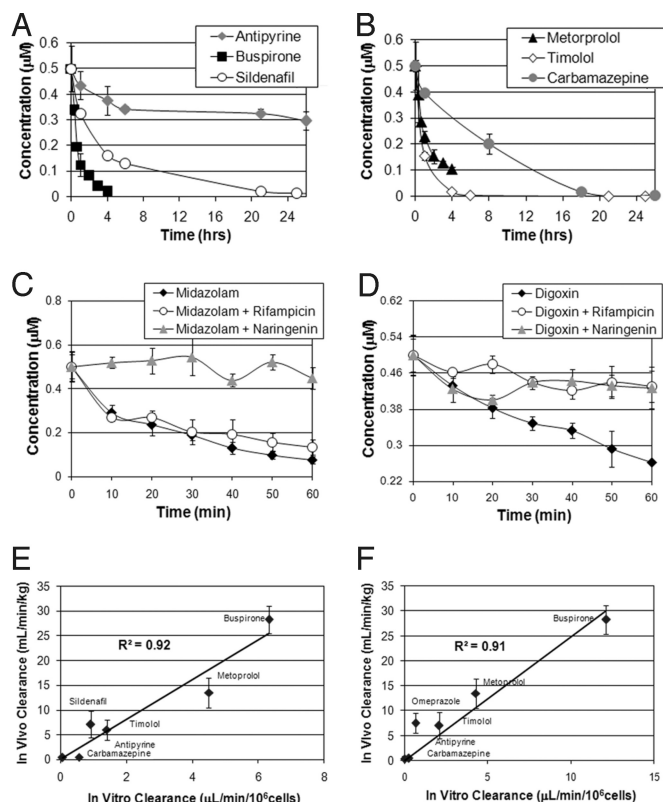


Fig. 5. Drug clearance and functional characterization of oxygenated co-cultures. (A and B) time course studies of the primary rat hepatocyte metabolism of rapidly clearing drugs, buspirone and metoprolol, medium clearing drugs, timolol and sildenafil, and slow clearing drugs, antipyrine and carbamazepine. (C) Time course of the metabolism of midazolam by oxygenated rat co-cultures following incubation with the oatp2 inhibitor rifampicin (100 μM) or the CYP3A4 and Pgp inhibitor naringenin (200 μM). (D) Time course of the metabolism of digoxin by oxygenated rat co-cultures following incubation with the oatp2 inhibitor rifampicin (100 μM) or the CYP3A4 and Pgp inhibitor naringenin (200 μM). (E) Comparison of in vitro rates of drug clearance measured in oxygenated co-cultures of cryopreserved human hepatocytes (day 1) with previously reported in vivo rates of hepatic clearance. The results are in excellent agreement with an R^2 of 0.92. (F) Comparison of in vitro rates of drug clearance measured in suspension cultures of cryopreserved human hepatocytes (day 0) with previously reported in vivo rates of hepatic clearance. For time course study in human cells see Fig. S6.

advantage of using endothelial cells over mouse 3T3-J2 fibroblasts, other than endothelial cells being species-specific. Therefore our results are readily extendable to other culture configurations including microfabricated co-cultures (14).

A significant element of our system is the serum-free media formulation. Such hormonally defined medium was originally reported to support gene transcription and gap junction communication in primary rat hepatocytes (23, 24). However, these serum-free cultures are traditionally carried out following cell seeding in serum-containing media. Here we demonstrate that the effects of serum are detrimental for hepatocyte function, even during a short overnight cell seeding. We suggest that positive effects of serum are mainly due to its ability to enhance cellular attachment, even on collagen-coated dishes. Enhancing cellular attachment in the absence of serum results in a significant increase in function and opens the door for oxygen-mediated enhancement.

While the transition to serum-free media demonstrated an increase in function, it is the transition to 95% oxygen that allowed the full metabolic potential of co-cultures to be realized. In vivo a mixture of venous and arterial blood supplies oxygen

to hepatocytes at a rate of 1.2 nmol/s/10⁶ cells (7). Fittingly, our prior work demonstrated that oxygen consumption of primary hepatocyte is 0.9 nmol/s/10⁶ cells during the first 24 h of culture (17). However, as the cells adapt to their new microenvironment, oxygen uptake rates drop to 0.4 nmol/s/10⁶ cells during long-term culture (17, 18). Not surprisingly, 0.4 nmol/s/10⁶ cells is also the upper limit of oxygen diffusion under atmospheric oxygen, but significantly less than hepatocyte demand during seeding (16). Metabolic flux models also suggest that hepatocytes in culture attempt to maximize their oxygen uptake (41). This suggests the oxygen supply is a limiting factor in the metabolic activity of hepatocytes. Therefore, increasing oxygen tension to 95%, which allows for oxygen supply in excess of 1.2 nmol/s/10⁶ cells to occur by passive diffusion, can reduce adaptation stress and allow for higher levels of metabolic activity.

Under these conditions, CYP1A activity, albumin secretion, and hepatocyte viability were, respectively, $70 \pm 7\%$, $90 \pm 18\%$, and $13 \pm 5\%$ higher in co-cultures seeded at 95% oxygen compared to co-cultures seeded at 21% oxygen. Drug clearance rates and gene transcription levels were similarly enhanced. These results stand in contrast to previous work which failed to find a significant enhancement of hepatocyte function at oxygen tensions greater than atmospheric (19, 21, 22). Our work clearly shows this is due to the effects of serum and in its presence the enhancement of function is minimal (Table 1 and Table S1). We note that oxygen supply is dependent on its rate of consumption and hence the global density of hepatocytes in culture. Reducing hepatocyte density to the point where oxygen is no longer limiting can have a similar effect to increasing oxygen tension. The Cho et al. demonstration of elevated oxygen uptake rates and enhanced synthetic function in low density cultures of rat hepatocytes supports this assertion (42). However, homotypic interactions are important in the maintenance of hepatocyte function, requiring the maintenance of high local cell density while global cell density is decreased. Micropatterned hepatocyte co-cultures, recently shown to support high levels of metabolic activity correspond to such a configuration. It may be interesting to test whether the elevated long-term activity in micropatterned cultures is due to increased oxygen availability.

The ability to quantify rates of hepatic clearance in a rapid and cost efficient manner represents a major advancement to the current state-of-the-art. Our work demonstrated that hepatocytes co-cultured under 95% oxygen demonstrate high levels of metabolic activity. In addition, long-term function allows the critical evaluation of slow clearing drugs as well as drug-drug interactions without the requirement for a long, work-intensive adaptation period. The functional polarization of the cells, demonstrated by active transporters, and proteoglycan expression, suggests this culture model can be particularly useful in the study of complex transporter dependent drug metabolism and perhaps even viral infection.

Materials and Methods

Hepatocyte Isolation and Culture. Primary rat hepatocytes were harvested from adult female Lewis rats purchased from Charles River Laboratories, weighing 150–200 g by a two-step in situ collagenase perfusion technique, modified by Dunn et al. 1991 (43). Hepatocyte viability after harvest was greater than 90% and purity was greater than 95%. All animals were treated in accordance with National Research Council guidelines and approved by the Subcommittee on Research Animal Care at the Massachusetts General Hospital. Primary human hepatocytes were obtained from BD Biosciences or were kindly provided by Dr. Stephen C. Strom, University of Pittsburgh. Cryopreserved human hepatocytes were purchased from either BD Biosciences or Celsis. Human cells were purified in 33% Percoll solution centrifuged at $500 \times g$ for 5 min before seeding. Cell viability post purification was greater than 90% and purity greater than 95%. Following purification hepatocytes were suspended in ice cold culture medium at 1×10^6 cells/mL and seeded on collagen-coated 6-, 12-, or 96-well plates as described. Unless otherwise noted seeding densities were 150,000 cells/cm². Hepatocyte cultures were main-

