High-Throughput Identification of Molecular Factors that Promote Phenotypic Stabilization of Primary Human Hepatocytes in vitro

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Liver disease is a leading cause of morbidity worldwide. The only therapy shown to directly alter mortality is organ transplantation, which is limited by a growing discrepancy between supply and demand of transplantable organs. Cell-based therapies have long held promise as alternatives to whole-organ transplantation, but have been hindered by the rapid loss of liver-specific functions in cultured hepatocytes. Within the stromal context of the liver in vivo, hepatocytes maintain their phenotype under the influence of cell-soluble, cell-matrix, and cell-cell interactions. In vitro, primary human hepatocytes can be maintained for several weeks through co-cultivation with a wide range of stromal cell types. The precise molecular mediators of this process remain unclear; and while effective at maintaining liver phenotype, the use of xenogenic stromal cells pose several challenges that ultimately limit the clinical applications of co-cultures. It is thus desirable to replace stromal cells with the acellular signaling components that are responsible for the co-culture effect. It is our hypothesis that, as in vivo, molecular signals from the stroma provide inductive cues to maintain liver phenotype in vitro, and that these stromal signals can be isolated and used to replace non-parenchymal cells in hepatic tissue engineering applications. In this thesis, we developed a high-throughput liver platform to conduct genetic knock down screens in two phases. Phase I, the validation screen, explored knock-down effects of 59 fibroblast genes that were theoretically predicted by previous gene expression profiling to be mediators of the co-culture effect. Results confirmed that at least 12 of these implicated factors are indeed important for hepatocyte functions, as evidenced by decreased albumin output in hepatocytes co-cultivated with fibroblasts lacking each of the 12 gene products. Importantly, none of the mutated co-cultures lost hepatocyte functions completely, suggesting that multiple signaling molecules are involved in the maintenance of hepatocyte phenotype ex vivo. We therefore conducted phase II, the combinatorial screen, to study the knock-down of 414 new stromal factors involved in cell-cell communications, in over 5000 two-way combinations. Overall, we identified 59 new positive mediators of the co-culture effect and observed that the knock down of multiple stromal factors synergized, leading to near-complete loss of hepatocyte phenotype. Supplementation of hit molecules, such as Activin A, into hepatocyte cultures improved cellular morphology and survival in vitro, and may be acting via signaling pathways that inhibit cell cycle progression and apoptosis to maintain hepatocyte functions in culture. These results represent important first steps in the elucidation of mechanisms instrumental to the functional maintenance of hepatocytes in vitro, and we hope this new insight will guide the assembly of a cocktail of recombinant acellular stromal products capable of replacing stromal cells in hepatic tissue engineering.
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GLOSSARY

BMP = Bone Morphogenetic Protein

CYP450 = Cytochrome P450

CV = Coefficient of Variation

FGF = Fibroblast Growth Factor

GO = Gene Ontology

MEF = Murine Embryonic Fibroblasts

MPCC = Micro-patterned Co-culture

ORF = Open Reading Frame

SBP = Spontaneous Bacterial Pertionitis

TIPS = Transjugular Intrahepatic Portosystemic Shunt
INTRODUCTION

Liver Disease and Current Therapies

Liver disease affects over 500 million individuals, 30 million in the United States alone, and leads to over 40,000 deaths annually\(^1\). There are generally two modes of liver failure: fulminant or acute injuries that lead to liver necrosis, and chronic disorders that result in hepatic cirrhosis.

Acute liver failure is relatively rare but exhibits a high mortality rate of ~28%. Common causes include drug overdose such as acetaminophen toxicity, infections (e.g. hepatitis A and B) and idiosyncratic drug reactions\(^3\). Fulminant hepatic failure is characterized by impaired liver synthetic functions and hepatic encephalopathy within 26 weeks of initial onset of jaundice. Associated clinical symptoms can additionally include microbial infections, coagulopathy and metabolic abnormalities. Spontaneous recovery from acute liver failure is possible, due to the powerful regenerative capacity of the liver, but this process is difficult to predict. Additionally, innate liver regeneration can be disabled if liver damage is too severe, which is often the case of etiologies such as acetaminophen overdose and hepatitis B.

Chronic liver failure is more common and a leading source of death in the United States. Etiologies range from hepatitis C virus (HCV) to fatty liver diseases, both alcohol-induced and nonalcoholic (NAFLD)\(^2\). Chronic liver disorders can progress to decompensated cirrhosis, with clinical symptoms such as ascites with or without spontaneous bacterial peritonitis (SBP), portal hypertension, variceal bleeding, encephalopathy, hepato-pulmonary and hepato-renal syndromes. Long-term inflammation from chronic hepatitis C also increases the risks of developing hepatocellular carcinoma. Liver cirrhosis resulting from HCV infection has historically been the number one reason for liver transplantation, accounting for 40-50% of transplant candidates\(^3\).

Current treatments for liver disease are largely palliative, including administration of fluids and serum proteins as well as management of the complications of liver failure, such as antibiotics for SBP prophylaxis, transjugular intrahepatic portosystemic shunt (TIPS) for variceal bleeding and Midodrine for hepato-renal syndrome. One notable exception is the recent development of new direct-acting antiviral agents, which have shown cure rates above 90% for HCV patients\(^4\). Outside of the HCV landscape, however, the only therapy proven to alter mortality is orthotopic liver transplantation. In an attempt to address the widening discrepancy between supply and demand of transplant-grade livers, several surgical options have been explored to maximize the utility of available organs. These include the use of non-beating-heart
donors and split liver transplants from both cadaveric and living sources. Split liver transplants attempt to harness the phenomenal regenerative capacity of the liver, which has been shown to replace up to two-thirds of lost liver mass without significant disturbances in clinically measureable markers of liver function. This regenerative response is enabled by the expansion of existing mature cells within the liver, led by hepatocytes, and followed by others such as bile duct epithelial cells. Studies including a recent in vivo RNAi screen identified MKK4 as a key regulator of liver regeneration. While powerful, liver regeneration is difficult to control clinically; biliary and vascular complications are major concerns in split liver transplants and the deaths of several donors from such complications have further raised significant ethical barriers. Finally, efficient utility of available organs alone is unlikely to address the widening gap between the number of patients requiring a transplant and the number of available organs. Therefore, alternative approaches are highly desired and are actively being pursued.

**Alternative Therapies for Liver Diseases**

Alternative approaches to liver disease include extracorporeal Bioartificial Liver (BAL) devices, which process the blood of liver failure patients ex vivo. These devices are designed primarily to provide transient support, serving as a bridge to transplantation or allowing time for innate liver regeneration in patients with acute or acute-on-chronic liver failure. Early designs employed mostly non-biological mechanisms such as hemodialysis, plasmapheresis, plasma exchange, molecular adsorbents recirculation, or hemoperfusion over charcoal or resins. These devices push patient blood through a charcoal column, which removes toxins and captures other useful metabolites. Of these various configurations of extracorporeal support devices, charcoal-based systems are the most extensively studied; they have been evaluated clinically in patients with acute liver failure, though no clear survival benefits were observed. This may be due to the limited range of functions served by each BAL device, particularly in contrast to the complex array of biochemical functions performed by a healthy liver. In response, newer generations of BAL devices are examining the addition of cell-based components. It is estimated that a minimum of 10% of total liver weight or 1 x 10^10 hepatocytes is needed to provide effective therapy; therefore, clinically impactful BAL devices will ultimately require a) renewable sources of functional human hepatocytes and b) scale-able systems capable of maintaining these large populations of functional human hepatocytes ex vivo.

In addition to the development of transient extracorporeal support, in vivo therapies aimed at the definitive replacement of damaged liver tissue are an active area of investigation. These include cellular transplantations as well as implants of tissue-engineered hepatocellular
constructs. Direct injection of hepatocytes into animal models of both acute and chronic liver failure as well as models of genetic metabolic defects have been found to improve host survival\(^{10-13}\). Clinically, different liver disorders demonstrate varying responses to cellular transplantation. Pediatric patients with liver-based metabolic diseases, particularly urea cycle defects, appear to benefit the most from direct hepatocyte injections. Even in best case scenarios, however, transplanted hepatocytes deteriorate over time and whole-organ transplantation is required by six months\(^{14}\). Progress in cell transplantation has been hindered by the lack of a renewable source of functional and safe human hepatocytes, which is aggravated by poor engraftment and survival of transplanted cells in the host, collectively reported at only 10-30% of injected cells\(^{15}\). Consequently, while hepatocyte transplantation therapy has been shown to possess excellent safety profiles, massive cell number requirements and inadequate survival benefits have limited its effectiveness as a clinical therapy\(^{16-18}\).

One approach that partially circumvents the challenges faced by direct cell transplantation is the development of tissue engineered hepatocellular constructs\(^{10,16}\). Various strategies have been developed to enhance hepatocyte survival and function in such constructs, including the use of biocompatible materials to provide physical support for normally adhesive cells like hepatocytes, as well as additional re-creations of the complex microenvironment that normally surround hepatocytes \textit{in vivo}\(^{19-32}\). Such re-creations introduce cell-cell, cell-matrix and cell-soluble factor interactions into engineered artificial liver implants to promote the generation of liver-like tissues \textit{in vitro} prior to \textit{in vivo} implantation. Overall, studies that improve cell delivery, survival, and integration with host have greatly enhanced the effectiveness of cell-based therapies, and will need the support of a renewable source of functional human hepatocytes for continued progress.

**Maintenance of Hepatocyte Functions**

\textit{In vivo}, hepatocytes exist within the complex architecture of the liver and interact with diverse extracellular matrix molecules, non-parenchymal cells and soluble factors such as hormones and oxygen. The liver is supplied by dual circulation: the hepatic artery brings oxygen-rich blood and accounts for one-third of the afferent blood supply while the portal vein brings nutrient- and hormone-rich blood from the digestive system, accounting for the remaining two-thirds. The liver itself is organized into functional units known as lobules, consisting mostly of hepatocytes (70% of total liver cells) aligned into cords and surrounded by different types of stromal cells. These include Stellate Cells that reside in the extracellular matrix known as the Space of Disse, fenestrated endothelium that line the liver sinusoids, Kupffer cells that roam the
blood and tissue compartments as well as cholangiocytes that interact with hepatocytes toward the end of the sinusoids. Homotypic cell-cell interactions between hepatocytes are mediated by gap junctions and tight junctions while bile canaliculi coordinate the excretion of bile to the gall bladder via bile ducts. Hepatocytes within liver lobules are divided into three zones along the length of the sinusoid, each with a unique morphology and function. How this zonation is established or maintained is not well understood but proposed mechanisms involve differential innervations, extracellular matrix composition and blood-borne factors (hormones, oxygen tension, pH levels etc.)\textsuperscript{33}. It is believed that this highly orchestrated microarchitecture enables precise cell-cell, cell-matrix and cell-soluble factor interactions and is critical for the proper functioning of the liver. A healthy liver is responsible for over 500 biochemical processes, which can be broadly categorized into detoxification of both endogenous (e.g. ammonia, bilirubin) and exogenous (e.g. environmental toxins, drugs) compounds, protein synthesis (e.g. clotting factors, albumin), energy and cholesterol metabolism and bile production. In addition to normal physiology, heterotypic cell-cell interactions between hepatocytes and their stromal neighbors are also known to have important roles \textit{in vivo} during liver development and in pathogenesis\textsuperscript{34-37}. During development, hepatic induction from endodermal foregut and mesenchymal vascular structures is believed to be directed by neighboring cardiac mesoderm through the secretion of FGF and the septum transversum via signaling with BMP. Further differentiation of hepatoblasts into either hepatocytes or biliary epithelium requires similar heterotypic cell-cell signaling from the septum transversum and neighboring endothelial cells. In the adult liver, Stellate Cells activated by TGF\(\beta\)-1 produce excessive amounts of extracellular matrix proteins (e.g. collagen), leading to liver fibrosis, which can progress to cirrhosis, portal hypertension and ultimately liver failure\textsuperscript{38}.

\textit{In vitro}, a majority of hepatocytes cultured alone on rigid collagen rapidly lose viability over hours in culture, while surviving cells lose liver-specific functions and adopt a fibroblastic morphology\textsuperscript{39}. The cause and underlying mechanisms of this process is not well understood but various strategies have been developed to impede this transformation, particularly since hepatocyte functions should ideally be maintained for the lifetime of the clinical intervention, which ranges from weeks for extracorporeal devices to years for \textit{in vivo} therapies. Classic approaches to address this problem manipulate the cellular environment, and include the culturing of hepatocytes in between 2 layers of collagen I gels or on matrigel substrates, the assembly of hepatocytes into 3D spheroids and the use of bioreactors\textsuperscript{40}. While many of these methods have been shown to maintain liver-specific functions \textit{in vitro} to some degree, their effects are transient and they are difficult to implement for clinical applications due to solute
transport and scaling limitations. In the 1980s, work pioneered by Guguen-Guillouzo and colleagues\textsuperscript{39} showed that the viability and liver-specific functions of hepatocytes from multiple species can be maintained for several weeks upon co-cultivation with stromal cell types. This phenomenon, known as the co-culture effect, can be observed with a large selection of non-parenchymal cells, both primary and immortalized, from intra-hepatic and extra-hepatic sources, and is preserved even across some species barriers\textsuperscript{39,41,42}. Hepatocytes in co-cultures, particularly with murine embryonic J2-3T3 fibroblasts, exhibit for weeks the polygonal morphology, well-demarcated cellular borders, distinct nuclei, and visible bile canaliculi network displayed by healthy cells in vivo. Co-cultures have since been utilized to investigate various physiologic and pathologic processes including host response to sepsis, mutagenesis, xenobiotic toxicity, oxidative stress, lipid metabolism, induction of acute phase response\textsuperscript{43-49}, and more recently, in the development of \textit{in vitro} liver models for pharmaceutical drug screening, disease modeling (e.g. HCV, HBV, malaria) and engineered hepatic tissues\textsuperscript{50-54}.

\textbf{Mechanisms Underlying the Co-Culture Effect}

In order to better understand the molecular mechanisms driving the co-culture effect, previous work from our group characterized the type and duration of heterotypic cell-cell interactions required to maintain hepatocyte functions \textit{in vitro}. Studies suggest that cell-cell contact between primary hepatocytes and non-parenchymal cells (i.e. murine embryonic 3T3 fibroblasts) is required for \textasciitilde18-24 hours, after which continuous stimulation with stromal-derived soluble signals alone over a range of \textasciitilde400um is sufficient\textsuperscript{55}. These critical soluble factors additionally appear to be constitutively expressed by 3T3 fibroblasts, independent of hepatocyte interactions and are not involved in any reciprocal signaling circuits between hepatocytes and the co-cultivated stromal cells.

While useful for \textit{in vitro} liver modeling, the use of stromal cells to induce hepatic functions poses several key challenges, including overgrowth of supportive cells leading to nutrient and oxygen depravation, surface area limitations, and difficulty in distinguishing the hepatic molecular signals from the stromal background. Therefore, for tissue engineering applications, it is desirable to replace stromal cells by coating biomaterials with recombinant cell contact molecules that can interact with hepatocytes to mimic initial heterotypic cell-cell signaling, followed by continuous stimulation with soluble factors. A handful of stromal-derived molecules have been identified to induce liver-specific functions in primary hepatocytes. These include liver regulating protein (LRP), E-cadherin, TGF-beta1 and T-cadherin\textsuperscript{50,56-60}. While effective at modulating hepatocyte functions \textit{in vitro}, none of these molecules are sufficient in
replacing the stromal cells nor are they all expressed by all cell types known to rescue hepatocyte phenotype. Using a gene expression profiling approach, we had previously identified additional candidate fibroblast genes that may play important roles in the maintenance of liver-specific functions. One such candidate is decorin\textsuperscript{50}, a chondroitin sulfate-dermatan sulfate proteoglycan that binds to collagen. As with its predecessors, decorin modulates hepatic functions in hepatocyte-fibroblast co-cultures, but it alone cannot maintain the liver phenotype of hepatocytes without supporting stromal cells. This suggests that other factors may be involved and that it is possible several stromal molecules may coordinate to enable the co-culture effect. Therefore, while these findings are promising, a complete picture of the mechanisms underlying the stabilizing effect of fibroblasts remains elusive, emphasizing the need to apply objective, systematic approaches to these studies.

It is our hypothesis that, as \textit{in vivo}, molecular signals from the stroma provide inductive cues to maintain hepatocyte phenotype \textit{in vitro}, and that these stromal signals can be isolated and used to replace the fibroblasts in maintaining hepatocytes. In this thesis, we aim to develop a high-throughput genetic screening platform for primary human hepatocytes in order to identify the most critical fibroblast gene products involved in the phenotypic stabilization of primary \textit{human} hepatocytes. Through this approach, we aim to elucidate key stromal-derived factors involved in heterotypic interactions between hepatocytes and J2-3T3 fibroblasts. We hope our findings will provide a more complete understanding of liver phenotype maintenance, with implications for basic research, drug development, molecular therapeutics and cell-based therapies.
METHODS

Cell Culture

**J2-3T3 Culture.** Passage 2 J2-3T3 fibroblasts were obtained from Howard Green (Harvard) and kept in liquid nitrogen until use. Cells were maintained under standard tissue culture conditions, in 1X DMEM media containing 10% BS and 1% Penicillin-streptomycin. Fibroblasts were grown in T-150 tissue culture flasks and passaged 1:10 using 0.25% Trypsin-EDTA when cells reached confluency. Experiments used J2-3T3s ranging in passage numbers from P7 to P9.

**Hepatocyte Culture.** Primary human hepatocytes were purchased in cryopreserved suspension from Celsis In vitro Technologies, and kept in liquid nitrogen until use. To thaw, cells were pelleted by centrifugation at 50g for 10 min. The supernatant was discarded before resuspension of cells in hepatocyte culture medium, which consisted of 1X DMEM supplemented with 10% fetal bovine serum (FBS), 15.6 µg/ml insulin, 7.5 µg/ml hydrocortisone, 16 ng/ml glucagon and 1% penicillin-streptomycin.

**Automated Cell Seeding.** Cell suspensions were diluted to the desired densities and kept in suspension using a magnetic stir bar. Thermo Combi robot was used to dispense cells into 384-well formats using speed setting low and standard cassette, 30ul/well.

Assay Validation

Assay readiness for high-throughput screening was assessed via z’-factor, which reflects both assay signal dynamic range and variation, and is mathematically defined:

\[ Z' = 1 - \left( \frac{3\sigma_{c+} + 3\sigma_{c-}}{|\mu_{c+} - \mu_{c-}|} \right) \]

where “c+”=positive control, “c-“=negative control, “σ”=standard deviation and “µ”=average. Assuming normal distribution, assays with positive z’-factors can separate 99.7% of the negative and positive control populations (i.e. the two populations, as defined by mean signal +/- 3 standard deviations, do not overlap), essentially separating signal from noise.

Functional Assays

**Albumin Competitive ELISA.** A saturating amount of human albumin (40ul/well of 50ug/mL albumin) was coated onto the walls of adsorptive 384-well plates (NUNC MaxiSorp plates, cat# NUNC 460372) at room temperature overnight under agitation. Sample supernatant was then introduced and competed with coated albumin for binding to HRP-
conjugated antibodies (MPBio Cat #55235). The amount of bound antibodies was then quantified via an ultra-sensitive chemiluminescent substrate (Thermo SuperSignal ELISA Pico Chemiluminescent Substrate, Cat # 37070).

**Biochemical Assays.** Urea concentration was quantified using a colorimetric assay that reacted diacetylmonoxime with acid and heat, following product instructions (Stanbio Labs Urea Nitrogen Test, Ref # 0580-250).

**Cytochrome-P450 Induction.** 7-benzyloxy-4-trifluoromethylcoumarin (BFC, BDGentest) was added to cultures at 50uM and incubated for 1 hr at 37C in phenol-red free media. Many different CYP450 isoforms process BFC into its fluorescent product of 7-hydroxy-4-trifluoromethylcoumarin (7-HFC), which was then quantified fluorometrically.

**Automated Plate Washing.** Washing for plates containing cells was done manually to prevent cell loss. Plate washing for ELISA was performed on the BioTek ELx-405 HT, using the following optimized settings:

- Prime: Prime_200 using DI water
- Wash: Named program HEPELISA
  - Method
    - Number of cycles = 02
    - Wash Format = Plate
    - Soak/Shake = Yes
    - Soak Duration = 010 sec
    - Shake before soak = yes
    - Shake Duration = 005 sec
    - Shake Intensity = 4 (18 cycles/sec)
    - Prime after soak = No
  - Disp
    - Dispense volume = 100ul/well
    - Dispense flow rate = 05
    - Dispense height = 120 (15.240 mm)
    - Horizontal X disp pos = 25 (1.143mm)
    - Horizontal Y disp pos = 20 (0.914mm)
    - Bottom wash 1st = no
    - Prime before start = no
  - Aspir
    - Aspir. Height = 020 (2.540 mm)
Automated Plate Reading. A Perkin Elmer Envision 2102 Multilabel Reader was used to quantify ELISA signal, integrated over 0.1 sec using luminescence 700 emission filter and measurement height of 6.5mm.

Fibroblast Viability Assay (AlamarBlue)
Plated J2-3T3 fibroblasts were incubated with the AlamarBlue (Thermo) reagent following manufacturer protocols. Stock solution was diluted 10x in culture medium and incubated with seeded J2-3T3 fibroblasts for 1hr at 37C. The level of fluorescence was then read using an excitation wavelength of 540–570 nm (peak excitation is 570 nm) and emission wavelength of 580–610 nm (peak emission is 585 nm). It is important that all reagents and media are pre-warmed to 37C prior to addition to cells; failure to do so leads to significant edge effects.

Hepatocyte Viability Assay (Imaging)

Image Acquisition. Cultures of hepatocytes and J2-3T3 fibroblasts were fixed using 4% paraformaldehyde (PFA) in black-walled, clear and flat-bottomed 384-well plates (Corning). Fixed samples were then stained with Hoechst 33342. It is important to note that the cell membrane is much more permeable to Hoechst 33342 than Hoechst 33258; thus an additional permeabilization step using 0.1% Triton-X for 30 min is necessary if visualizing nuclei with Hoechst 33258. Images of fluorescently labeled nuclei were acquired and digitized using a high-throughput screening microscope (Molecular Devices IXM) coupled to a barcode reader and robotic arm (Thermo) for automated plate loading. The microscope was configured to self-focus, first using lasers to identify the bottom of wells via differences in the refractive index of
plastic and fluids, then using image-based focusing algorithms that scan through a z-stack of ~200 µm in ~50µm steps in search of the plane with the sharpest images. Accurate examination of nuclear morphology required image acquisition at 20x magnification. 50% of the well area was sampled in a checkerboard fashion, imaging a total of 21 sites per well.

Image Processing and Nuclei Identification. We used the freely available open-source software CellProfiler for all image analysis 62, and the configured image analysis pipelines are provided online (http://cellprofiler.org/published_pipelines.html). All images underwent illumination correction, as the background intensities varied by up to 1.5 fold across a field of view and often caused unacceptable intensity artifact. The illumination correction algorithm averaged all acquired images per plate to identify and normalize consistent discrepancies in the staining intensities across the field of view 63. All corrected images then underwent a custom image analysis pipeline for nuclei identification (segmentation), by smoothing and using relative peaks in intensity to separate overlapping nuclei. For each identified nucleus, we measured a large number of features to construct a nuclear profile, including measures of nuclear size, shape, and texture, and the number of punctate sub-nuclear spots. This per-cell data profile was stored in a MySQL database (Oracle, Inc.) and was subsequently used to train supervised machine learning algorithms to automatically classify nuclei as hepatocytes or fibroblasts.

Nuclei Classification and Quantification. The nuclear profiles generated by CellProfiler were loaded into CellProfiler Analyst 64 for training of supervised machine learning algorithms to distinguish and count hepatocyte nuclei. Manually created training sets of representative hepatocytes and fibroblasts (50 example objects each) generate a preliminary set of rules for nuclei classification using the GentleBoosting algorithm applied to regression stumps 64. This rule set was used by CellProfiler Analyst to classify a new batch of nuclei, outputting the results for manual error correction. Iteration of this process refined the rule set until an acceptable accuracy plateau was reached. The final rule set was then applied to the profiles of every nucleus in every image acquired to classify each object as a hepatocyte or fibroblast before outputting a count of each nucleus type per well. The final training set contained 577 objects with an accuracy plateau using a total of 100 rules. We observed that the single feature of nuclear morphology that most effectively distinguished fibroblast nuclei from hepatocyte nuclei was the punctate sub-nuclear structures present in fibroblasts but absent in hepatocytes, due to the murine origin of the former, which is known to have more textured chromatin. The main pipeline, illumination correction pipeline, and the classifier rules are all available at http://cellprofiler.org/published_pipelines.html.
**shRNA Library**

The custom shRNA library was synthesized at the Broad Institute RNAi platform and contained a U6 promoter for constitutive expression, a cPPT domain that increased efficacy and a PGK promoter to drive PAC conveying puromycin resistance. RRE served as the binding site for reverse transcriptase and Psi served as the lentiviral packaging site where helper plasmids bound. Two LTR domains flanked this section of the vector to enable integration into the host genome. Other components on the vector were included to enable viral generation by bacteria and include F1 phage origin of replication, pUCori for bacterial replication of plasmid DNA and AmpR for selection of bacteria successfully inserted with plasmid DNA through electroporation.

**Validation Screen**

The workflow of the high-throughput screen to validate implicated factors is summarized in Figure 1. 384-well screening plates (Corning) were incubated with a solution of type-I collagen in water (100 mg/ml, BD Biosciences) for 1 h at 37°C. A feeder layer of J2-3T3 fibroblasts were robotically plated onto the collagen at a density of 4,000 cells/well and allowed to acclimate over 24 hours. Polybrene at 8ug/mL and a library of lentiviruses carrying 384 shRNAs representing the 59 genes of interest as well as control vectors were added in duplicates, centrifuged for 30 min at 37°C and allowed to incubate for 24 hours prior to selection with 5ug/mL of Puromycin over 2 days. In order to avoid donor-to-donor variability, human primary hepatocytes from a single donor were plated onto successfully transduced fibroblasts at a density of 4,000 cells/well and maintained under standard culture conditions with daily replacement of hepatocyte medium for 7 days, during which time the sample plates were kept in metal stacks with uniform air buffers between each plate in order to provide uniform gas and heat exchange. Additionally, breathable membranes and extra water reservoirs were employed to minimize edge effects arising from fluid evaporation. On day 7 of co-culture, culture supernatants were collected for automated ELISA analysis, and cells were fixed in 4% PFA for imaging and analysis, as described under “Functional Assays” and “Hepatocyte Viability Assay”.

**Combinatorial Screening**

The workflow of the combinatorial genetic screen is summarized in figure 1. 12 mutant J2-3T3 fibroblast cell lines were generated, each harboring knock down of one of the 12 hit candidates identified via the validation screen. Knock-down was achieved using lentivirus-delivered shRNAs shown to consistently and effectively knock down target genes during validation screening, following previously optimized protocols detailed above. The resultant
mutant J2-3T3 fibroblasts were robotically plated onto 384-well screening plates (Corning) adsorbed with type-I collagen (100 mg/ml, BD Biosciences), at a density of 4,000 cells/well and allowed to acclimate over 24 hours. A library of lentiviruses carrying shRNAs representing 414 hand-selected genes involved in cell-cell communications were added in duplicates to mutant J2-3T3 fibroblasts to achieve two-way combinatorial knock-down following the same screening workflow outlined under “Validation Screen”, now with blasticidin selection. Human primary hepatocytes from a single donor were plated onto successfully transduced fibroblasts at a density of 4,000 cells/well and maintained under standard culture conditions with daily replacement of hepatocyte medium for 7 days. On day 7, cultures underwent ELISA analyses and image-based hepatocyte cell counting, as described under “Functional Assays” and “Hepatocyte Viability Assay”.

**Western Analysis**

Samples from transduced J2-3T3 fibroblasts were lysed in RIPA buffer (Upstate Biotechnology, Waltham, MA) with protease inhibitors cocktail (Roche, Indianapolis, IN) and analyzed by Western Blot as previously described. The following primary antibodies were used: Decorin, GAPDH (Cell Signaling, San Jose, CA).

**Immunofluorescent Staining**

Samples were fixed in 4% PFA for 15 min at room temperature, permeabilized with 0.1% Triton-X for 30 min at room temperature, and blocked with 3% FBS-PBS for 1 hr at room temperature. Primary antibodies for various target proteins were added to cultures at manufacturer-recommended concentrations and incubated at 4C overnight on rocker. Secondary antibodies (BD Biosciences) were added to cultures at 1:400 dilution and incubated at room temperature for 45 min. Nuclei were visualized through Hoechst Staining.

**Statistical Analyses**

The z-scores were calculated as the deviation of the mean of a single shRNA to the mean of all controls found on the same plate, normalized to the standard deviation of all controls. Hit candidates from the screen were selected according to their z-score, as described under “Hit Selection”.

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RESULTS

Liver Platform Development

To best represent normal human physiology in our liver model and to maximize the model’s ability to predict clinical outcome, we opted to employ primary human hepatocytes in our in vitro platform. All cells were sourced from a single donor in order to eliminate innate genetic variations that exist within all populations. Eight different donors of cryopreserved human hepatocytes were tested in total, from which we selected one donor based on the cells’ adhesive properties, age and baseline liver functions. The chosen donor, GHA, is a one-year-old Caucasian female who died from dry drowning, whose hepatocytes attached well to rigid collagen and demonstrated good synthetic, detoxification and metabolic functions.

To maintain GHA hepatocytes in culture, we co-cultivated them with murine embryonic J2-3T3 fibroblasts, which we had previously found to be the most effective non-parenchymal cell type at transiently stabilizing hepatocyte phenotype in vitro. The platform contained a sub-confluent population of hepatocytes on top of a confluent layer of J2-3T3s within 384-well plates coated with collagen type I at 100ug/mL for enhanced cell attachment (Figure 3A). This design maintained hepatocyte functions for at least 9 days and is amenable to genetic manipulation of fibroblast populations in 384-well formats. The number of fibroblasts seeded per well was empirically optimized to 4,000 cells/well in order to establish a confluent feeder layer while minimizing the risks of phenotypic transformations due to overcrowding at the end of a four-day transduction process. The number of hepatocytes seeded per well was empirically optimized to 4,000 cells/well to ensure that signals from both control and experimental groups were within the linear detection range (Figure 3C) of all accompanying assays. Similarly, the amount of media used per well was empirically optimized to 30ul, which balanced opposing requirements of nutrient supply and gas diffusion. All cells were robotically seeded at the lowest possible speed setting in order to minimize shear stresses on the cellular membrane.

High-throughput Assay Development

To assess cell fates in this platform, we developed three separate high-throughput readouts. The commercially available AlamarBlue assay was employed to quantify viability of the fibroblast feeder layer, measure transduction efficiency and optimize transduction procedures. Two additional assays were developed to measure hepatocyte functions and cell numbers after one week of heterotypic fibroblast-hepatocyte interactions in the screening platform.
A number of commercial assays exist to measure cell viability in culture, including fluorescent-based live/dead cell stains and quantifications of cellular enzyme activity as surrogate markers of cell number. Many of these assays, however, are not amenable to automation due to cumbersome workflow and/or have cytotoxic properties that restrict them to end-point usage only. After testing a number of candidates, including CellTiter-Glo, Ki67 staining and the MTT assay, we chose AlamarBlue to quantify fibroblast viability and transduction efficiency. AlamarBlue is based on the conversion of the molecules resazurin to resorufin through the reducing power of living cells. Resazurin is blue in color and minimally fluorescent while Resorufin produces bright red fluorescence, thus providing both a colorimetric and fluorescent distinction between parent and daughter compounds. AlamarBlue yielded linear relationships between fluorescence and fibroblast cell numbers when tested on J2-3T3 fibroblasts in 384-well formats, with an effective range of 2,000 to 16,000 cells and a coefficient of determination of $R^2 = 0.98$. The incubation period was empirically optimized to one hour, with acceptable window between one and five hours, during which the assay showed excellent signal to noise ratio, with good reproducibility across wells, plates and batches (Figure 3C). Using the AlamarBlue assay, we automated and optimized lentiviral transduction conditions for delivering shRNAs to J2-3T3 fibroblasts in 384-well formats. Parameters examined include polybrene concentration for neutralization of cell surface charges, viral titers needed for effective transduction as well as the concentration of puromycin required for selection of successfully transduced cells. Our results showed that a higher polybrene concentration of 8ug/mL provided superior transduction than 4ug/mL of polybrene. Similarly, puromycin was tested at a concentration range between 0ug/mL and 8ug/mL, and found to be optimal at 5ug/mL (Figure 3D). Using these transduction parameters, we were able to obtain a median infection efficiency of 79.3% (Figure 3D); knockdowns of select target genes were confirmed via Western analysis (Figure 3E), RT-PCR or immunofluorescent staining.

In order to assess hepatocyte phenotype, we equipped the high-throughput liver platform with three functional assays. Due to the immense repertoire of the hundreds of documented and yet unidentified biochemical functions of the liver, there does not exist a single gold-standard assay for examining hepatocyte functions. Therefore, we sampled 3 different types of key liver processes: 1) albumin output through a competitive ELISA as a surrogate marker for protein synthesis functions of the liver, 2) urea generation through a colorimetric assay as a surrogate marker for amino acid metabolism, and 3) cytochrome P450 activity through an enzyme activity assay as a surrogate marker for detoxification functions of the liver. For all three assays, we altered parameters such as reagent type, volume and concentration to develop
them into assays compatible with high-throughput screening. Validation data showed that these assays can confidently (Z'>0) detect two-fold changes in hepatocyte populations with low variance (CV<10%) and good reproducibility (Figure 3B). For genetic screening, we chose the ELISA-based albumin quantification as the functional readout. The most common form of the ELISA assay is a sandwich ELISA that captures the antigen of interest in between 2 layers of antibodies. This assay is difficult to adapt to high-throughput screening due to a cumbersome protocol, which is difficult to program robotically, thus limiting throughput. Therefore, for our liver platform, we employed a competitive ELISA assay, which reduced the length of the workflow by approximately one third. When coupled with the optimized fibroblast and hepatocyte cell numbers as well as transduction procedure, the platform and assays are able to easily identify hepatocytes that have lost their liver-specific synthetic functions (Figure 4A).

In addition to assessing the functional output of hepatocytes, we developed an image-based proliferation assay that uses nuclear morphology to quantify hepatocyte nuclei numbers in co-culture. This allowed us to measure hepatocyte viability and to determine hepatocyte synthetic functions on a per cell basis. When visualized with Hoechst stain, hepatocyte nuclei are relatively uniform in texture while fibroblast nuclei are speckled (Figure 2C). The assay utilized this distinction to isolate hepatocytes from stromal background and provided a count of the number of hepatocyte nuclei in culture. Imaging of a series of 384-well plates containing control hepatocyte-fibroblast co-cultures showed that the image-based readout could confidently (Z'>0) detect two-fold changes in the number of hepatocyte nuclei, with low variance (CV<15%) and good reproducibility (Figure 3B).

**Validation Screening**

*Selection of genetic factors for systematic knock down*

A large number of non-parenchymal cell types are known to support the co-culture phenomenon. They differ, however, in the degree of hepatic functions that they are able to induce. J2-3T3 fibroblasts, for example, are able to induce physiological or even supra-physiological levels of liver-specific functions in co-cultivated hepatocytes, while the closely related NIH-3T3 fibroblasts and the primary mouse embryonic fibroblasts (MEFs) from whence these cell lines were originally derived are much lower inducers of the co-culture effect. Previously, Khetani et. al. conducted gene expression profiling of these three fibroblast populations using Affymetrix GeneChips. Here, we analyzed the data to identify 59 genes whose levels of expression correlated strongly with the induction of hepatocyte functions in vitro.
All 59 genes exhibited a minimum of five-fold difference in levels of expression between the high inducer J2-3T3s and low inducer MEFs. We prioritized molecules with known functions in cell-cell communications, such as various cell surface (e.g. Delta-like homolog 1), extracellular matrix (e.g. Decorin) and secreted factors (e.g. VEGF-D, ceruloplasmin) and eliminated genes that were flagged for having poor quality-control data on the Affymetrix GeneChips. We also added factors that had been implicated in the co-culture effect through other previous studies (e.g. T-cadherin). Among these 59 genes were 31 genes whose differential levels of expression correlated positively with the pattern of hepatocyte induction observed (positive inducers); the remaining 33 factors showed expression patterns that correlated negatively with hepatocyte functions (negative inducers). All 59 genes are listed in Table 1 and were used to conduct loss-of-function studies using lentivirus-delivered shRNAs in our high-throughput liver platform to determine if these stromal factors are mediators of the co-culture phenomenon.

Validation screen and hit selection

During validation screening, we conducted genetic knock-down studies in J2-3T3 fibroblasts, targeting each of the 59 selected genes through a custom shRNA library. The library contained 4 to 10 independent shRNAs for each gene of interest, a redundancy designed to distinguish real biological effects of gene knock-down from artifacts such as off-target effects, and the disruption of potentially critical cellular functions by the random insertion of lentiviral genes into the host genome. Only genes represented by two or more different shRNAs were included in the final hit list, though exceptions were made for those with particularly profound effects (z>4). All shRNAs were tested in duplicate along with various controls (1. empty virus without vector 2. vectors with low infection efficiency, and 3. vectors that conveyed puromycin resistance without gene knockdown). The resultant mutant J2-3T3 fibroblasts were then co-cultivated with primary human hepatocytes in the high-throughput liver platform and their ability to induce liver-specific functions was examined via the ELISA-based functional assay and the image-based hepatocyte viability assay.

Individual shRNAs were considered biologically active and selected as a hit for further analysis if they significantly impaired the overall functional output of the hepatocytes on either a population or individual cellular level. Population level data was directly measured by the ELISA assay and any shRNA with a z score greater than 3 was considered active (p<0.001). This z score yields a false positive rate of 0.1%, which, in our library of 350 shRNAs representing 59 genes, is negligible at <1 false positive shRNA. Cellular level data (i.e. albumin output per cell) was calculated through the integration of ELISA and image-based assays. Regression analyses
of control cultures containing wild type fibroblasts revealed the following relationship between ELISA z scores (z_ELISA) and imaging z scores (z_HepCount):

\[ z_{\text{ELISA}} = (-0.37 \times z_{\text{HepCount}}) \]

Applying to this equation the same hit selection criteria of z score greater than 3 (p<0.001), cellular level hits were identified via the equation:

\[ z_{\text{ELISA}} > (-0.37 \times z_{\text{HepCount}}) + 3 \]

Additionally, any shRNA with infection efficiency below 20% was eliminated from analyses. The final hit list contained a total of 12 genes, listed in Table 2, validating previous hypotheses that these molecules play an important role in the maintenance of hepatocyte phenotype in vitro. The magnitude and consistency of effect of some active shRNAs in the final hit list is plotted in Figure 4B.

**Hit analysis**

As a first step to analysis of the validation screening results, we examined the effect of Decorin, a putative positive control, in our screen. Decorin’s role as a positive mediator of the co-culture effect was not only theoretically predicted by gene expression profiling, but also confirmed via studies that showed up-regulation of hepatic functions in vitro when cultivated on adsorbed Decorin in a dose-dependent manner. In our screen, two active shRNAs were found to represent Decorin and both showed a decrease in hepatocyte functions upon knock-down, which is consistent with prior findings.

Knock down of the 12 hit genes perturbed the co-culture effect to varying degrees. The most active single shRNA targeted Inhba, which encodes for inhibin beta-A, a subunit of both activin A and inhibin A, both secreted growth factors involved in a myriad of biological processes including cell cycle regulations. Inhba knockdown via this shRNA resulted in ELISA z scores of 9.9-11.4 and was reproduced by both biological replicates.

Genes represented by the highest number of active shRNAs were H2-K1 (6 active hairpins) and Ttk (4 active hairpins). Both exhibited good consistency between biological replicates. H2-K1 encodes for the K region of a histocompatibility protein with a human ortholog of major histocompatibility complex, class I, A (HLA-A). While it is expressed within the liver during both embryonic and adult stages, its main function is in mediating immune responses and has no obvious connections to liver homeostasis. Ttk encodes for a serine / threonine dual specificity protein kinase with important roles in promoting chromosomal stability. It was also recently reported to promote G1 progression in hepatocellular carcinoma. The knock-down of either H2-K1 or Ttk significantly (p<0.001) decreased hepatocyte functions on both a population
and cellular level, suggesting that they are positive mediators of the co-culture effect. This is consistent with early findings that their levels of expression is high in J2-3T3 fibroblasts and low in primary MEFs. In fact, H2-K1 is expressed only in J2-3T3 fibroblasts and undetectable in both low inducers NIH-3T3 and primary MEFs.

In contrast to H2-K1 and Ttk, the observed impact of Shc1, Sfrs3 and Rock2 knock down during this screen contradicted their previously predicted roles. Shc1 encodes for the signaling molecule src homology 2 domain-containing transforming protein c1, which has documented roles in cell differentiation and has been localized to the plasma membrane. Sfrs3 encodes for the pre-mRNA splicing factor serine/arginine-rich splicing factor 3 while Rock2 encodes for the protein kinase Rho-associated coiled-coil containing protein kinase 2. These 3 genes were predicted to be negative inducers of the co-culture effect through expression profiles but their knock down in vitro actually significantly deteriorated hepatocyte phenotype during validation screening. Shc1, Sfrs3 and Rock2 were each represented by 4, 2 and 3 active shRNAs respectively, all consistently showing them as positive mediators of the co-culture effect. While unexpected, these findings demonstrate the importance of testing theoretical prediction models with empirical studies.

Not all hit candidates demonstrated consistent effects within the screen: Cd44, Ssb and Tsnax were each represented by 4, 3 and 4 active shRNAs, divided into two different populations: those that significantly decreased hepatocyte functions upon knockdown, versus others that enhanced hepatocyte synthetic output (Figure 4). Cd44 encodes for a cell surface antigen that is expressed in virtually all organ systems. Ssb encodes for the ribonucleoprotein sjogren syndrome antigen B, a marker used clinically to diagnose the autoimmune disease Sjogren Syndrome. Tsnax encodes for the DNA-binding protein translin-associated factor X, an intracellular protein involved in cell differentiation. Neither Ssb nor Tsnax has documented expression in the liver and biliary system. While contradictory at first glance, this dichotomy of effects is actually often observed in genetic screens. One possible explanation is that the effect of these genes is highly dose-dependent, such that two different shRNAs with varying knock down efficiencies elicits opposite effects in the same cell population.

The final group of hit candidates, Rgnef, Pkp2 and Mlf1 were each represented by 3 active shRNAs and demonstrated good consistency of effect both within the screen and with their previously hypothesized roles as positive inducers of hepatocyte functions. Rgnef encodes Rho guanine nucleotide exchange factor 28 while Mlf1 encodes the signaling molecule myeloid leukemia factor 1; both are intracellular proteins with documented expression mainly in the
nervous system. Pkp2 encodes the intermediate filament binding protein plakophilin 2, another intracellular factor, involved in signaling and found predominantly in the cardiovascular system.

**High-throughput Identification of New Stromal Factors**

*Selection of new stromal factors*

While knockdown of the 12 hit candidates each had significant effects on hepatocyte functions, none completely abolished the co-culture effect, as evidenced by significantly higher albumin output from mutant co-cultures when compared to hepatocytes cultured alone, without fibroblast support (Figure 4). This suggests that multiple signaling molecules are involved in the maintenance of hepatocyte phenotype *ex vivo*, and that a cocktail of stromal factors will ultimately be needed to replace non-parenchymal cells in hepatic tissue-engineering applications. We therefore conducted a two-way combinatorial knock-down screen, coupling each of the 12 hit candidates with 414 new stromal factors to systematically identify combinations of molecules critical for the phenotype maintenance of primary human hepatocytes *in vitro* (Figure 1). The list of new stromal factors was manually generated using Gene Ontology (GO) annotations cross-referenced with murine fibroblast expression data. All genes in GO categories involved in the initiation, conduction or regulation of cell-cell signaling were included, along with factors noted to be secreted or have extracellular domains. The resulting list was then filtered to include only those found in mouse and those that have documented expression in fibroblasts. The final list of new stromal factors contained 414 genes, listed in Table 3.

*Combinatorial screen and hit selection*

The workflow of the two-way combinatorial screen is summarized in Figure 1. Similar to the validation screen, each of the 414 new stromal factors was targeted by 4 to 10 different shRNAs, all tested in duplicates. This new library of shRNAs, unlike the validation screen, was introduced to 12 mutant J2-3T3 cell lines, each already harboring knock down of one of the 12 hit candidates identified via the validation screen. All 12 mutant J2-3T3 lines were generated using lentivirus-delivered shRNAs previously shown to consistently and effectively knock down target genes during validation screening. Additionally, knock down of select target genes was confirmed via RT-PCR, RNA-seq, immunofluorescent staining, and Western analysis (Figure 3E). In total, 12 x 414, or over 5,000 different two-way combinations of stromal factors were examined, and the impact of their 2-way knock-down on hepatocytes in co-culture is plotted in
Figure 5. Individual shRNAs were considered active if they significantly ($z>2$, $p<0.02$) impacted the overall functional output of the hepatocytes as measured by the albumin ELISA assay. Any shRNA with infection efficiency below 50% were eliminated from analyses. The final hit list contained a total of 75 different two-way combinations, all listed in Table 4.

Hit analysis

In examining the putative positive control Decorin, the data showed an overall shift of the entire population along the ELISA z-score axis (Figures 4, 5), suggesting that the albumin output of hepatocytes supported by fibroblasts lacking decorin more closely resembled those of hepatocytes in mono-culture without stromal support. This finding is consistent with decorin’s hypothesized role as an important positive modulator of the co-culture effect. In fact, this decline in liver-specific functions on a population level was observed with all 12 mutant J2-3T3 fibroblast cell lines, confirming findings from the validation screen that these 12 molecules play critical roles in maintaining the liver-specific functions of primary human hepatocytes in vitro.

Each of the 12 mutant lines yielded effective two-way combinations with a distinct set of new stromal factors (Table 4). A total of 59 new factors were found to contribute significantly ($p<0.02$) to the co-culture effect through 75 different two-combinations. 13 stromal factors (Fras1, Pax4, Tdgf1, Vegfb, Ckb, Cxcl12, Dag1, Ifnb1, Lamb1, Pea15a, Ptger3, Ptx3, Slit3) were involved in more than one active combination. Of the 12 validated genes, Inhba yielded the most number of effective combinations, identifying 29 new stromal factors. This suggests that the loss of inhibin, beta A renders the resulting hepatocytes particularly susceptible to additional losses of supportive factors. The two consistently strong performers, H2-K1 and Ttk, each yielded 7 and 5 effective two-way combinations respectively. Interestingly, Ttk shared 3 (Vegfb, Ifnb1 and Cxcl12) out of its 5 hit combinations with Rgnef, which represented 100% of the Rgnef hit factors; Inhba and Shc1 shared 3 hit factors (Pax4, Pea15a and Ptger3) while H2-K1 shared factors with Rock2, Ssb and Sfrs3. Network analysis conducted based on gene ontology revealed existing pathway connections between H2K1 and Rock2 via Cd44 as well as links between Sfrs3 and Ssb. These results suggest that our hit candidates may share common signaling pathways through which the co-culture effect is mediated. Ongoing work in pathway analyses has utilized RNA sequencing to determine the global gene expression state of mutant fibroblasts with aims of identifying common affected pathways. We are also examining cocktails of recombinant hit factors towards eventual replacement of stromal cells by acellular products for the maintenance of human hepatocytes in vitro.
Replacement of Non-parenchymal Cells with Stromal Products

**Adsorption of Activin A improves hepatocyte morphology**

The strongest reproducible shRNA from validation screen mediated knock-down of Inhba (z_ELISA = 9.9 – 11.4, p << 1E-5). Inhba encodes for the alpha subunit of activin A, which has roles in early liver development as well as cell cycle regulations. To explore its utility in replacing stromal support cells for liver tissue-engineering applications, we conducted preliminary studies to investigate the effects of Activin A on hepatocyte maintenance in vitro. Primary human hepatocytes were cultured without stromal support on surfaces with co-adsorbed collagen I (included to promote cellular attachment to tissue culture plastic) and activin A. Phase contrast images after 16 days in culture showed that adsorption of Activin A better preserved hepatocyte morphology when compared to hepatocytes cultured on rigid collagen I alone. Activin A treated monocultures of hepatocytes exhibited more polygonal cell shape, well-defined networks of bile canniliculi and less cellular debris than the untreated control (Figure 6A).

**Mechanisms of hepatocyte phenotype maintenance in vitro**

Activin A is a member of the TGF-beta superfamily that inhibits the progression of cell cycle through the G1 phase. To explore whether this mode of action has a role in hepatocyte maintenance, we explored the effect of other cell cycle inhibitors on hepatocyte functions in culture (Figure 6B). One such inhibitor is the small molecule Lovastatin, which arrests cell cycle in the G1 phase via upregulation of p27. Primary hepatocytes treated with Lovastatin were observed to have enhanced viability and morphology as well as increased albumin output in a dose-dependent manner (Figure 6C). Similar effects were seen with an inhibitor of PI3K (LY294002), which acts upstream of p27 to inhibit cell cycle progression. Unpublished work done by March et. al., have characterized the effects of LY294002 on hepatocyte functions in vitro. Albumin secretion and intracellular levels, urea production, and CYP450 activity were all higher in the group treated with the PI3K inhibitor than in the DMSO-treated control groups. At day 6 post-treatment, hepatocytes in the control group (DMSO) lost their polygonal shape and bile canaliculi structures, while hepatocytes treated with LY294002 maintained both features, including functional transporters such as MRP2. At day 13 post-seeding, the number of hepatocytes in the DMSO-treated group had decreased by 85%, while only 10% of the cells were lost in hepatocyte cultures treated with LY294002. This dramatic loss of hepatocytes is due to apoptosis, as demonstrated by caspase activation as well as a significantly higher
number of apoptotic cells in control cultures (88% in DMSO-treated group vs. 2% in LY294002 treated cultures). Both LY294002 and Lovastatin inhibited BrdU incorporation and upregulated p27 protein levels (Figure 6D) to mediate cell cycle arrest. Their beneficial effects on hepatocytes in culture were both sensitive to Mevalonate, a molecule known to rescue cells from Lovastatin-mediated G1 arrest (Figure 6E), suggesting that LY294002 and Lovastatin share a common pathway and that this pathway contributes to the maintenance of hepatocyte phenotype in vitro.
DISCUSSIONS

Sourcing of hepatic cells is a fundamental challenge for many fields of liver research, often compelling the use of xenogeneic sources such as porcine and rodent hepatocytes, or immortalized human cell lines. Animal hepatocytes have been extensively studied and are easily obtained, but exhibit significant species-specific differences in hepatocellular functions, including expression and activity of phase I detoxification enzymes, metabolic regulation of cholesterol and apolipoprotein expression. Human hepatocyte cell lines, while easily renewable, contain mutations and exhibit an abnormal repertoire of liver functions that limit their clinical impact. Primary human hepatocytes present the best range of hepatocellular function and recent advances in cryopreservation technologies have additionally enabled the storage of entire livers of human hepatocytes, enough to power multiple batches of phenotypic screens. This enables hundreds of thousands of knockdown or overexpression studies on a constant genetic background over a period of several months. Extensive characterizations have shown that appropriately cryopreserved primary human hepatocytes exhibit phenotypes that are comparable to fresh hepatocytes and are thus suitable for in vitro liver studies; our findings here, however, indicate that not all donors are suitable for use in phenotypic screens. Therefore, we propose that empirical characterization of each donor of cryopreserved primary human hepatocytes is an indispensable first step to their use in liver research.

Maintenance of cryopreserved primary human hepatocytes in vitro can be achieved transiently via co-cultivation with a variety of non-parenchymal cells. There exist multiple configurations of co-cultures, with a wide range of architectural organization. The most basic form contains a random mixture of hepatocytes and J2-3T3 fibroblasts in a co-planar distribution on a rigid matrix of type I collagen. More sophisticated designs utilize semiconductor-driven microtechnology to organize hepatocytes into colonies of empirically optimized sizes and shapes, surrounded by J2-3T3 fibroblasts (MPCC). All configurations of the hepatocyte-J2 co-culture were shown to maintain primary human hepatocyte functions in vitro for at least 9 days. Generally, a higher degree of architectural organization prolongs maintenance of hepatocyte functions in vitro, with MPCC being the most optimal configuration, enabling stabilization of hepatocyte phenotype for 4-6 weeks. However, such segregation of hepatocyte and fibroblast populations limits the number of hepatocytes that engage in heterotypic cell-cell interactions. Additionally, MPCCs are difficult to miniaturize beyond 96-well platforms and months of functional hepatocyte cultures are neither necessary nor practical for most whole-cell screens. We thus designed our high-throughput liver platform to assume a feeder layer co-
culture configuration, which ensures that every hepatocyte has access to a fibroblast and can participate in heterotypic cell-cell signaling.

Significant efforts were dedicated to the development of an image-based hepatocyte viability assay. The co-existence of two different cell types in each well renders their differentiation challenging. Existing measurements of cell numbers, such as AlamarBlue, CellTiter-Glo and Live/Dead stains all reflect the joint state of the whole well, which allows behavior of the more populous J2-3T3 fibroblasts to mask that of hepatocytes in culture. Therefore, we needed to develop a custom readout in order to isolate and quantify the hepatocyte subpopulation. Human hepatocytes in culture can be distinguished from the underlying J2-3T3 fibroblasts through a variety of methods, including bright field microscopy, fluorescent staining of hepatocyte-specific markers, and striking differences in nuclear morphology. Phase contrast images are easy to acquire but are difficult to quantify, particularly in a high-throughput manner. Immunofluorescent staining of specific antigens, while easy to quantify, are difficult and expensive to execute in 384-well and smaller formats with often unacceptable signal to noise ratios. Therefore, we chose to leverage differences in nuclear morphology. We did note that the highly textured nature of fibroblast nuclei rendered their segmentation difficult, often leading to the breakup of a single nucleus into multiple nuclei. Therefore, while the assay reports numbers for both fibroblasts and hepatocytes, it is optimized for accurate detection of hepatocyte nuclei only. We point to our later work, which yielded an image analysis pipeline for more accurate measurement of the number of fibroblasts in co-culture. Additionally, we recognize that nuclei numbers do not always equate cell numbers. Hepatocytes in particular are well known for their ability to assume polyploid states and can sometimes contain 8 or more nuclei. While nuclear proliferation does not always translate into cellular proliferation, cell death is always accompanied by a decrease in nuclei numbers. Therefore, nuclei number represents a viable surrogate marker for cell number, albeit without a strict one to one correlation. We noted during analyses that close clustering of more than 2 nuclei is rare and that polyploidy can be detected visually in images during follow-up analysis of hits.

Analyses of the validation screen focused on finding only positive inducers of the co-culture effect and neglected to examine negative mediators, whose knockdown actually enhanced hepatocyte functions. This decision was made because J2-3T3 fibroblasts are strong inducers of the co-culture effect, often supporting physiological to supra-physiological levels of liver-specific synthetic functions in vitro. We thus believe that it would not be rewarding to look for negative mediators in a cell type that is inherently a high inducer of hepatocyte functions. We
also prioritized the search for positive mediators because they have the potential to substitute for non-parenchymal cells and directly advance the development of fibroblast-free tissue-engineered hepatic systems. Although negative mediators are less useful in this regard, studies of such factors can provide insight into the signaling pathways involved in the phenotypic maintenance of hepatocytes ex vivo and should be conducted in the future. Such studies are best suited for either overexpression experiments using open reading frame (ORF) libraries in J2-3T3 fibroblasts or knock down studies in non-parenchymal cells known to be low inducers of the co-culture effect, such as primary MEFs.

We note that our analyses during high-throughput screening sampled only one of the 500+ biochemical functions served by the liver. Due to the immense scope and diversity of liver functions, there lacks a “gold standard” assay that can accurately reflect the functional state of a hepatocyte in its entirety. Therefore, we typically measure surrogate markers that each examines a major category of hepatocyte functions: albumin output for protein synthesis functions, urea processing for protein metabolism, CYP450 activity for detoxification of innate and external toxins. Of these, we found albumin output to be the most reliable and easiest to quantify in high-throughput. As the goal of high-throughput screens is to identify candidates for further study, we reserved in-depth analyses for follow-up studies. On-going investigations of screening hits will re-expand characterization of hepatocyte functions to include aspects of liver biology beyond protein synthesis and may further include gene expression profiling to obtain a more global perspective on the phenotypic state of these cells.

During follow up studies of the validation screen, we noted that two strong hits, Inhba and Ttk, induced opposite responses in cultured hepatocytes when adsorbed onto tissue culture plates (Figure 6A). Inhba enhanced hepatocyte survival and morphology while Ttk accelerated the phenotypic decline and death of primary human hepatocytes in vitro. Both genes encode for proteins implicated in cell cycle progression: Activin A inhibits G1 progression while Ttk promotes it. This led us to hypothesize that the loss of hepatocyte phenotype in vitro may actually be the result of a failed regenerative process. We understand from extensive animal studies that liver regeneration is initiated in part through the disruption of the liver extracellular matrix, which releases buried growth factors important for hepatocyte proliferation. Collagenase perfusion during standard cell isolation protocols provides such disruption, triggering a regenerative response whereby hepatocytes enter active cell cycle. This is supported by cyclin A immunofluorescent staining, which showed that a significant subpopulation of hepatocytes progress beyond the G1 restriction point within 24 hrs of seeding in culture (Figure 6F). A smaller percentage of these cells further undergo active DNA synthesis, as indicated by BrdU
incorporation (Figure 6D). Cellular proliferation is not usually completed, however, possibly because the \textit{in vitro} environment lacks critical molecular signals that allow proper cell cycling. Once cells have progressed past the G1 restriction point, they are unable to revert to a quiescent state and the fate of failed replication attempts in such cells is apoptosis. This is consistent with our observations that hepatocytes seeded in confluent mono-layers, and thus prevented from entering the cell cycle through contact inhibition, are able to better maintain their liver-specific phenotype. This hypothesis is further supported by data showing enhanced survival and liver phenotype in hepatocytes treated with cell cycle inhibitors including LY294002 and Lovastatin. Such rescue is best seen when hepatocytes are arrested within 24 hours of seeding in culture, before they progress past the G1 restriction point, and has been demonstrated to be due to a reduction of apoptosis, as shown by lower levels of caspase-9 activation in treated cultures.

The seemingly dichotomous effects of Ttk may be the result of the same mode of action on two different cell populations. During validation screening, Ttk was knocked down in J2-3T3s, which were then used to co-cultivate primary human hepatocytes. This gene encodes for a protein that is essential for chromosome alignment at the centromere during mitosis and is a critical mitotic checkpoint factor. Its knockdown in fibroblasts may thus have compromised fibroblast proliferation and survival. Therefore we cannot rule out the possibility that the observed decrease in hepatocyte numbers and function maybe the indirect result of decreased fibroblast numbers rather than the direct effect of lost Ttk signaling. This inability to separate direct and indirect signaling effects on hepatocytes in co-culture is a limitation innate to all platforms containing multiple cell types and a long-standing motivation for the replacement of supportive cells by their protein products. We have now improved our image-based assay to accurately quantify fibroblast nuclei in addition to hepatocyte numbers, and will re-examine the screening data to determine if Ttk knock down significantly decreased fibroblast numbers in co-culture. Ultimately, only extensive follow up studies conducted using a single cell type can confidently separate the direct and indirect effects of the various signaling molecules. We hope that the tools we have developed here as well as the pilot work conducted using them can serve as a roadmap to guide such endeavors in the future.
CONCLUSIONS

The overall goal of this thesis was to take initial steps towards the systematic identification of genetic factors involved in hepatocyte phenotype maintenance *in vitro* in order to help generate a source of functional human hepatocytes for studying liver biology and treating liver disease. It is our hypothesis that, as *in vivo*, molecular signals from the stroma provide inductive cues to maintain liver-specific functions *in vitro*, and that these stromal signals can be isolated and used to replace non-parenchymal cells in stabilizing primary human hepatocytes. We reported here the development of a high-throughput human liver model and attendant automatable assays capable of reflecting human liver physiology *in vitro*. These tools were used to conduct genetic knock down screens of over 450 stromal factors in over 5000 two-way combinations in order to identify molecules important for the maintenance of hepatocyte phenotype *in vitro*. Overall, we identified 72 positive inducers of hepatocyte functions. Through combinatorial screening, we further determined that knock down of multiple stromal factors often synergized, with some two-way combinations leading to a near-complete loss of liver-specific synthetic output. This suggests that multiple signaling molecules are involved in stromal-mediated stabilization of hepatocytes *ex vivo*. Adsorption of hit molecules such as Activin A onto tissue culture plastic improved hepatocyte survival and morphology, and may be acting via signaling pathways that inhibit cell cycle progression and apoptosis. These results represent important first steps in the elucidation of mechanisms instrumental to the functional maintenance of hepatocytes *in vitro*, and we hope this new insight will guide the assembly of a cocktail of recombinant acellular stromal products capable of replacing stromal cells in hepatic tissue engineering applications.
Figure 1. Overview of Research Plan. A high-throughput liver platform consisting of primary human hepatocytes on a confluent feeder layer of J2-3T3 fibroblasts was developed along with three high-throughput assays to quantify fibroblast viability, hepatocyte viability and hepatocyte functions in culture. This platform was used to conduct genetic knock down screens in two phases. Phase I, the validation screen, examined knock-down effects of 59 fibroblast genes that were theoretically predicted by previous gene expression profiling to be inducers of the co-culture effect. Phase II, the combinatorial screen, studied knock-down of 414 new stromal factors involved in cell-cell communications, in over 5000 two-way combinations. Ultimately, we hope our findings will represent the first steps towards the assembly of a cocktail of recombinant acellular stromal products capable of replacing stromal cells in hepatic tissue engineering applications.
Figure 2. Overview of Screening Methods. Hepatocyte functions were measured via a competitive ELISA assay quantifying albumin content in culture media (A). shRNA library vector design (B). Hepatocytes were distinguished from J2-3T3 fibroblasts in co-culture via nuclear morphology: fibroblast nuclei were larger, more irregularly shaped and exhibited punctated texture (C). Image processing algorithm to identify hepatocyte nuclei via an automated process, which includes illumination correction and nuclei segmentation (D). Machine learning algorithm to classify objects in every image as hepatocyte or fibroblast, enabling quantification of hepatocyte nuclei in co-culture with J2-3T3 fibroblasts (E).
Figure 3. Platform and Assay Development. Platform design (A). Assay quality control; CV = coefficient of variation (B). Brightfield image of hepatocyte-fibroblast feeder layer co-culture; Assay validations - image-based hepatocyte viability assay; AlamarBlue fibroblast viability assay; Albumin ELISA assay; Urea colorimetric assay; Cytochrome P450 activity assay (C). Transduction validations – puromycin selection curve; infection efficiencies; hepatocyte functional output (D). Protein knock down validation (E). Error bars represent standard deviation.
Figure 4. Validation screen -- high-throughput identification of previously implicated stromal factors. Primary screening data; hits were selected based on
decreased albumin secretion, on either a population or individual cell basis (A). Representative hit candidates (B).
Figure 5. Combinatorial Screen. ELISA z score reflective of hepatocyte functional output upon knock down of 414 stromal factors in >5000 two-way combinations with each of the 12 hit candidates from validation screen. Green = active shRNAs. Yellow = empty vectors.
Figure 6. Maintenance of Hepatocytes in vitro Without Support From Non-parenchymal Cells. Effect of adsorbed protein hits on hepatocyte morphology 16 days post initiation of culture; Activin A enhanced hepatocyte survival and morphology while Ttk accelerated cellular decline of culture hepatocytes (A). Modes of action of Lovastatin, Ly294002 and Mevalonate; both Lovastatin and Ly294002 inhibits cell cycle in G1 phase via p27 upregulation (B). Cell cycle inhibitors enhanced hepatocyte survival and function in a dose dependent manner (C). Lovastatin and Ly294002 suppressed DNA synthesis and upregulated p27 (D). Ly294002 and Lovastatin act via common pathways, perturbed by the addition of Mevalonate (E). Untreated primary hepatocytes contain high levels of cyclin A, suggesting cell cycle progression beyond the G1 restriction point. Such progression is suppressed in Ly294002 and Lovastatin treated cells (F).
Table 1. 59 genes previously implicated in the co-culture effect, tested in validation screen.

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|      |      | - Multiple independent hairpins  
|      |      | - Good reproducibility  
|      | Ttk  | - KD effects are consistent within screen and with prior predictions |
| 2    | Rgnesf | Multiple hairpins  
|      | Pkp2 | Effects are consistent within screen and with prior predictions  
|      | Mlf1 |  |
| 3    | CD44 | Multiple hairpins  
|      | Ssb  | Effects are mostly consistent within screen and with prior prediction  
|      | Tsnax |  |
| 4    | Shc1 | Multiple hairpins  
|      | Inhba | Effects are consistent within screen  
|      | Sfrs3 |  
|      | Rock2 |  |
Table 3. 414 new stromal factors tested in combinatorial screen. All genes were selected for their role in cell-cell signaling or for being secreted / having an extracellular domain that interacts with the extracellular environment.

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Table 4. Hit candidates from combinatorial screen. 59 new stromal factors were found during 2-way combinatorial screen, in 75 pairings.
REFERENCES


45 Lebreton, J. P. et al. LONG-TERM BIOSYNTHESIS OF COMPLEMENT COMPONENT C-3 AND ALPHA-1 ACID GLYCOPROTEIN BY ADULT-RAT HEPATOCYTES IN A


