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Generation of a Scaffold-Free Three-Dimensional Liver Tissue via a Rapid Cell-to-Cell Click Assembly Process

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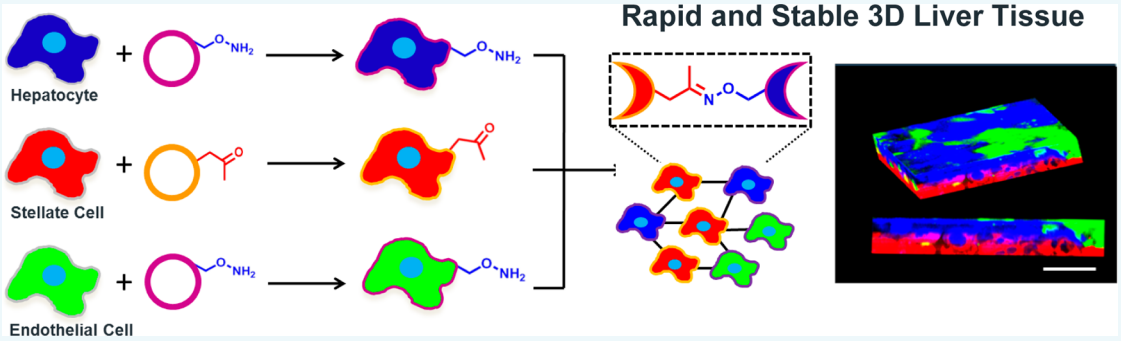

ABSTRACT: There has been tremendous interest in constructing in vitro liver organ models for a range of fundamental studies of cell signaling, metabolism, and infectious diseases, and as a commercial system to evaluate therapeutic drug discovery prioritization and toxicity. Although there has been progress toward studying two-dimensional hepatic function in vitro, there remain challenging obstacles to generate rapid and efficient scaffold-free three-dimensional multiple cell line coculture tissue models of liver. Herein, we develop and employ a strategy to induce specific and stable cell−cell contacts among multiple hepatic cell lines to generate 3D tissues through cell-surface engineering based on liposome delivery and fusion to display bio-orthogonal functional groups from cell membranes. We generate, for the first time, a three cell line coculture 3D liver tissue model by assembling hepatocytes, hepatic endothelial cells, and hepatic stellate cells via a rapid intercell click ligation process. We compare and analyze the function of the superior 3D liver tissue chips with 2D coculture monolayer by assessing mitochondrial metabolic activity and evaluating drug toxicity.

INTRODUCTION

Recent strategies to assemble complex tissues in vitro have revolutionized biomaterial and bioengineering research and are now a central design feature for drug discovery and organ engineering research efforts to improve human health.[1−5] In particular, the human liver is a complex multicellular organ containing a range of different cell types including hepatocytes as the key parenchymal cells and hepatic sinusoidal endothelial cells (HSEC), hepatic stellate cells (HSC), and Kupffer and pit cells as nonparenchymal cells.[6,7] The liver organ is responsible for many diverse functions, including protein, carbohydrate, and lipid metabolism, detoxification of endogenous and exogenous compounds, storage of glucose, production of bile and cholesterol, secretion of albumin and clotting factors, production of urea, and many other vital processes.[8] Due to its central importance, any liver function impairment may lead to a range of deleterious illnesses and diseases.[9,10] Furthermore, many therapeutic drugs discovered in cell based assays result in hepatotoxicity in animal trials leading to high failure rates in drug approvals. This directly impacts the tremendous cost associated with drug discovery and may also result in the retrieval of existing drugs from the pharmaceutical market due to liver toxicity.[11,12]

Despite the critical significance of establishing in vitro liver tissue models, many challenges limit the exploration and development of the field of liver tissue engineering.[13] For example, in order to evaluate liver toxicity of drug molecules before animal studies or clinical trials, a functional liver tissue model system established in the laboratory would be highly desirable to assign drug priority.[14] However, for in vitro models, it is very difficult to maintain hepatocyte cells, which rapidly lose liver-specific functions and stop growth under in vitro conditions.[15] In order to address this severe cell culture limitation, many liver model systems containing cocultures of parenchymal hepatocytes with other nonparenchymal cells have been investigated.[16] The current state of the art for 2D liver tissue is based on coculturing nonparenchymal cells with hepatocytes in specific ratios or patterns.[17] Pioneering research discovered that hepatocytes could survive and maintain liver-specific functions for much longer periods when surrounded by certain (maintenance) cell lines.[18] These artificial coculture

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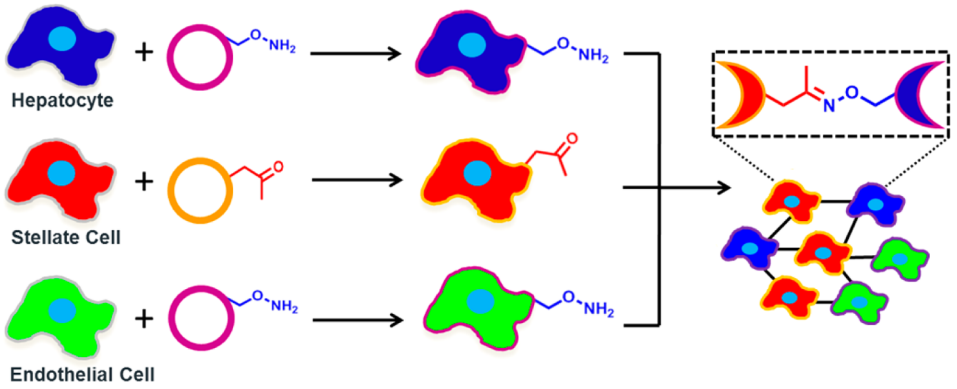

Figure 1. General schematic process of engineering cell surfaces with bio-orthogonal chemistry groups for the programmable assembly of multiple cell lines into complex coculture spheroids and tissues. The bio-orthogonal groups were delivered to parenchymal and nonparenchymal liver cells via a straightforward liposome fusion strategy. The cells were surface-engineered to present oxyamine and ketone functional groups. The tailored cells rapidly assemble on demand through an interfacial oxime ligation.

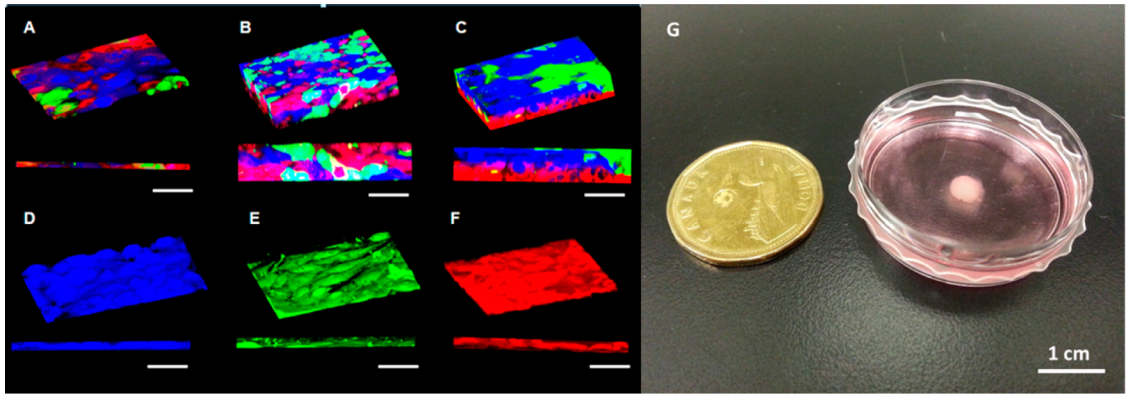

Figure 2. Fluorescent images and digital photograph of single layer and three-dimensional liver tissue. (A) 3D and sideview confocal image of a three cell type coculture as a single layer. The cells do not have bio-orthogonal groups and therefore only form a mixed monolayer. (B) Confocal image of three-dimensional tissue containing the three cells types engineered with ketone and oxyamine groups via liposome fusion. The three cell types formed mixed multilayers. (C) Confocal images of surface-engineered liver tissue assembly with orientation control. Stellate cells (red) were at the bottom, and mixed hepatocytes (blue) and endothelial cells (green) were on the top. Hepatocytes, hepatic endothelial cells, and hepatic stellate cells were live-stained as blue (D), green (E), and red (F), respectively. (D–F) show monolayers of the individual cells used to construct the liver tissue. Scale bar (A–F) represents 50 μm. (G) Digital camera photograph of macroscale liver tissue that was fabricated based on surface-engineered hepatocytes, stellate cells, and hepatic endothelial cells.

systems have greatly expanded the range of liver studies possible and as an in vitro drug screening platform.[19]

While two-dimensional cocultured hepatocytes were used in various drug analyses to help researchers estimate liver function and drug responses, most of these systems remain as traditional cell monolayer systems (two-dimensional systems).[14] The two-dimensional tissue on a chip systems have advanced in vitro liver model systems, but there remain many challenges to generating appropriate functional three-dimensional in vitro tissue models with more than two liver specific cell types. These 3D coculture systems would be better mimics of real liver tissue and may expand liver cell viral and toxicity studies, which in turn may lead to potential new liver organ assays and functional liver construction.

To achieve 3D cell culture and tissue construction, a variety of synthetic scaffolds were developed to support cell adhesion and growth and to study cell behavior in 3D. These scaffolds usually contain synthetic polymers such as poly(ε-caprolactone) (PCL), poly(L-lactic acid) (PLLA), polyglycolic acid (PGA), and poly(lactic-*co*-glycolic acid) (PLGA) or biological materials such as collagen, gelatin, chitosan, alginate, and agarose.[20,21] While synthetic scaffolds were widely studied to support cell proliferation and construct 3D tissues, many limitations remained for cell based research and applications, including the inherent stability of the scaffolds, toxicity of degradation byproducts, potential inflammation and immune responses, interference with cell–cell interactions, duration for cell saturation in the scaffold, and unpredictable impact on signaling pathways.[22,23] Although some scaffolds are biocompatible and capable of mimicking certain features of the extracellular matrix, their long-term safety and side effects are still unclear. Therefore, scaffold-free tissues may present an alternative and complementary system for future applications in tissue engineering and regenerative medicine.

Herein, we introduce a scaffold-free platform system to generate rapid, efficient, and controlled multiple cell type coculture cell assemblies of 3D liver tissues. The strategy relies on mildly rewiring cell surfaces in order to click cells together to form stable complex structures without the use of polymer scaffolds or encapsulating gel materials.[24] This system is based on integrating a universal cell surface engineering method to install chemoselective and bioorthogonal lipid-like groups onto cell membranes to which interfacial click reactions among different cell type membranes can be induced.[25] Upon this

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multivalent interfacial ligation, cells can be clicked together and thus result in rapid cell−cell assembly with high cell economy. With simple manipulation, cell orientation and positioning can be potentially achieved to construct multicellular tissues approaching the complexity of organs.[26]

RESULTS AND DISCUSSION

As shown in Figure 1, parenchymal (hepatocytes) and nonparenchymal (stellate and endothelial) liver cells can be surface-engineered via functionalized liposomes presenting ketone or oxyamine groups. After cell surface engineering, these ketone or oxyamine-tailored cells can be chemoselectively ligated together through interfacial multivalent oxime bonding. The oxime conjugation is stable, bioorthogonal, and instantaneous through polyvalency under physiological conditions.[27−33] Moreover, it does not require any catalyst or produce any toxic side product. These velcro-like features on the cell surface allow for the installation of only minimal amounts (several thousand) of ketone or oxymaine groups onto cells surfaces to initiate the multivalent oxime conjugation, while ensuring no cytotoxic impact on cells. Previous studies have shown that the liposome fusion delivery of cargo and bio-orthogonal lipids is a transient transfection that helps to initiate the cell-to-cell assembly process.[24−39] Over time, the cells proliferate and dilute the bio-orthogonal lipids; however, during this time period the cells excrete new extracellular matrix which then holds the assemblies and eventually tissues together with high cell density.[40]

Based on the cell surface engineering method, human immortalized hepatocytes Fa2N-4, human hepatic stellate cells (HSC), and human hepatic sinusoidal endothelial cells (HSEC) were surface-engineered to present oxyamine or ketone molecules, respectively. Fa2N-4 hepatocytes are noncancerous and widely used in liver model systems due to their ease of maintenance and similar expression profile of liver-specific functions.[41] As shown in Figure 2, through the interfacial oxime conjugation between oxyamine and ketone, these surface-engineered liver cells, when mixed, rapidly assemble into 3D tissue on demand. To easily distinguish among the three cell types in coculture, hepatocytes, hepatic endothelial cells, and hepatic stellate cells were live-stained as blue, green, and red with vital dyes, respectively. Without the surface engineering process, mixing the three cell types only resulted in a 2D monolayer (Figure 2A). In contrast, 3D multilayer liver tissues were easily and rapidly constructed when the three bio-orthogonal cell surface engineered cells were mixed (Figure 2B). Due to the interfacial ligation, the surface-engineered cells may also be oriented depending on the manner of sequential addition. For example, stellate cells (red) were seeded first to form a bottom layer to which mixed hepatocytes (blue) and endothelial cells (green) were seeded to form an oriented multilayer structure (Figure 2C). Without the surface engineering procedure the cells only formed 2D monolayers (Figure 2D−F). This controllable cell assembly and orientation system can only occur with surface-engineered cells. This unique system provides researchers the opportunity for truly scaffold-free construction of any tissue in a range of orientations. Furthermore, the cell ligation assembly method allows for large-scale tissues to be generated (Figure 2G) rapidly with efficient cell economy. We found the assembled tissues were stable for many weeks with no change in liver specific functions. Future studies combined with 3D printing technology may afford complex tissues approaching the complexity of organs. As a representative example shown in Figure 2G, a macroscale liver tissue was constructed in 4 h through the efficient bio-orthogonal tissue click method.

To demonstrate that the coculture assemblies excrete their own extracellular matrix (ECM) after initial adhesion through the interfacial click ligation method we examined over time the production of collagen and elastin. Figure 3 shows fluorescent images of ECM production over time for 2D and 3D coculture assemblies of the three cell lines. The cell nuclei were stained blue with DAPI and the secretion of ECM is visualized over time as green through fluorescent small molecule Col-F

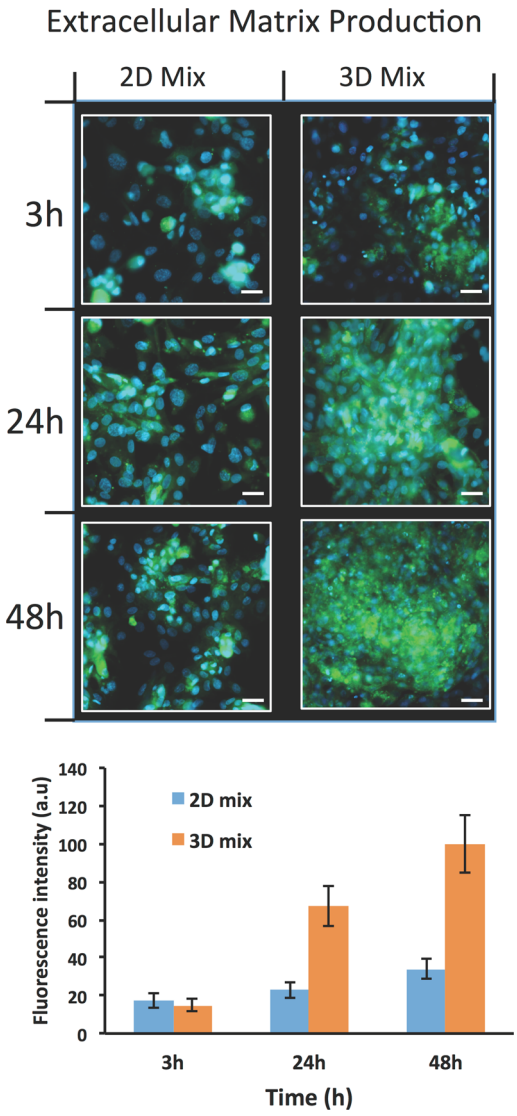

Figure 3. Fluorescent image comparisons of extracellular matrix (ECM) production over time after two-dimensional coculture monolayer and three-dimension multilayer tissue assembly. (Top) Nuclei of the three cell lines are stained blue and the production of ECM is stained green with a small molecule probe for collagen and elastin. After initial rapid 3D cell assembly via bio-orthogonal ligation, the cells soon excrete extracellular matrix to further support cell adhesion. Scale bar = 40 μm. (Bottom) Secretion of extracellular matrix over time by 2D monolayers and 3D tissues of mixed cells. Relative fluorescent intensity produced by Col-F staining of collagen and elastin was measured at 3, 24, and 48 h and expressed in arbitrary units (a.u). n = 15, p < 0.05. All fluorescent intensity images were relative to the 48 h time point image (assigned 100 arbitrary units).

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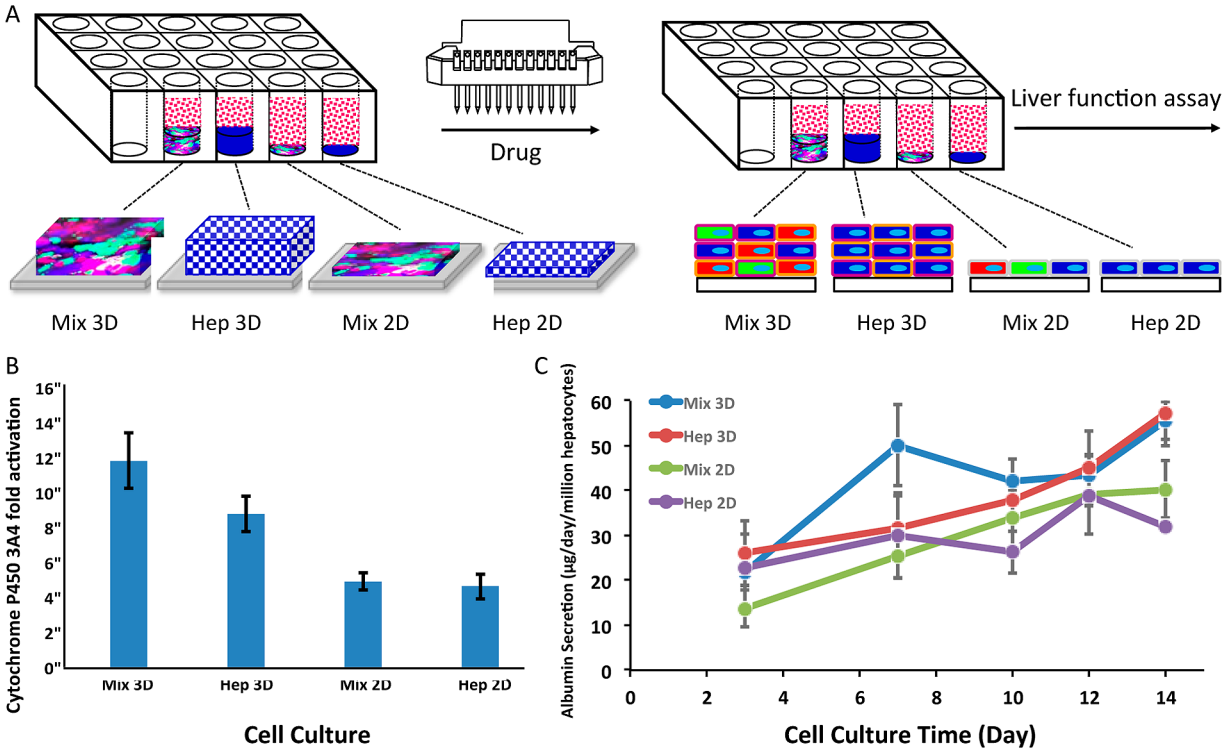

Figure 4. Fabrication and analysis of various types of liver chips generated via an intercell bioorthogonal ligation strategy. (A) Schematic of different types of liver chips for liver function analysis. (B) Study of cytochrome P450 3A 4-fold activation in different liver tissues treated with 10 μM rifampin over 72 h. (C) Rates of albumin secretion by different liver tissues over 14 days. Error bars represent standard error of the mean.

staining of collage and elastin. It is clear that the initial cell-to-cell adhesion process is rapidly induced through oxime ligation which is then re-enforced over time through the production of ECM.

To further demonstrate the utility and scope of the system, four representative liver tissues were fabricated and then analyzed and compared for various liver specific functions (Figure 4A). As a comparison, 3D coculture liver tissue (Mix 3D − containing all three cell lines), 3D hepatocyte liver tissue (Hep 3D − containing only the hepatocyte cell line), 2D coculture liver tissue (Mix 2D − containing all 3 cell types, and 2D hepatocytes liver tissue (Hep 2D − containing only hepatocyte cell line) were studied for enzyme activity of cytochrome P450 3A4—a well-known enzyme responsible for drug metabolism in hepatocytes (Figure 4B).[42] These liver tissues were treated with rifampin, a common cytochrome p450 3A4 activator, for 72 h. It was observed that the 3D coculture liver chip (Mix 3D) showed highest activation (~4-fold), and 2D hepatocytes tissue (Hep 2D) showed the lowest activation. It is well-known that the albumin secretion is an important liver-specific function and therefore we performed an albumin secretion assay over 14 days. According to the results from the assays, 3D coculture (Mix 3D) and 3D hepatocyte (Hep 3D) liver tissues showed excellent liver function over 14 days. These results provide clear evidence that the scaffold-free 3D liver tissue system has tremendous potential in liver tissue engineering and liver on a chip fabrication and applications.

Liver toxicity is one of the primary reasons for withdrawal of drugs from the market as well as from lengthy and expensive clinical trials.[14] Therefore, drug screening of model livers or liver chips before clinical trials is considered a critical step during the evaluation and prioritization of drug candidates. In order to highlight the potential of the tissue click system described for drug screening application, several representative drugs were tested on the various liver tissue chips fabricated (Figure 5). It has been shown that cellular interactions between hepatocytes and other nonparenchymal cells such as HSC and HSEC modulate the physiological response of liver to drugs in 2D monolayer coculture systems. Thus, when modeling the drug response in vitro it is important to account for interactions of parenchymal and nonparenchymal cells in a complex 3D tissue environment. In this work, we compared the responses of four different liver tissue chips to the treatments with common hepatotoxic compounds including troglitazone, rosiglitazone, and cyclophosphamide. These various liver chips were treated with 10 μM troglitazone, 150 μM rosiglitazone, or 7.5 mM cyclophosphamide, respectively, over 16 h to screen for acute liver toxicity (Figure 5B−D). To evaluate the responses, a standard MTT assay for analyzing mitochondrial activity was performed after 16 h drug treatment. It was found that the 3D coculture liver chip showed the highest mitochondrial activity in response to these drugs while 2D hepatocytes showed the lowest mitochondrial activity. These results further support that 3D cocultured liver tissue containing parenchymal and nonparenchymal liver cells displayed the best liver function and highest resistance to drug toxicity.

CONCLUSION

In this report, we established for the first time a scaffold-free 3D tissue construction system based on a straightforward bioorthogonal cell surface engineering tissue click ligation and applied it to liver fabrication and study. Based on this strategy we were able to rapidly construct a range of complex 3D liver tissues containing multiple parenchymal hepatocytes and nonparenchymal liver cell lines and evaluate their ability to recapitulate liver function through a variety of metabolic and

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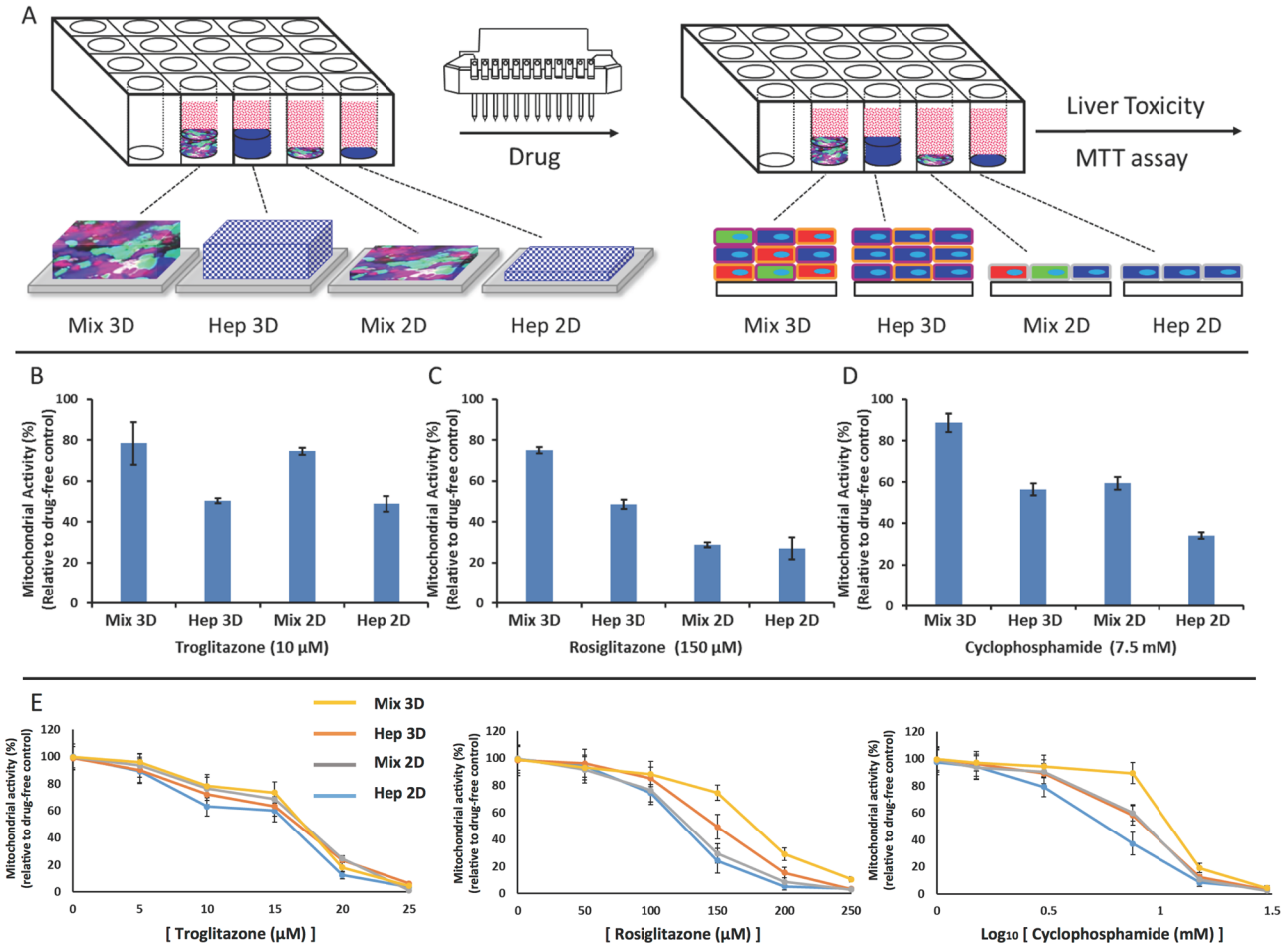

Figure 5. Study of mitochondrial activity for evaluation of liver toxicity in different types of liver constructed chips. (A) Drug screening of fabricated liver chips based on MTT assay after treating tissues for 16 h with (B) 10 μM troglitazone, (C) 150 μM rosiglitazone, and (D) 7.5 mM cyclophosphamide. (E) Plots of MTT assay for a range of drug concentrations for various tissue constructs.

drug assays. To the best of our knowledge, this is the first example of using more than two liver specific cell lines in a liver coculture scaffold free 3D system. The 3D coculture liver tissue showed similar function to a real liver organ, and expressed excellent liver-specific functions. Furthermore, the 3D liver chip fabricated provides a new platform to generate a wide range of complex tissues with multiple cell types for a variety of future drug screening and tissue specific assays. The method can be used to generate a range of length scales of oriented 3D cocultures and multilayer tissues with many cell type components. Since the cells are the scaffold, no polymers or encapsulating materials are required to generate or stabilize the 3D tissues. The bioorthogonal ligation between cells initiates the assembly process, which is then further enhanced (held together) over time by the production of extracellular matrix. This strategy is also amenable to bioprinting and microfluidic methods to assemble stable spheroids and tissues in flow without post-encapsulation materials and may be combined with 3D printing and biodegradable polymers for the generation of many complex tissues.[43−47] Due to the efficient cell assembly (cell economy) process this method in combination with polymer strategies may allow for filling of polymer molds or decellularized scaffolds rapidly for bioreactor applications since the cell types attach to each other and the polymer to rapidly generate thick tissues in a range of length scales. Finally, many complex thick tissues may be generated in 3D for organ on a chip type drug screens or viral assay screens, or as model systems for paracrine and autocrine signaling.

MATERIALS AND METHODS

Tissue Culture. Immortalized human hepatocytes Fa2N-4 were obtained from Xenotech, KS. The cells were thawed and cultured on collagen I-coated plates in multifunction enhancing (MFE) plating medium containing 10% newborn calf serum (Xenotech, KS), and 1% penicillin/streptomycin solution (Sigma). At 24 h upon plating, the medium was replaced with MFE serum-free support medium containing component A. The medium was replaced every 48 h. The cells were passaged upon reaching confluence (every 3−4 days).

Primary human hepatic stellate cells (HSC) were obtained from Sciencell, CA. The cells were cultured on poly(L-lysine)-coated plates in stellate cell medium (SteCM) consisting of 500 mL basal medium, 5 mL of stellate cell growth supplements (SteCGS), 10 mL of fetal bovine serum (FBS), and 5 mL of penicillin/streptomycin solution. The cells were passaged upon reaching 95% confluence. The medium was changed every 48 h.

Primary human hepatic sinusoidal endothelial cells (HSEC) and their medium were purchased from Sciencell, CA. The cells were cultured on fibronectin-coated plastic plates in endothelial cell medium (ECM) consisting of 500 mL of basal medium, 5 mL of endothelial cell growth supplement (ECGS), 25 mL FBS, and 5 mL penicillin streptomycin solution. The cells were

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passaged upon reaching 95% confluence (every 2−3 days) and the medium was changed every 48 h.

All cells were maintained at 37 °C, 5% CO_2.

Preparation of Liposomes. To prepare oxyamine and ketone-tethered liposomes, chloroform solutions of palmitoyl-oleoylphosphatidylcholine (POPC) and 1,2 dioleoyl-3-trimethyammonium-propane (DOTAP) were mixed with *O*-dodecyloxyamine (for oxyamine-tethered liposomes) or dodecanone (for ketone-tethered liposomes on the following ratios: POPC (430 μL,10 mg/mL in $CHCl_3$ at 86 mol %); DOTAP (10 μL, 10 mg/mL in $CHCl_3$ at 2 mol %); and *O*-dodecyloxyamine or dodecanone (60 μL, 10 mM in $CHCl_3$ at 12 mol %). The mixtures of lipids were thoroughly dried and then resuspended in 3 mL of phosphate-buffered saline (PBS). The suspension was then sonicated with a tip sonicator for 20 min until it was clear.

Liver Tissue Assembly. Prior to tissue assembly the cells were allowed to reach 85−95% confluence. Cells were then treated with liposome solution (50 μL of liposomes/1 mL of medium) and incubated at 37 °C for 4 h. The medium was discarded and the cells were washed with PBS twice. The ketone- and oxyamine-tethered cells were removed from the plate with 0.25% trypsin and centrifuged at 800 rpm. The medium was discarded and the oxyamine- and ketone-labeled hepatocytes, HSC and HSEC, were mixed in a small volume of medium (2×10^6 cells/mL). Small drops of concentrated resuspended cell solutions were then placed in 12-well (3.7 cm^2) collagen I-coated plates and given a slight shake to induce cells to aggregate and assemble. Each mixed (coculture) 2D or 3D sample contained 1×10^5 of Fa2N-4 hepatocytes, 1×10^5 of HSEC and 5×10^4 of HSC. Each 2D and 3D hepatocyte-only culture contained 1×10^5 of Fa2N-4 hepatocytes. The cells were then incubated at 37 °C and 5% CO_2 for 16 h to achieve full spreading of cells in the tissues. Upon spreading of cells, fresh MFE plating medium was added into the plates. After 24 h, the medium was exchanged with a 1:1 mixture of MFE support medium and endothelial cell medium (ECM). Cells in the control samples were treated with nonfunctionalized liposomes.

Fluorescent Staining for Collagen and Elastin in 2D and 3D Assemblies. To visualize the secretion of ECM by tissues over time, the cells were stained with the green fluorescent probe Col-F (Immunochemistry Technologies, MN), which binds collagen and elastin. A 20 mM stock solution of Col-F reagent in DMSO was prepared. The stock solution was dissolved in the medium to give a final 20 μM concentration. The tissues were treated with the medium containing 20 μM of Col-F reagent and incubated for 1 h at 37 °C. The medium was removed and the cells were washed twice with PBS. The cells were then fixed with 10% formalin for 15 min and visualized via fluorescent microscopy with excitation of 488 nm and emission of 520 nm.

Immunohistochemistry and Confocal Microscopy. Prior to tissue assembly cells were incubated in serum-free medium containing fluorescent live stain (Life Technologies). Fa2N-4 hepatocytes, HSEC, and HSC were treated with 25 μM CellTracker Blue CMAC (7-amino-4-chloromethylcoumarin), 25 μM CellTracker Green CMFDA (5-chloromethylfluorescein diacetate), and 25 μM CellTracker Red CMTPX, respectively. The cells were incubated for 45 min, after which they were washed thoroughly with PBS and incubated in serum-free medium for an additional 45 min. Cells were then assembled into 3D tissues and incubated for 24 h. Subsequently, the tissues were fixed with 10% formalin for 15 min and visualized with LSM-700 (Zeiss) confocal microscope.

Activation of Cytochrome P450 3A4. To induce cytochrome P450 3A4, the 3D tissue samples were incubated in MFE support medium containing 10 μM Rifampin (Sigma) for 72 h at 37 °C, 5% CO_2. The control samples were treated with DMSO vehicle only for calculation of fold activation. The medium was changed every 24 h. The samples were analyzed via P450-Glo CYP3A4 Assay Kit (Luciferin-IPA) (Promega) by using the manufacturer's instructions for the nonlytic cycle. The sample's medium was exchanged with fresh MFE support medium containing 3 μM Luciferin-IPA and the samples were incubated for 50 min. 50 μL of medium was then transferred into a white 96-well plate. 50 μL of the detection reagent was then added into each well. The samples were covered with aluminum foil and incubated for 20 min at room temperature. The luminescence was read with a Biotek Synergy Multi-detection Plate Reader (Biotek).

Albumin Analysis. Culture medium was collected from the various samples and the albumin content was measured via Human Albumin Pincer Assay kit (Mediomics). New medium (1:1 MFE support medium and endothelial cell medium (ECM)) was added 24 h prior to collection for albumin quantification.

Toxicity Assays. Liver tissues were incubated with 10 μM troglitazone, 150 μM rosiglitazone, or 7.5 μM cyclophosphamide dissolved in MFE support medium for 16 h. The control samples were treated with DMSO vehicle. Subsequently, cell viability was analyzed by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma), where the tetrazolium ring is cleaved by mitochondrial dehydrogenase enzymes. The samples were then incubated in DMEM phenol red-free medium containing MTT reagent for 1 h resulting in production of a purple precipitate which was subsequently dissolved in a 1:1 solution of isopropanol and DMSO. The absorbance was measured at 570 nm using a Synergy Biotek assay device.

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Notes

The authors declare the following competing financial interest(s): Prof. Yousaf has equity in OrganoLinX Inc.

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