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Review

Generation of Patterned Cocultures in 2D and 3D: State of the Art

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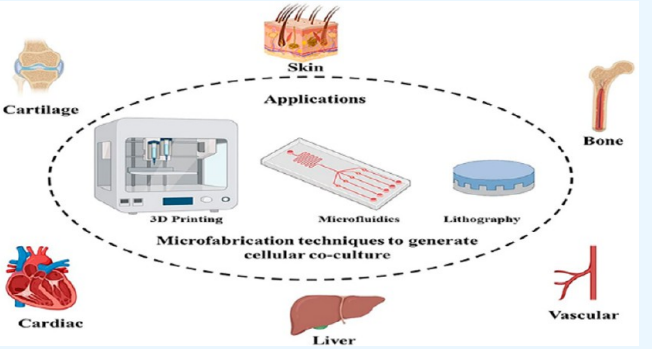
ABSTRACT: Cells inside the body are embedded into a highly structured microenvironment that consists of cells that lie in direct or close contact with other cell types that regulate the overall tissue function. Therefore, coculture models are versatile tools that can generate tissue engineering constructs with improved mimicking of *in vivo* conditions. While there are many reviews that have focused on pattering a single cell type, very few reviews have been focused on techniques for coculturing multiple cell types on a single substrate with precise control. In this regard, this Review covers various technologies that have been utilized for the development of these patterned coculture models while mentioning the limitations associated with each of them. Further, the application of these models to various tissue engineering applications has been discussed.



1. INTRODUCTION

Cell fate, including proliferation, migration, and differentiation, is greatly influenced by the cellular microenvironment and interactions between individual cells.[1−3] Organs or tissues consist of cells that are embedded in a highly structured microenvironment made up of an extracellular matrix (ECM) and nearby cells.[4,5] Cells integrate and interact with a microenvironment compromised of a milieu of biochemical, biomechanical, and bioelectrical signals derived from surrounding cells, the extracellular matrix (ECM), and soluble factors.

Cell−cell interactions that occur through direct cell−cell contact or exchange of soluble factors play a vital role in determining the cell fate *in vivo*.[6−9] These interactions are not only important in various regenerative processes but also crucial for the generation of functional tissues *in vitro*.[10−12] This is one of the main reasons for the loss of cell functionality once the cells are isolated from the host and cultured *in vitro*. With no heterotypic cellular interactions and a uniform substrate that is flat and inflexible in a Petri dish, cells have very little in common with *in vivo* properties. Such systems have been used for years to maintain and promote cell growth, but they are still highly artifactual.

In contrast, coculture techniques provide us with a tool to mimic these *in vivo* conditions for *in vitro* cell culture studies.[13−15] Natural tissues exist in the physiological environment as multicellular systems made up of two or more cell types that interact with one another to perform diverse biological functions. Due to recent progress in the field, researchers are now concentrating more on coculture models, since these systems more accurately mimic original tissue from a physical and biological standpoint through interactions between various cell types. One of the bottlenecks in developing coculture platforms is the precise control of the cells. Conventional techniques such as surface modification, trans-well inserts, Petri dishes, gels, microarrays, and bioreactors have been successful in the generation of these coculture models.[16,17] Different synthetic biology-inspired devices have been fabricated to gain insights into how the cells communicate with each other, which were helpful in understanding the organization of multicellular organisms.[18] Yamato and colleagues utilized thermoresponsive polymer chemistry to generate a model to culture two cell types.[19] Further, studies have utilized bioreactor cultures for culturing different cell types and spheroids to study the effect of cocultures over monocultures.[20,21] Although these techniques were successful, precise control over cell placement was difficult to achieve using these techniques to study the different homotypic and heterotypic cellular interactions.[22−24] These challenges have led to the exploration of more sophisticated systems where cells can be patterned, termed as patterned coculture. Patterned coculture models are mainly focused on broadly utilizing two approaches: the first approach is based on printing, whereas the second approach utilizes the concept of

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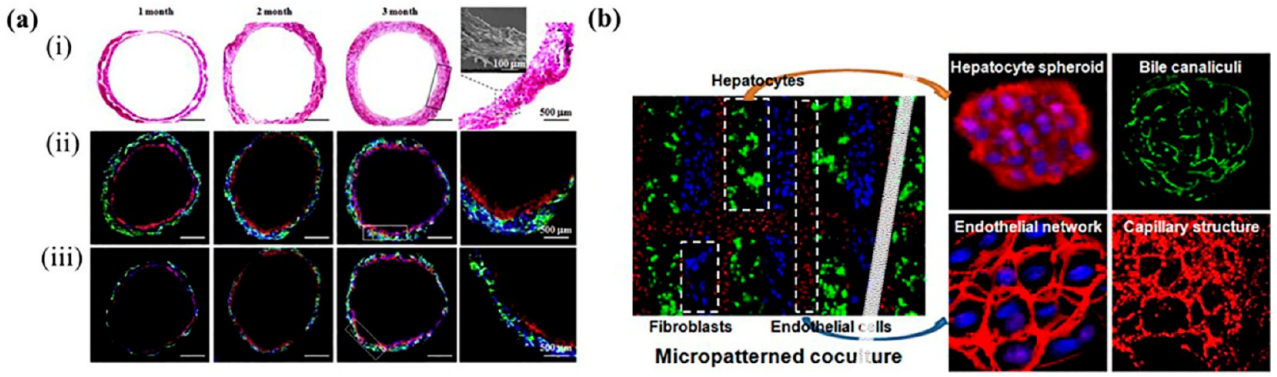

Figure 1. Different coculture models generated using photolithography. (a) (i) H&E-stained images of the engineered vessel after coculture for 1, 2, and 3 months; (ii) IF images for collagen IV (EC) and α-SMA (SMC) after coculturing for 1, 2, and 3 months; and (iii) IF images for laminin (EC, red) and collagen I (SMC, green) after coculturing for 1, 2, and 3 months.[40] Reprinted with permission from ref 40. Copyright 2015 Elsevier. (b) Overall process of hepatocyte coculture with endothelial cells and fibroblasts.[43] Reprinted with permission from ref 43. Copyright 2017 Royal Society of Chemistry.

photolithography.[25−29] These techniques give us a powerful tool to tackle some of the core problems in cell biology and tissue engineering, such as cell survival, proliferation, and differentiation and the interaction between various cell types. The current Review will focus on the various approaches involved in 2D and 3D for the development of these patterned models while highlighting the primary limitations associated with each of these methods. The use of these coculture models in the field of tissue engineering will also be emphasized. The Review concludes with possible future developments that can be helpful in dealing with associated challenges in current advancements.

2. OVERVIEW OF DIFFERENT TECHNIQUES FOR COCULTURING CELLS

A variety of techniques have been utilized in previous research for generating coculture models. For instance, a breakthrough in the field of patterning cells came with the introduction of photolithography in biology. This technique though is well studied and established because of its application in the electronics industry, but it requires expensive instruments and clean rooms, which are generally not available in an average biology lab.[30,31] To overcome these drawbacks, soft lithography for patterning cells was utilized. In this method, photolithography is employed to create a soft, patterned elastic material that is then used for cell patterning.[32,33] Although this technique was highly efficient in generating cellular patterns, studying cellular behavior under dynamic conditions was not possible with this technique, which led to the use of microfluidic devices for generating cellular patterns. This technique was highly efficient for generating coculture models even under dynamic conditions, but one of the drawbacks was that most of these techniques were restricted to 2D platforms.[34,35] *In vivo*, all the tissues and organs in our body are three-dimensional; hence, to better mimic the *in vivo* conditions, researchers have started moving toward 3D to generate coculture models, which were highly efficient in mimicking the *in vivo* conditions for *in vitro* cell cultures.[36−38] Despite rapid advancement in the field, conventional techniques for the generation of patterned coculture models are still limited by challenges such as poor shape and dimensions of the scaffolds and spatial patterning of multiple cell types. In fact, for generating 3D models, now the rapid, precisely controlled 3D printing technique is preferred, as that allows the assembly of multiple types of cells, materials, and biological cues in a predecided structure; hence, this Review mainly focuses on utilizing 3D printing technologies for the generation of these coculture models.

2.1. Photolithography. Photolithography is the method of creating patterns on a surface coated with a light-sensitive polymer or resin (photoresist) by exposing it to high-intensity UV light through a photomask. According to the application, a positive or negative photoresist is first applied to the substrate to be patterned. A negative photoresist cross-links and does not dissolve in the developer after UV exposure, whereas a positive photoresist becomes soluble in the developer after UV exposure. The appropriate patterns are then transferred onto the substrate by placing a photomask over it, exposing it to UV light, and developing it. Since the introduction of this method in the field of tissue engineering, many different cellular patterns have been created using this technique for studying various cellular interactions.[39] This technique has recently been used to create coculture models to explore various homotypic and heterotypic cell interactions *in vitro*. For instance, Liu et al. developed an engineered blood vessel through micropatterned coculturing of vascular endothelial and smooth muscle cells on bilayered electrospun fibrous mats with pDNA inoculation.[40] The authors created a micropatterned fibrous mat and seeded smooth muscle cells (SMCs) onto these sheets (Figure 1a). The coculture system was created by overlaying these SMCs with vascular endothelial cells (ECs) seeded on flat fibrous mats, simulating the bilayered structure of a vessel. The efficacy of the developed coculture system was investigated *in vitro* by quantifying the overall ECM production. The ECs cocultured with SMCs showed a twofold increase in the collagen-IV bands and higher levels of laminin when compared with ECs cultured alone. After three months of coculture, an engineered vessel with compact EC and SMC layers was created by wrapping the layered fibrous mats into a cylinder. Similarly, Kang et al. generated micropatterned stripes of photosensitive poly(allylamine) (LPAN3) and poly(methyl methacrylate) (PMMA) for coculturing hepatocytes and fibroblasts.[41] Coculturing on the stripe patterned substrate was carried out by first seeding hepatocytes, followed by fibroblast seeding. The cocultured cells produced extracellular matrix such as fibronectin, suggesting a biological function.

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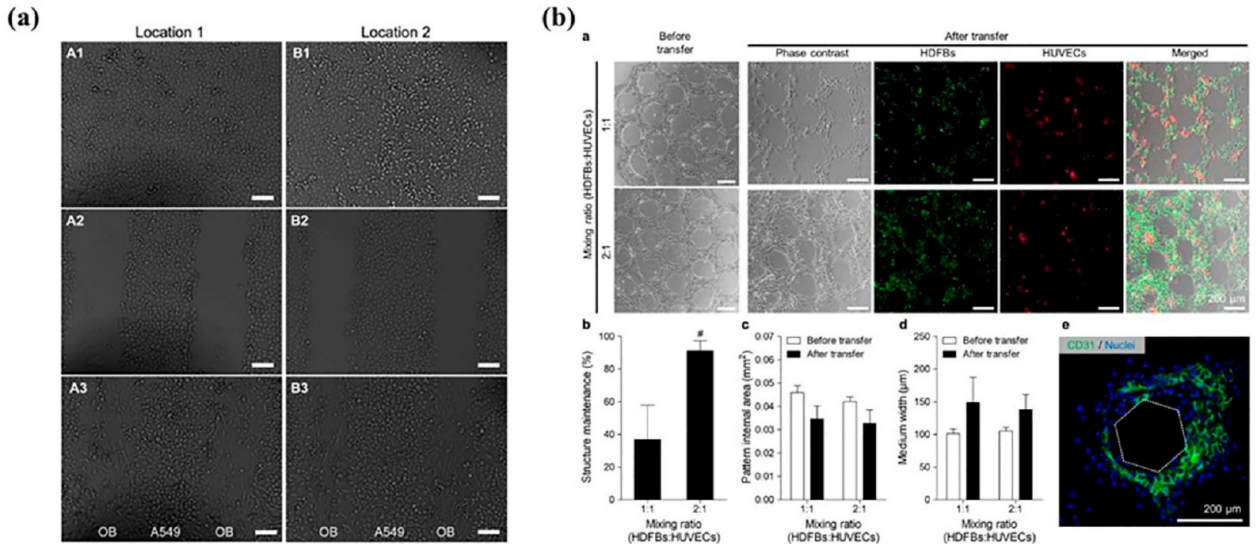

Figure 2. Coculture models generated using soft lithography. (a) Bright field images showing the coculture of OB/A549 cells.[45] Reprinted from ref 45 with license under CC BY 3.0 (https://creativecommons.org/licenses/by-nc/3.0/). Copyright 2017 Royal Society of Chemistry. No changes were made to the copyrighted material. (b) Bright field and fluorescence images of cocultured microtissue before and after delivery.[47] Reprinted with permission from ref 47. Copyright 2021 Elsevier.

Further, Hong and colleagues utilized multilayer photolithography to develop a hydrogel-based coculture system to study the effect of phenotypical changes of macrophages in the presence of fibroblasts. The presence of fibroblasts not only promoted the proliferation and migration of macrophages but also had a higher polarization when compared with cultures without fibroblasts. The study successfully demonstrated how the presence of fibroblasts influenced the macrophage activities.[42] These coculture models have not only been utilized for studying basic biology. Recent research has exploited these models for developing drug toxicity models, as they resemble the native tissue *in vitro*. For instance, Liu et al. developed micropatterned fibrous scaffolds to establish a cardiac myocyte coculture system with cardiac fibroblasts and endothelial cells for the predictive screening of cardiotoxicities *in vitro*.[43] The CMs cocultured with ECs and CFs showed a higher elongation ratio, strong Cx-43 expression, strong smA-α staining, and higher beating rates when compared to CMs cultured on aligned fibers (Figure 1b). Their study demonstrated the capabilities of a micropatterned coculture of cardiac myocytes over a monoculture to establish the cardiac function as a reproducible and reliable platform for screening cardiac side effects of drugs. In another research by a similar group, micropatterned coculture of hepatocytes was developed as a potential *in vitro* model for predictive drug metabolism. Compared to hepatocytes cultured alone, coculture with either fibroblasts or ECs significantly enhanced the liver specific functions. Their study demonstrated how coculture of hepatocytes with fibroblast and endothelial cells enhanced the liver specific functions and can be used as an *in vitro* testing model to study the drug metabolism *in vivo*.[44]

Although this technique was highly efficient in generating coculture models to study various cellular interactions and developing models for drug screening, the advancement in the field has led researchers to utilize soft-lithography techniques for the generation of these models.

2.2. Soft Lithography. Since the use of photolithography was expensive and required multiple steps to generate these coculture systems, to overcome these drawbacks, researchers have started utilizing the technique of soft lithography to pattern cells, as it is inexpensive and easy to replicate. The technique creates a soft, elastic material that is patterned using photolithographic techniques and is then used as a stamp, mold, or mask to pattern a substrate. To this end, extensive research has been done to develop coculture models utilizing this method. In a research by Zhong et al., a coculture model to stimulate lung cancer bone metastasis for anticancer drug evaluation was developed.[45] The authors utilized a microeraser technique to coculture A549 lung cancer cells and osteoblast (OB) cells to develop a model to study cell−cell interactions (Figure 2a), and further the model was utilized for antidrug cancer evaluation and to study the efficacy of an anticancer drug doxorubicin. In another study by March et al., a coculture model of primary hepatocytes and supporting cells was developed for the study of hepatotropic pathogens.[46] The model was developed to replicate the *in vitro* hepatic cycles for hepatitis B and C viruses. Further, these coculture models have been utilized for the delivery of microtissues with complex architectures. In a work by Kim et al., a temperature-responsive hydrogel surface with honeycomb patterns was created using the microcontact printing technique. Human dermal fibroblasts (HDFBs) and human umbilical vein endothelial cells (HUVECs) implanted on the hydrogel surface spontaneously generated honeycomb-shaped microtissues (Figure 2b). Additionally, the produced microtissues can be successfully transferred to the target region while maintaining their original honeycomb structures. The work showed how temperature-sensitive hydrogels with micropatterned patterns might be used as a productive way to deliver complex tissues for tissue engineering applications. Overall, this method proved quite effective in producing a range of coculture models for a range of static tissue engineering applications.[47]

Microfluidics. Because it was challenging to create coculture models under dynamic conditions using soft lithography-based systems, researchers began taking advantage of microfluidic-based systems to produce these models and study the impact of coculture in dynamic environments. In a study by Jie et al., an integrated microfluidic system for cellular coculture and

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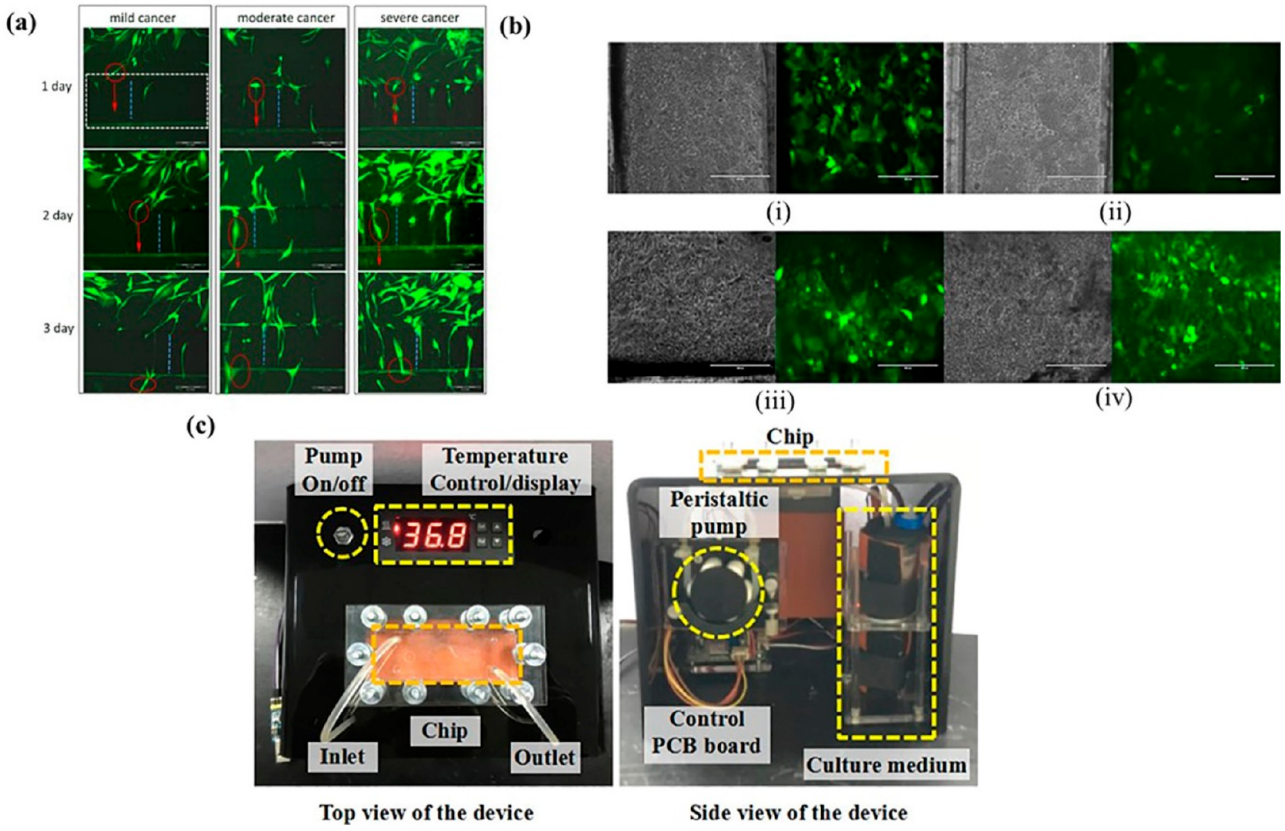

Figure 3. Coculture systems generated under dynamic conditions. (a) Image showing cellular migration of different models at the same location.[49] Reprinted from ref 49 with license under CC BY 4.0 (https://creativecommons.org/licenses/by-nc/4.0/). Copyright 2016 Nature Publishing Group. No changes were made to the copyrighted material. (b) Image of PRHs infected with AdGFP or AdGFP-HBV using the liver sinusoid on a chip.[51] Reprinted with permission from ref 51. Copyright 2015 Wiley-VCH. (c) Image showing the hardware of the culture platform.[52] Reprinted from ref 52 with license under CC BY 4.0 (https://creativecommons.org/licenses/by-nc/4.0/). Copyright 2020 Nature Publishing Group. No changes were made to the copyrighted material.

simulation of drug metabolism was developed.[48] To achieve this, the authors cocultured Caco-2, HEPG2, and U251 cells as mimics of the intestine, liver, and glioblastoma, respectively. The developed dynamic system provided a high-throughput platform for drug screening and personalized cancer therapy. In another study by Mi et al., a coculture model using human mammary epithelial cells (HMEpiC) and MDA-MB-231 breast cancer cells was developed to study cancer cell migration and anticancer drug screening applications.[49] The developed system showed high cell viability and demonstrated that the density of cancer cells determined the probability of the occurrence of metastatic cells and that the induction of normal cells affected the metastatic velocity of each cancer cell (Figure 3a). The study further demonstrated the role of IL-6 in increasing the migration ability of MDA-MD-231 cells cocultured with HMEpiC cells. Overall, the developed system was successful in the quantification of the migratory capability of the cells. Further, soft lithography has been utilized for the generation of coculture models to coculture microbiome with human cells to study the interactions between microbiome with the host cells.[50] Additionally, the technique has also been further utilized for the development of organs on a chip model for various tissue engineering applications. Kang et al. developed a liver sinusoid on a chip model using these microfluidic platforms. Primary rat hepatocytes (PRHs) and endothelial cells were cocultured in layers in single and dual microchannel configurations with or without continuous perfusion.[51] The study showed that hepatocytes retained their normal morphology and generated urea for at least 30 days when cocultured with endothelial cells in a dual microchannel with continuous perfusion (Figure 3b). The developed system closely resembles the *in vivo* hepatic sinusoid and can be used for many liver biology experiments. In another study by Yin et al., a coculture kidney chip was developed for efficient drug screening and nephrotoxicity assessment.[52] The system consists of a device and a three-layered microfluidic chip that provides a simulated environment for kidney organs (Figure 3c). When compared to cells in a monoculture, cells cultured in the developed system exhibited higher performance for drug nephrotoxicity evaluations and can be further utilized for the development of reliable kidney drugs.

In yet another study, Zhou et al. developed a liver injury on a chip model with integrated biosensors for monitoring cell signaling during injury.[53] The primary emphasis of the study was on how alcohol-related damage impacts TGF-β signaling between hepatocytes and stellate cells. The study showed higher levels of stellate cell activation in microfluidic-based cocultures in response to alcohol liver injury when compared to conditioned media and trans-well tests, emphasizing the significance of the developed method. Additionally, a micro-system with five chambers was created, and the system was successful in tracking the paracrine crosstalk between the two cell types that were connected by the same signaling molecule, TGF-β.

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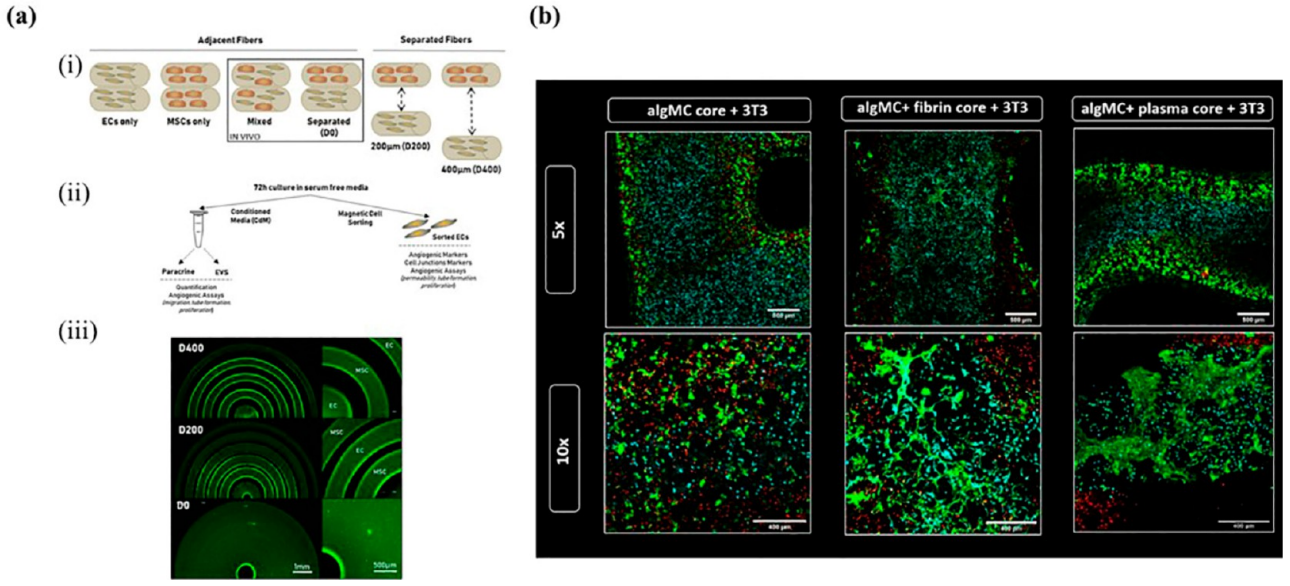

Figure 4. Different coculture models developed using 3D printing. (a) (i) Figure showing endothelial Cells (ECs) and mesenchymal stem cells (MSCs), mixed and separated. (ii) After printing, 3D printed samples were incubated for 48 h. (iii) Micrographs of 3D printed samples (D0, D200, and D400).[55] Reprinted with permission from ref 55. Copyright 2019 Elsevier. (b) Fibroblast network formation in the core compartment at day 7 of coculture.[58] Reprinted from ref 58 with license under CC BY 4.0 (https://creativecommons.org/licenses/by-nc/4.0/). Copyright 2021 Nature Publishing Group. No changes were made to the copyrighted material.

3D Printing. The above-mentioned techniques were highly successful in generating coculture models in 2D, but since a tissue or organ is a three-dimensional structure, researchers have started utilizing 3D printing techniques for the generation of these coculture systems to better mimic the *in vivo* conditions. In a study by Giglio et al., 3D printing technique was utilized for the development of an *in vitro* coculture osteogenic model.[54] A multicompartment structure in PCL that can house endothelium and stem cells was created utilizing the fused filament fabrication process. Human TERT mesenchymal stem cells (TERT-hMSCs) were cocultured with human umbilical vein endothelial cells (HUVECs), which increased the expression of several osteogenic markers and improved cellular survival. The outcomes demonstrate that the developed system was efficient in studying osteogenesis *in vitro*.

In another study by Piard et al., a 3D printed scaffold consisting of coculture of human umbilical vein endothelial cells (HUVECs) and human mesenchymal stem cells (hMSCs) was developed and the effect of cell patterning and distance between cell populations on their crosstalk was studied.[55] The expressions of VEGF, FGF-2, and ITGA3 (integrins) were upregulated in HUVECs cultured apart from hMSCs, but VE-Cadherin and Ang-1 showed a lower fold change. On the other hand, HUVECs cultured close to hMSCs displayed tighter monolayers and an increase in Ang-1 and VE-cadherin expression. Conditioned media collected from this promoted tube formation, a later stage of angiogenesis (Figure 4a). Finally, after 12 weeks of implantation in a rat cranial lesion, *in vivo* tests revealed an increase in blood vessels and new bone thickness, indicating a higher efficacy of the EC/MSC interaction in boosting the angiogenesis of local tissues and improved bone regeneration. In yet another study, our group utilized extrusion-based 3D printing to control the differentiation of the encapsulated human mesenchymal stem cells simply by modifying the ECM of the cells. To prove this hypothesis, a smart cell instructive scaffold was fabricated where the presence of carboxylic groups enhanced the chondrogenic differentiation and the presence of phosphate groups enhanced the osteogenic differentiation of the seeded hMSCs. Overall, the study demonstrated the use of 3D printing in the development of clinical viable osteo-chondral grafts.[56] Further, these 3D printing techniques have also been utilized for the development of various drug screening models. In a study by Zhang et al., the ink jet printing technique was utilized for cell patterning in microfluidic chips, followed by cell coculture for the detection of drug metabolism and diffusion.[57] The developed approach presented a feasible way to integrate inkjet cell printing and microfluidic chips, which can be applied to tissue engineering- and drug testing-related areas. In another study by Taymour et al., an extrusion-based 3D printing technique was utilized to coculture hepatocytes with fibroblasts to develop an artificial *in vitro* model.[58] The presence of fibroblasts acted as a support for the hepatocytes and enhanced the expression of albumin, which is a biomarker for hepatocytes (Figure 4b). Recently, 3D printing has been utilized for the development of spheroids, as these spheroids can recapitulate the key features of native tissue more realistically.[59−63] Hong and colleagues utilized extrusion-based bioprinting for producing hepatic-lobule-like microtissue spheroids using hepatic and endothelial cells. The developed spheroids not only allowed long-term structural integrity but also showed higher expression for MRP2, albumin, and CD31 when compared with nonstructured spheroids.[64] Further, Heo and colleagues developed heterogeneous spheroids using MSCs and ECs, which could be bioprinted to form complex shapes and had enhanced osteogenic properties.[65] In yet another study, hepatocytes and mouse fibroblasts were cocultured to form spheroids and can be used for bioprinting of liver tissues.[66]

Although discussion occurred on recent advancements for patterned coculture systems, certain challenges need to be considered before these systems can be effectively utilized for mimicking certain tissue types. Table 1 highlights various

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Table 1. Various Associated Challenges while Modeling Coculture Systems

issue	challenges	refs
medium optimization	which growth medium to add for different cell types in the coculture system	42−45, 54, 59, 67, 69, 76−78, 80, 86
cell ratio	ratio of cell added for the coculture	45, 46, 49, 51, 53−55, 58, 67, 68, 70, 74−78, 81
static vs dynamic	effect of dynamic conditions over static in the coculture system	39−47, 54, 55, 58, 67−70, 74−81, 86−88
2D vs 3D	effects of dimensions on cellular behavior	40, 43−49, 51−53, 67, 68, 74, 81
cell type/origin	the origin of the cells affects the coculture system and hence an appropriate cell type should be chosen	43, 46, 54, 58, 70
distinguish between whether the effects are due to cell−cell or paracrine signaling	strategic experiments should be designed to distinguish between the cell−cell contact vs paracrine mediated effects	40, 42−44, 49, 54, 55, 57, 62, 67, 76−78, 80

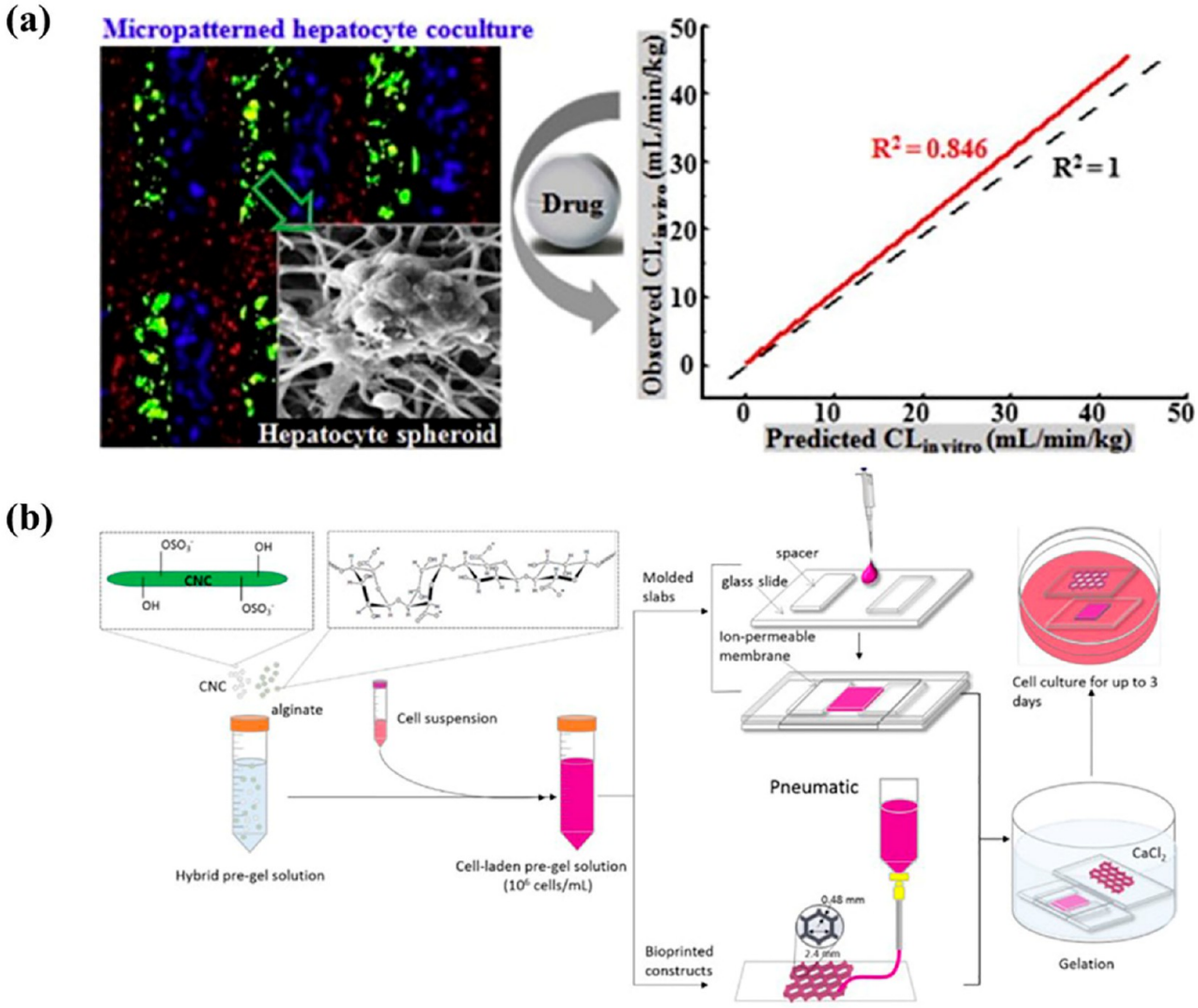

Figure 5. (a) Coculture of hepatocytes with fibroblasts and endothelial cells (Hep-Fib-EC) on micropatterned fibrous scaffolds.[69] Reprinted with permission from ref 69. Copyright 2016 Elsevier. (b) 3D bioprinting of liver-mimetic structures with hybrid bioink.[71] Reprinted with permission from ref 71. Copyright 2018 Elsevier.

challenges associated with utilizing these advanced systems for coculturing multiple cells.

Application of Coculturing in Liver Tissue Engineering. The liver is a special organ that performs a variety of tasks, including the metabolism of glucose, detoxification, urea generation, and secretion.[67,68] Although the liver can greatly replenish itself in the body, maintaining hepatocytes *in vitro* has proven challenging. Coculturing hepatocytes with other types of cells has been proven to increase the stabilization and maintenance of liver-specific function.

In a study by Liu et al., an *in vitro* model for predictive drug metabolism was developed by coculturing hepatocytes with fibroblast and endothelial cells on micropatterned eletrospun fibers.[69] Liver-specific functions were well maintained in the

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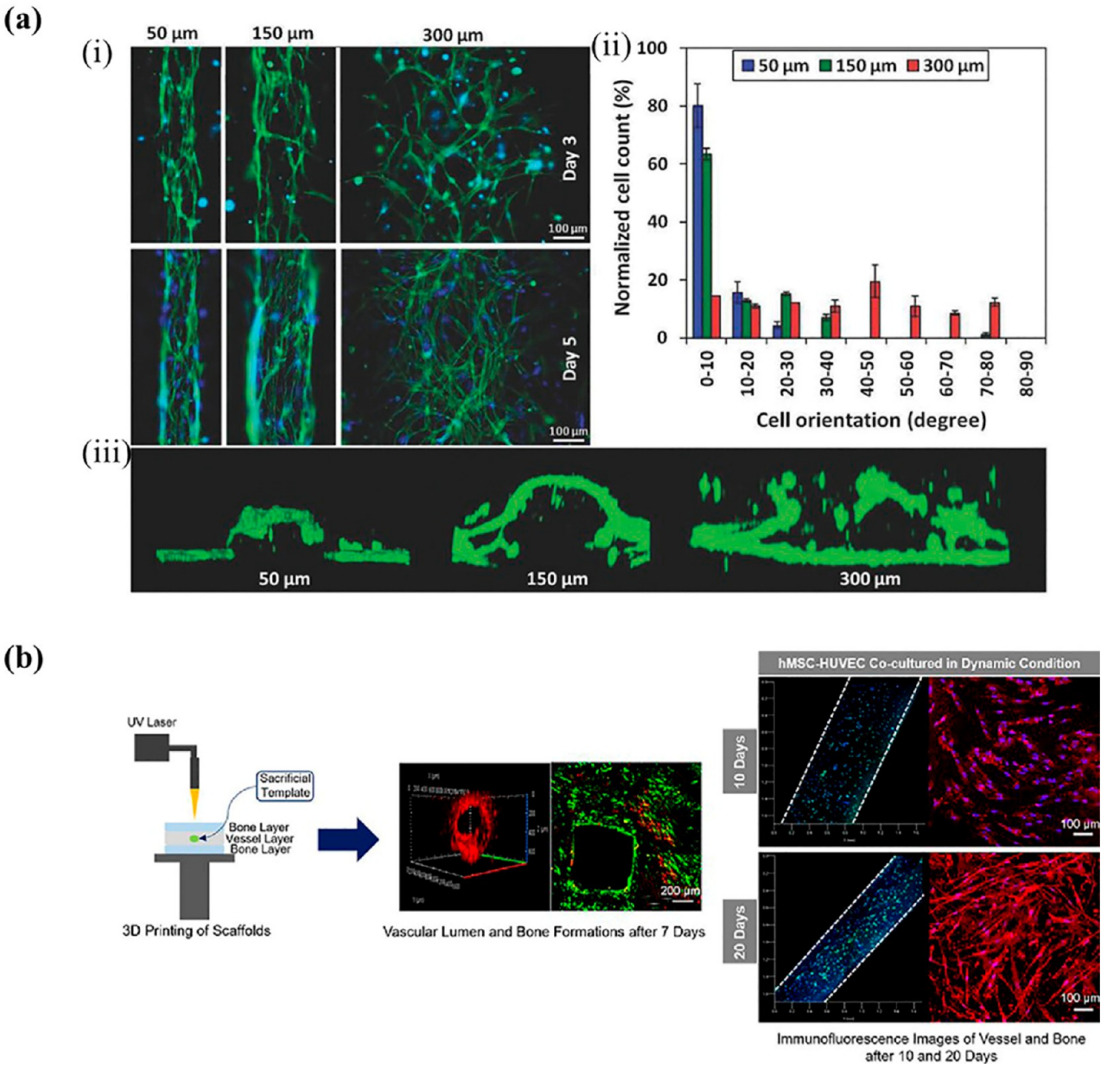

Figure 6. (a) 3D cord formation and alignment of actin filaments (green) and DAPI (blue) stained HUVEC/hMSC in the micropattenred GelMA hydrogel.[76] Reprinted with permission from ref 76. Copyright 2017 Wiley-VCH. (b) Overall process of dual 3D printing for vascularized bone tissue regeneration.[77] Reprinted with permission from ref 77. Copyright 2021 Elsevier.

coculture system, accompanied by a rapid formation of multicellular hepatocyte spheroids. Additionally, the Hep-Fib-EC coculture model demonstrated sensitive responsiveness to the inducers and inhibitors of metabolizing enzymes in the enzyme activity and drug clearance rates of hepatocytes (Figure 5a). These findings showed that micropatterned hepatocyte cocultures are a viable *in vitro* testing option for predicting *in vivo* drug metabolism.

In another study by Jeong et al., the lithography technique was utilized for coculturing hepatocytes and endothelial cells in a ECM-based micro honeycomb to create a 3D liver model that accurately replicates the ultrastructure of the liver and improves liver function.[70] The PDMS structures demonstrated an excellent ability to load cells or drugs. A three-dimensional (3D) liver model with compact cell spheroids and vessel-like structures that provided improved liver functioning was created by coculturing hepatocyte and endothelial cells in the ECM hydrogel microhoneycomb structures.

Further, Wu et al., and group utilized a 3D bioprinting technique to fabricate a liver mimetic construct using alginate and cellulose nanocrystal hybrid bioink.[71] The bioink can be efficiently extruded via the nozzle, has good initial shape fidelity, and has outstanding shear-thinning properties (Figure 5b). Next, using this bioink, we created a 3D-printed liver-inspired honeycomb structure that contained fibroblast and hepatoma cells. $CaCl_2$ was used to cross-link the constructs, which were then cultured for 3 days.

In yet another study by Janani et al., 3D bioprinting technique was utilized to mimic the native liver lobule structure *in vitro* for drug toxicity and drug screening applications.[72] The HLC/HUVEC/HHSC-loaded liver model exhibits improved albumin production, urea synthesis,

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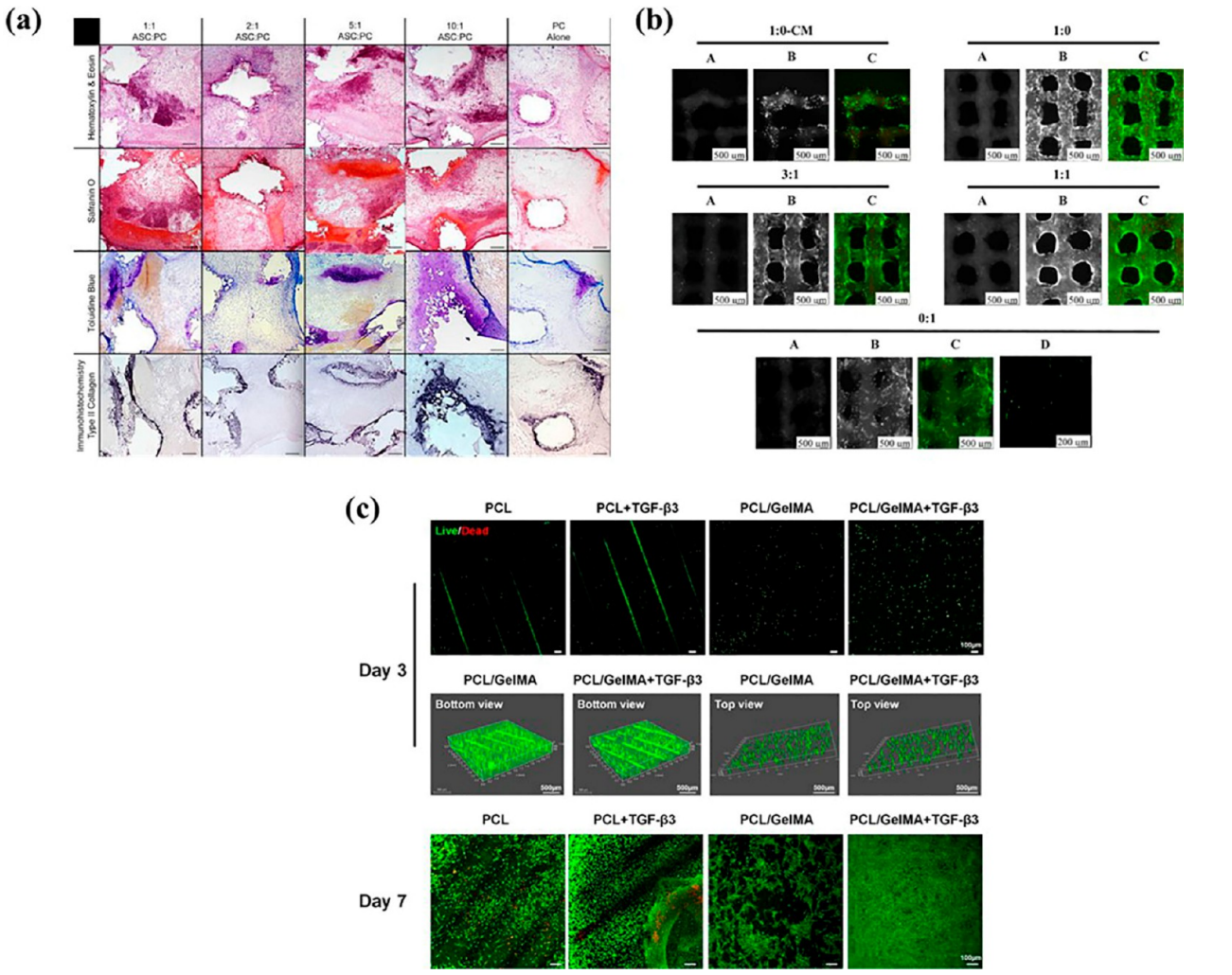

Figure 7. (a) 40× histologic and immunohistochemical results of coculture experimental groups at differing ratios of ASCs-to-chondrocytes after 4 weeks of *in vitro* culture followed by 4 weeks of *in vivo* culture.[79] Reprinted with permission from ref 79. Copyright 2018 Wiley-VCH. (b) Live/dead images of coculture mixtures at day 35 (n = 3)[80]. Reprinted from 80. Copyright 2022 American Chemical Society. (c) Confocal images of scaffolds after live/dead assay showing cell viability on the scaffolds after 3 and 7 days of culture.[81] Reprinted with permission from ref 81. Copyright 2021 Elsevier.

and cytochrome P450 (CPR) activity and resembles native alternating cords of hepatocytes with a functioning sinusoidal lumen-like network in both horizontal and vertical orientations. These findings proved that the proposed clinically relevant vascularized liver model is a cost-effective and reliable platform for hepatotoxicity testing.

Application of Coculturing in Bone Tissue Engineering. Bone has a remarkable ability to repair and restructure without leaving a scar, since it is a dynamic, highly vascularized tissue.[73] These characteristics make it the ideal smart material, together with its ability to quickly mobilize mineral stores in response to the metabolic demand. Its primary function is to give the body structural support. The skeleton also protects interior organs and acts as mineral storage while also supporting muscular contractions that provide mobility. Despite significant advancements in bone regenerative medicine over the years, engineering bone tissue requires the generation of a highly organized vasculature. To achieve this, research has started moving toward coculture systems for generating bone tissues.[74,75]

In a study by Narbat et al., photolithography was utilized to engineer a 3D construct with tunable angiogenic and osteogenic niches and to study its potential for the formation of vascularized bone tissue.[76] Using a two-step photolithography technique, the stiffness of the hydrogel and the distribution of cells within the patterned hydrogel were regulated (Figure 6a). Additionally, osteoinductive nanoparticles were used to stimulate osteogenesis. The development of mineralized zones surrounded by well-organized vasculature was achieved by the coexistence of angiogenic and osteogenic niches within the same construct. Overall, the strategy mentioned here forth can be utilized for engineering constructs that can be used to treat bone deformities.

Hann et al. and colleagues utilized 3D printing for the generation of a vascularized bone tissue by combining stereolithography (SLA) and fused deposition method (FDM) 3D printing techniques.[77] The authors developed a GelMA based bioink incorporating nanocrystalline hydroxyapatite to coculture human bone marrow mesenchymal stem cells (hMSCs) and human umbilical vein endothelial cells (HUVECS) within the same construct (Figure 6b). Coculturing of HUVECS with hMSCs not only promoted angiogenesis but also enhanced the process of bone regeneration, as confirmed by various osteogenic markers. The study high-

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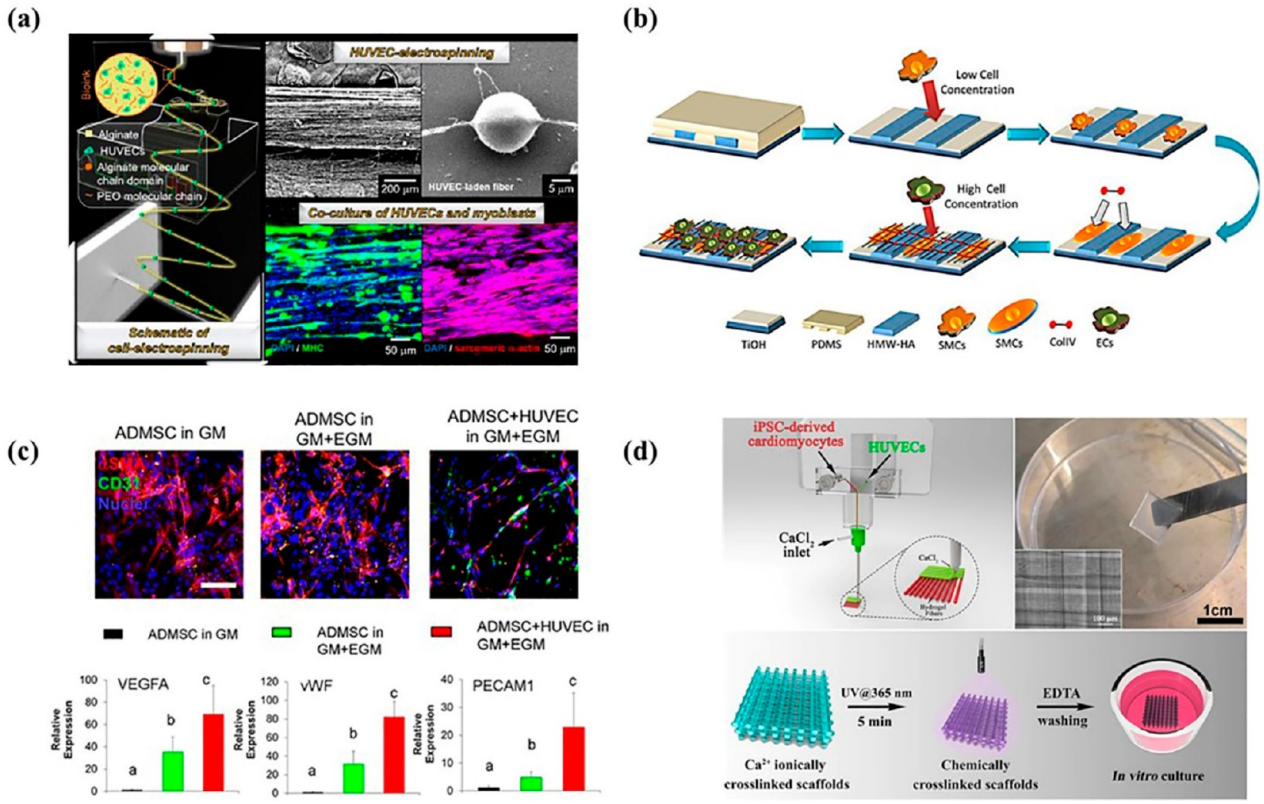

Figure 8. (a) Overall process of developing a platform for coculturing HUVEC's and C2C12 cells using cell electrospinning and 3D bioprinting.[83] Reprinted with permission from ref 83. Copyright 2015 Elsevier. (b) Novel coculture model of vascular endothelial cells and low smooth muscle cells built on the titanium surface.[88] Reprinted with permission from ref 88. Copyright 2018 Wiley-VCH. (c) 3D coculture of ADMSC and HUVEC within the hydrogel-promoted capillary network formation and vascularization gene expression.[89] Reprinted from ref 89 with license under CC BY 4.0 (https://creativecommons.org/licenses/by-nc/4.0/). Copyright 2018 Nature Publishing Group. No changes were made to the copyrighted material. (d) Schematic representation of the microfluidic printing head (MPH) coupled to a coaxial nozzle extruder employed to simultaneously bioprint iPSC-derived cardiomyocytes and HUVEC cells.[90] Reprinted with permission from ref 90. Copyright 2016 Wiley-VCH.

lighted the importance of coculture systems over monoculture in enhancing the process of bone regeneration.

Further, Goushki et al. and colleagues utilized 3D printing to develop a model to generate a direct coculture between preosteoblasts (OBs) and pro-inflammatory macrophages (M1s) to study the effect of immune response toward osteogenic differentiation. The presence of macrophages upregulated the expression of pro-inflammatory markers when compared with monocultures, which diminished the process of differentiation. The study successfully demonstrated the effect of inflammatory response toward the differentiation of osteoblasts.

Application of Coculturing in Cartilage Tissue Engineering. The bony surfaces of joints are lined by complex, living tissue called articular cartilage. The primary purpose of articular cartilage is to offer a low-friction surface so that the joint can support weight during the various motions required for daily activities as well as athletic endeavors. Nevertheless, articular cartilage has little to no ability to heal itself, necessitating frequent surgical intervention for any damage. Although the use of monoculture of chondrocytes or mesenchymal stem cells has been successful, most technique often struggle to fully restore the damaged cartilage's total functionality. Therefore, there is a critical need for appropriate model systems that can recapitulate the native cartilage microenvironment. Toward this, research has started moving toward generation of coculture models, as these models have been proven to address many issues encountered by monocultures in cartilage tissue engineering.

In a study by Morrison et al., a 3D printing scaffold was fabricated for coculturing adipose derived stem cells and chondrocyte toward cartilage tissue engineering.[79] Without the use of exogenous growth factors, successful cartilage formation was achieved employing a coculture model of chondrocytes and adipose-derived stem cells (Figure 7a). All experimental ratios at the post-*in vivo* time point showed cartilage growth according to histology, which was supported by type II collagen immunohistochemistry. Sulfated-glycosaminoglycan synthesis did not differ across the experimental groups.

Posniak et al. fabricated a 3D bioprinted scaffold of gelatin methacryloyl and methacrylated hyaluronic acid to study the effect of coculture of human septal chondrocytes and human bone marrow-derived mesenchymal stem cells toward cartilage regeneration.[80] Results showed that the combination of MSCs and PCs elicited chondrogenic expression that mimicked PCs as well as cell proliferation that mimicked MSCs (Figure 7b). Overall, the study indicated that healthy septal PCs and BM-hMSC cocultures might be used in place of monocultures in chondrogenic investigations for cartilage regeneration.

Cao et al. developed 3D printed PCL/GELMA biphasic scaffolds to coculture mesenchymal cells and chondrocytes to study the effects of coculture in the process of cartilage regeneration.[81] (Figure 7c). The developed scaffolds enhanced the process of cartilage regeneration, which was confirmed

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Table 2

cells	source	refs
endothelial cells	Dr. P. Ravi Selvaganapathy's lab at McMaster University, American Type Culture Collection (Rockville, MD), Lonza Biosciences, Switzerland, Life Technologies, Invitrogen Corporation	35, 43, 44, 54, 55, 70, 77, 83, 89
hepatocytes	adult SD rats, DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), HMCS1SA, Life Technologies or HUCSD, Triangle Research Laboratories	41, 44, 46, 51, 53, 58, 66, 69, 70, 72
human mesenchymal stem cells	Sciencell Research Laboratories, Carlsbad, CA, USA, Prof P. Genever (York University), RoosterBio, Frederick, MD, Lonza	45, 54−56, 66, 77, 80
fibroblasts	Korean Cell Line Bank (KCLB 21658, Korea), ATCC, American Type Culture Collection (Rockville, MD), DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany)	41−44,46, 58, 69, 70, 72
osteoblasts	Laboratory Animal Center of Sun Yat-Sen University	45, 78
macrophage	ATCC, J774A.1, passage 15, Merck KGaA, Germany	42, 78
cardiomyocytes	Sprague−Dawley rats, CDI, Madison, WI,	43, 61, 89
chondrocytes	porcine, human nasal septal cartilage (PC, male, age 43), SD rat	79−81

through a series of *in vitro* experiments. Further, the *in vivo* implantation of the scaffolds in a rat osteo-chondral defect demonstrated the excellent capability of these cocultured scaffolds to enhance the process of cartilage regeneration.

Application of Coculturing in Other Tissue Types. Apart from the above-mentioned tissues, these cocultured models have been utilized to mimic other tissue types. In a study by Yeo et al., 3D printing and cell electrospinning techniques were combined to fabricate micro/nanohierarchical scaffolds for coculturing myoblasts and HUVECs to induce myoblast alignment and differentiation.[82] The cocultured scaffolds showed enhanced expression of various myogenic markers in comparison to the scaffolds that included only myoblasts.

In another study by Li et al., a coculture model of high-density vascular endothelial cells and micropatterned low-density smooth muscle cells was developed for enhancing the functions of the attached endothelial cells.[83] Vascular smooth muscle cells (SMCs) were cultured on a titanium surface with a hyaluronic acid (HA) microstrip pattern at a low density to mimic the EC pericyte environment (Figure 8a). Then, the EC number and each of its functional components, such as nitric oxide (NO), prostacyclin (PGI2), tissue factor pathway inhibitor (TFPI), thrombomodulin (TM), and the inflammatory-induced component endothelial leukocyte adhesion molecule-1 (E-selectin), were measured. The blood flow shear stress (BFSS) action was used to evaluate the antishedding property. The findings demonstrated that the innovative coculture model outperformed the monoculture in terms of EC coverage, functional factor release, and antishedding abilities.

Further these coculture models have also been utilized for *in vitro* prevascularization of scaffolds.[84−87] For instance, in a study by Kuss et al., a bioactive hydrogel coated on porous 3D-printed PCL/HA scaffolds using a combination of human umbilical vein ECs (HUVECs) and adipose-derived mesenchymal stem cells (ADMSCs) showed the ability to create capillary-like networks within the scaffold (Figure 8b).[88] Upon subcutaneous implantation, the hydrogel systems encouraged anastomosis between host mouse vasculature and human-originated vascular networks while promoting microvascular and lumen development. In another study by Maiullari et al., a vascularized heart tissue was fabricated by coculturing human umbilical vein endothelial cells (HUVECs) and induced pluripotent cell -derived cardiomyocytes (iPSC-CMs) using a multicellular 3D bioprinting approach (Figure 8c).[89] Yet in another study, Gong et al., developed a vascular graft by using photolithography.[90] The study demonstrated the importance of micropatterns in *in vitro* cocultures of endothelial cells and smooth muscle cells. Further *in vivo* analysis was performed by implanting the graft in the rabbit carotid artery, and the results showed the effectiveness of the developed graft in regeneration of new blood vessels (Figure 8d). Table 2 summarizes the various cells used for the development of coculture models.

CONCLUSION

This Review has examined several techniques that have been developed for coculturing cells. Although various methods for creating cocultures and the biological outcomes for cell−cell interactions in cocultured systems have been discussed, the techniques utilized and the coculture models developed are not beyond limitations. For instance, although the micropatterning techniques discussed in this Review were successful in developing coculture models, the design of such multicellular patterns poses quite a few challenges. This is because after patterning an initial cell type on a substrate, to naturally mimic the *in vivo* conditions, a second cell pattern must be created without utilizing any exogenous cues often employed in most of the patterning techniques that have been utilized until date, hence the natural phenomenon of cell adhesion still remains unclear. A study by our group was successful in developing a model to coculture cells using lithography technique without the use of any exogenous cues.[15] Our finding showed how the cell−material interactions can be enhanced at the edges of the patterns by fabricating micropatterns of micrometer depths, which force the cells to migrate and align along the edges, thus creating voids that can be utilized for culture of another cell type. However, it was difficult for us to retrieve the two phenotypes of cells precisely from the center and edges of these patterns. To overcome this challenge, we can utilize the advantage of stimuli -responsive polymer coatings (temperature sensitive) in the future, which can change from adhesive to nonadhesive substrates in response to the stimuli. This will allow the growth of cells in the pattern followed by simple peeling-off upon exposure to simple cyto-compatible stimuli such as temperature changes.[91] To seek application in tissue engineering, our group extended these studies and developed a micropatterned wound dressing to show the effect of these microfeatures in wound healing applications. The developed microfeatures not only enhanced the adhesion and cellular migration of the cells involved in the process of healing but also can be loaded with growth factors or drugs, which can further enhance the process of healing.[92] Further, the 3D printing techniques for generation of these coculture models also have limitations because most printing techniques are

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sluggish, which restricts scale-up, and there are few material options that can be printed into complex structures with good resolution and fidelity. This drawback highlights the need for a more concentrated study on the synthesis of new materials or the modification of current materials for improved printability and resolution. Overall, micropatterned cocultures have many benefits for use in regenerative medicine. These coculture techniques are expected to open new opportunities for creating artificial tissues.

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Notes

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