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Engineering Liver

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Abstract

Interest in “engineering liver” arises from multiple communities: therapeutic replacement; mechanistic models of human processes; and drug safety and efficacy studies. An explosion of micro- and nano-fabrication, biomaterials, microfluidic, and other technologies potentially afford unprecedented opportunity to create microphysiological models of human liver, but engineering design principles for how to deploy these tools effectively towards specific applications, including how to define the essential constraints of any given application (including available sources of cells, acceptable cost, and user-friendliness) are still emerging. Arguably less appreciated is the parallel growth in computational systems biology approaches towards these same problems – particularly, in parsing complex disease processes from clinical material, building models of response networks, and in how to interpret the growing compendium of data on drug efficacy and toxicology in patient populations. Here, we provide insight into how the complementary paths of “engineering liver” – experimental and computational – are beginning to interplay towards greater illumination of human disease states and technologies for drug development.

Therapeutic tissue engineering

The field of therapeutic tissue engineering took off about 25 years ago, with early demonstrations showing how combining donor cells with synthetic degradable polymer scaffolds could lead to regeneration of tissue, such as cartilage in the shape of a human ear (1). This sparked excitement that similar approaches could be applied to either transplant hepatocytes on scaffolds or to build livers ex vivo for transplantation. In the ensuing two decades, lab-grown bladders and tracheas have made it to the clinic – why not liver? Intuitively, both the degree of structural complexity, with finely interwoven vascular, biliary, and lymph networks, as well as its sheer size and vascularity make ex vivo liver engineering vastly more challenging. Scaffolds for growing trachea and bladder can be made as simple stacked layers and nurtured in bioreactors that flow fluid over the tissue. In contrast, in addition to the intricate structural complexity, the functions of liver depend on intimate close contact of hepatocytes with local flow of blood, hence scaffold complexity

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and local tissue microperfusion are intimately intertwined in creating and maintaining functional “liver”. Presuming the availability of the relevant constituent source cells, “engineering liver” thus requires not only more sophisticated tools – biomaterials, methods of scaffold fabrication, bioreactors – but also more sophisticated quantitative design principles for how to use the tools to drive creation of tissue (2).

While it is debatable whether building lab-grown livers for transplant remains a realistic goal, the vision of doing so helped drive development of myriad biomaterials and microfabrication tools. We first take stock of the state of the art solid free-form fabrication (SFF) technologies for building 3D scaffolds (1,3) (see Supplemental Material for details of methods and caveats) and then discuss biomaterials and bioreactors. All SFF methods build complex 3D objects as a series of thin (10-200 um) layers, guided by computer programs that direct creation of complex features within each layer (4-6). Most methods make scaffolds or sacrificial molds, though they can also be used to manipulate the cells themselves, maintaining cell viability with varying degrees of success(6). Examples of these processes include: 3D Printing, which involves depositing material from a nozzle into the “build” layer, where the layer may contain either a fine powder that is gelled or bonded by a printed liquid or may contain a support material to catch cells printed directly from the nozzle; stereolithography, a layer-by-layer process in which (UV) light is used to polymerize macromers by illuminating only selected regions of a thin layer of precursor solution (which may contain cells); and variations combinations of these (5-7). Although SFF methods are permeating many consumer product domains from fashion to guns, tissue engineering applications remain highly demanding due to the desire for control over both very small length scales (~10 um) and larger scales (100-1000 um) in the same object, the inverse relationship between how long it takes to build an object and the fineness of the length scale, and the sensitivity of cells to polymerization processes and movement through nozzles. For example, while direct printing of cells is conceptually attractive, and an approach based on printing dense liver cell suspensions supported by inert agarose co-printed as a physical support to guide formation of 3D tissues results in formation of viable tissue structures, the structures are relatively large (300 um) (8). Methods to develop finer structures by (for example) perfusion in bioreactors are still evolving, as the challenges in directing morphogenesis of fine features along with providing appropriate distribution of oxygen and signaling molecules are not trivial (2). A path to accomplishing the fine scale has recently been applied to creating microscale perfused vascular liver structures by extrusion of arrays of molten sugar cylinders, which can subsequently be infused with gel suspensions containing cells, dissolving the sugar to yield perfusable channels that can be lined with endothelial cells and has shown promise for creating vascularized structures in vitro that can be transplanted in vivo (9).

Arguably, the vision that spurred development of new tissue engineering technologies – creation of transplantable livers - is being eclipsed by the goal of deploying tissue engineering to understand, prevent, and cure liver injury and disease. Many confluent forces have pushed the field incrementally in this direction. On the therapeutic front, steady progress is being made on understanding and treating liver diseases that ultimately cause liver failure, and in stabilizing patients with late-stage disease. Although extracorporeal support devices have not yet shown a strong benefit in improving patient survival (10),

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hepatocyte transplantation to treat cirrhosis and other diseases is moving closer to clinical reality (11). At least one human clinical trial using donor-derived hepatocytes showing that the procedure is not only safe but can have a beneficial effect on cirrhotic patients (12). A side consequence of therapeutic cell transplantation is development of protocols for creating mice with humanized livers, which despite certain limitations as a model for liver pathophysiology and drug development broadly, are gaining traction for study of infectious disease(13). At the same time that new drugs and drug regimens for managing progression of hepatitis may chip away at some of the clinical need, the tremendous progress in understanding fibrotic processes in general, and liver in particular, stokes optimism that together with earlier diagnosis, new therapies for fibrotic diseases that cause so much clinical devastation may truly be in sight (14,15). The major push, though, is the increased demand for human in vitro models in all phases of the drug discovery and development process (1).

In Vitro Models

The mouse is increasingly under attack for failing as an adequate model of human physiology and disease, let alone for assessing drug metabolism and toxicity, responses to chemotherapy agents and biotherapeutics – not to mention for being an expensive, high maintenance tool for research and discovery. Enter “tissue engineered” human in vitro models – which are still quite nascent and limited for widespread use, but which have promising futures. Realization of complex models is enabled in part by increasingly sophisticated experimental tools for constructing controlled cell and tissue micro-environments, but also a growing appreciation for the quantitative rules that govern how cells (and tissues) integrate chemical and mechanical cues across many length and time scales to generate a basal phenotype and to respond to microenvironmental perturbations (2,16). Indeed, relatively straightforward experimental tools can be transformed by quantitative analysis into in vitro models that are high impact because they are both accessible to a wide community (i.e., biology labs) and they conceptually capture a complex in vivo situation. An elegant example is the study of fibrosis, where a popular, accessible “tissue engineering tool” – 2D polyacrylamide gel substrates that can be tuned to specific bulk stiffness and modified with various ECM molecules – is being deployed to illuminate how matrix stiffness interplays with chemical cues to drive fibrotic phenotype of stellate cells as illustrated in Figure 1 (17). Matrix stiffness, together with biophysical presentation mode of epidermal growth factor (EGF) in concert with integrin ligands also influences morphogenesis, proliferation, and differentiation of primary hepatocytes in quasi 2-D culture (18). Moving into more physiological 3D environments requires attention to multiple variables at once: degradability, stiffness (at bulk and molecular scales), permeability, and bioactive ligands. Synthetic and semi-synthetic gels for controlling such variables are slowly moving from specialty labs that develop them into use in liver tissue engineering through iterative processes of design, phenomenological impact on phenotype, and translation to commercial availability (see Supplemental Material). An example of this process is the recent demonstration, using commercially-available hyaluronan gels, that 3D matrix stiffness influences differentiation of hepatic progenitors (19). A host of molecular-to-macroscale design principles are emerging from the biomaterials community to further

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improve how materials function for controlling dynamic processes such as migration and morphogenesis. For example, nanoscale clustering of ligands, compared to random presentation, can dramatically influence cell migration speed (20), the stiffness of tethers between adjacent FNIII 9-10 domains can influence integrin specificity and epithelial differentiation (21), localized gel degradation and ensuing enhanced traction can change differentiation fate of stem cells independent of matrix stiffness (16), and gradients in matrix stiffness can direct capillary morphogenesis in vitro as illustrated in Figure 1 (22). Further, ECM also acts as a depot for autocrine ligands in a sort of “cell sonar” fashion, a property that is now being productively exploited by incorporation of specific growth factor binding domains to control localized concentrations of factors (23). These design principles are being incorporated together into synthetic and semisynthetic gels in systematic fashion to allow translation to the general biology community (24,25). Isolated liver cells can also use geometric cues from macroporous 3D scaffolds to self-organize into 3D tissue-like structures, guided by the balance of cell-cell and cell-substrate adhesion (26). A more detailed summary of how these approaches are being applied to liver cell and tissue culture is provided in the online Supplemental Material Section B and in other recent reviews (4,27).

How close are we to a true 3D sinusoid model of fibrosis? If we presume that the constituent cells are available and simply need to be assembled properly, a combination of biomaterials and microreactor technologies are converging to make this at least theoretically possible, though making all the parts work together quantitatively – and in an accessible format – is an evolutionary process. Arguably, a starting point is the work with 100-250 um thick liver slices, which contain all the constituent non-parenchymal cells in a relatively quiescent state and can recapitulate stellate cell activation and other early inflammatory responses to pro-fibrotic stimuli (28-30) as well as complex mechanisms of metabolism and toxicological responses to drugs (31). Although slice cultures likely have enduring utility due to their high functionality and the reproducibility of creating large numbers of slices from scarce donor material, their survival is typically limited to a few days, (presumably due to limitations in oxygen and regulatory molecule diffusion) even when placed in an environment with controlled perfusion around the slice and further physically stabilized in the flow by addition of matrigel, which may also enhance the phenotypic stability of cells at the slice surface (32). It is possible that the missing essential ingredient is local sinusoidal microperfusion through the slice, which serves not only to distribute oxygen and nutrients but also provides essential mechanical stimuli to tissues in part through interstitial flow from capillaries to lymph(2). It is interesting to speculate that, if placed in a flow upstream of a suitable biomaterial gel, microvessels in slices might shift to align with microperfusion and demonstrate tissue outgrowth, though matching the time scales for tissue survival with those for such tissue remodeling, may be a barrier. Hence, approaches based on tissue assembly from isolated cells together with microfluidics are a more commonly-pursued approach to make tissue structures. Classic microfluidics approaches employ simple molding methods to create intricate designs of interconnected channels and chambers in silicone rubber (PDMS) slabs bonded to glass, with feature sizes ranging 1 um - 10 mm, allowing precise control of fluid flow and associated molecular transport between adjacent fluid streams and compartments (33) (see Supplement Section C). A microfluidic device that creates sinusoid-

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size (55 × 80 × 3000 um) quasi-3D cords of hepatocytes separated from a fluidic channel by a porous PDMS “sinusoid membrane”, enabling maintenance of hepatocellular functions (34) (Figure 2) has been adapted to a multi-well format commercially, with a caveat that PDMS is highly absorptive to many lipophilic drugs and steroid hormones. A spectrum of related designs that organize hepatocytes into cord-like structures surrounded by microchannels that control molecular and fluid transport and maintain tissue-like structure/function have been described (33,35) (Figure 2). These hepatocentric models were geared toward applications in drug development – metabolism, toxicity, and possibly bile transport or small molecule drugs (“ADME-tox”) – though are now being deployed to create organoid-like hepatocyte-NPC structures (36). Further, we are still learning about how subtle changes in parameters like interstitial-level fluid flows influence the biology of endothelium and the underlying tissues, hence comparison of the desired phenotype against in vivo counterparts in an iterative fashion is crucial (2,37).

As with microfabrication methods, the goal of building therapeutic technologies has driven innovation in design of bioreactors for maintaining highly metabolically-active liver tissue, for example in scaling down clinical-size hollow fiber reactors to miniature versions, though these remain complex for general use (38) (Figure 2). The limitations of PDMS and microfluidics has also led to development of other user-friendly microscale liver reactors that foster long term viable co-cultures of hepatic tissue-like structures, including liver sinusoidal endothelial cells in the absence of VEGF, and that are made from material with less propensity to adsorb hydrophobic molecules than PDMS and with an open platform to facilitate cell seeding and to accommodate larger tissue structures (39) (for a comprehensive review of liver bioreactors, see (40)). This increased tissue mass and depth allows studies to extend beyond liver-only pathologies, for example, study of micrometastasis homeostasis in human tissue (41). Another example of a tissue engineered disease model is the use of 2D micropatterned hepatocyte-fibroblast cultures for analysis of HCV life cycle(42), where fibroblasts stabilize liver function. This format is commercially available though has not yet been widely adapted for in vitro analysis of HCV, perhaps due to the complexity of questions regarding immune function contributions and other factors that require idiosyncratic development of in vitro models of HCV to address specific questions including the role of cell polarization(43,44). Thus, the goals of “engineering liver” have moved beyond even the secondary development of ex vivo relevant models of liver function, to aspirations of understanding of systemic diseases.

Assemble it or let it develop?

Most tissue engineering approaches involve reconstructing tissues using dissociated cells. More recently, organogenesis from stem cells is emerging as a viable alternative route (45). A dramatic example is generation of human intestinal organoids, from iPS cells, comprising all the intestinal epithelial cells types plus intestinal mesenchyme and smooth muscle, villus-like structures with CDX2 expression, secretory and excretion function (46). These organoids exhibit substantial function without vascular perfusion, but liver is likely more intimately co-dependent on vascular development and perfusion as embryonic development of the liver bud requires signals from endothelial cells (47), and efficient protocols for in vitro differentiation of ES cells to hepatocytes result in co-appearance of endothelial cells

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(48). Encouragingly, spontaneous formation of vascularized “liver bud” structures have been observed when human iPS-derived hepatic-specified cells (immature endomermal cells destined to track to the hepatic cell fate) are combined in vitro with human umbilical vein endothelial cells (HUVEC), and these structures form patent vascularized vessels when transplanted in vivo, improving liver-specific differentiation (49). Combinations of microscale perfusion technologies and biomaterials may help drive morphogenesis into a functional perfused microtissue in vitro, if the morphogenesis cues can be quantitatively presented (45,50). Nascent efforts to capture similar interactions in terms of computational modeling morphogenesis of endothelial-cell dependent developmental differentiation is being applied to pancreas (51) and other stem cell differentiation processes (45). A tremendous challenge in computational modeling of developmental morphogenesis is capturing the interplay between processes wherein co-dependent intracellular and extracellular chemical and mechanical cues are integrated in dynamic fashion, and understanding how faithfully these models recapitulate the process requires experimental validation. While these systems (liver and pancreas) offer substantial experimental challenges at the present time, dramatic examples of how mathematical models can capture complex developmental processes have recently been described in more tractable experimental systems, such as the development of mammalian teeth (52) and whole organismal differentiation of drosophila (53). The merging of quantitative insights from both traditional tissue engineering approaches and developmental biology is just starting and will likely foster substantial evolution in the design of experimental tools.

What to model? Tissue engineering meets systems biology

The methods for making tissue ever more complex are evolving in tandem with approaches for deconstructing complex problems and making them ever more tractable experimentally. Engineering almost always invokes images of “building” (or designing and building), but engineering is equally about framing problems in ways that allow them to be solved with the minimal possible experimentation, by using mathematical analysis to understand complex systems.

One of the biggest demands for in vitro liver models is in assessing drug-induced liver injury (DILI) early in the development process before proceeding to clinical trials. A variety of new computational models built in part on mining clinical data and in part on designing mechanistic experiments based on patient data are continually shaping the landscape of what is desirable experimentally (54,55). For example, one of the most difficult types of DILI to predict is idiosyncratic toxicity, which appears late in clinical trials. Among the mechanisms that might give rise to such adverse events in humans include rare alleles for metabolic or transport enzymes, hypersensitive immune network responses, and interaction of drug metabolism with mild liver inflammation that arises from transient increases in gut permeability and specific microbiome products. While the liver slice models described above capture facets of these NPC-mediated events that are not represented in standard hepatocyte cultures (31), computational modeling of inflammation provides insights into experimental conditions that can drive hepatocentric experimental models into inflammatory states, offering additional high-throughput approaches to screening (56). The in vivo situation is complex especially when including the invasion of immune cells, a high-

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throughput 96-well plate assay based on dosing primary hepatocytes with combinations of cytokines and drugs captured a significant fraction of idiosyncratic toxins from a list of 90 drugs with known clinical outcomes; moreover, consensus signaling pathways were identified in toxic outcomes by computational analysis of time-dependent activity of multiple kinase signaling nodes (56), and the approach was extended into a 3D co-culture model as a step toward incorporating greater complexity of the immune response(57). Immune system signatures have also been revealed in genome-wide association studies of idiosyncratic toxicity of several drugs (58), suggesting mechanisms that would like be difficult to detect with any current in vitro assay. A powerful new approach for analyzing post-market surveillance data to account for sparse information and co-variation of factors in large clinical trials that permits the identification of drug targets, prediction of drug indications, and discovery of drug-class interactions may allow further inroads in understanding DILI (59).

Finally, multiscale systems biology approaches applied in an iterative fashion to patient samples, animal models, and in vitro data, are making inroads on the complex immune networks that underlie chronic inflammatory diseases and pointing the way to therapeutic intervention at non-intuitive targets. A recent example is the first illustration of how quantitative analysis of extracellular cytokine networks, intracellular signaling networks, and temporal evolution in cell phenotypic behaviors (apoptosis, proliferation, immune cell invasion, cascades of cytokine secretion by immune and epithelial cells) sensitizes the intestinal epithelium to TNF-α-driven apoptosis in vivo (60). While it is unclear precisely where the inferences from such multiscale analysis of in vivo data will drive the development of human microphysiological systems, it underscores the necessity of including trafficking of various circulating cells into and out of the tissue analogues – as well as cross talk with other organ systems through cytokines and other types of molecular signals. Many of the drugs that might intervene in this cascade are biologics – underscoring that many of the emerging challenges in both efficacy and toxicology center on predicting behavior of biologics, particularly how they affect the non-target cells in both direct and indirect (paracrine-mediated) manner.

Future Challenges and Opportunities

Three common threads running through the technical analysis above point to barriers in “engineering liver” to solve the problems outlined in the introduction: (i) there are many diverse applications with different technical needs, creating a fragmented set of approaches with only partial overlap in efforts toward common problems in the field (ii) any complex model requires a team with substantial breadth of scientific and technical expertise (and related financial resources) to design, build and test the model and (iii) bridging the gap between proof-of-principle that a model or therapy works in a research setting and that it works in the ultimate user community usually requires substantial commercial investment to “productize” and disseminate the approach, and is usually not until the model is test run in multiple different environments that the true utility and economic sustainability is known, and in the process, the model made be made partially redundant or obsolete by other simpler, more cost-effective approaches. Extracorporeal liver support bioreactors are an example of well-engineered technology that has not shown a strong, clear-cut advantage

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over less costly alternatives. The application landscape is still sorting out for several microfabricated and microfluidic-type liver culture devices that are commercially available or are in development, including the Hμrel chip (monolayer micropattered co-culture), HepatoPac (monolayer micropatterned co-cultures), In Sphero (hanging drop spheroid culture), Regenemed (3D scaffold-supported culture), CN Bio Innovations (3D microscale reactor), the Pearl system by CellAsics/Millipore (device for microfluidic culture in a sinusoid configuration), and OrgoNova scaffold-free tissue. Although many of the envisioned applications of these technologies are in pre-clinical drug development for ADME/Tox, the advantages they offer are still sorting out (see recent reviews (27,40)); for example, although the extended functional longevity of engineered systems appears conceptually attractive as a way to measure clearance of drugs that are metabolized very slowly, clearance of a substantial fraction of such low clearance compounds can be captured using sequential incubations with more traditional hepatocyte preparations (61). A clear challenge shared by all engineered systems is the availability of various liver cells as "reagents" that are broadly accessible (commercially or otherwise), reproducible, and cost-effective to deploy. All cell lines, while useful for certain targeted purposes, have some drawbacks in representing liver physiology broadly (powers), and of the many cell types in liver, only hepatocytes and Kupffer cells are routinely commercially available in cryopreserved form; many cells, particularly the sinusoidal endothelial cells, are relatively fragile and resistant to cryopreservation approaches. The tremendous progress in deriving hepatocyte-like cells from stem cells appears on the verge of yielding robust supplies, but if the argument that engineered systems are tailored for illuminating more complex physiologies, particularly those involving immune function or higher-order interactions among hepatocytes and NPC, then reliable sources of NPC and immune cells are also needed.

It is interesting to speculate that the organogenesis approaches may ultimately yield at least partial breakthroughs – indeed, co-evolution of hepatocytes and NPC in an organoid format may foster more robust maturation of hepatocyte-like cells, and at the same time provide a source of at least some NPC; though the origins of some NPC are debated (62). It is also interesting to further speculate that engineered devices and biomaterials – together with quantitative "design principles" – may boost the prospects of success in organoid development, by providing control of external cues, particularly mechanical forces including flow.

Finally, regardless of the evolution in sophistication of experimental systems, continued progress is needed on the "reverse engineering" of complex cell-cell communication and signaling networks in patient samples; i.e., to build quantitative models of disease pathogenesis so that the best targets in complex networks can be identified.

In summary, the combined computational and experimental approaches to "engineering liver" will surely continue to yield rich new insights, at least incrementally. There is great potential for synergy between these that will accelerate the pace, and we look forward optimistically to the continued blending of these two ends of the "liver engineering" spectrum.

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Supplementary Material

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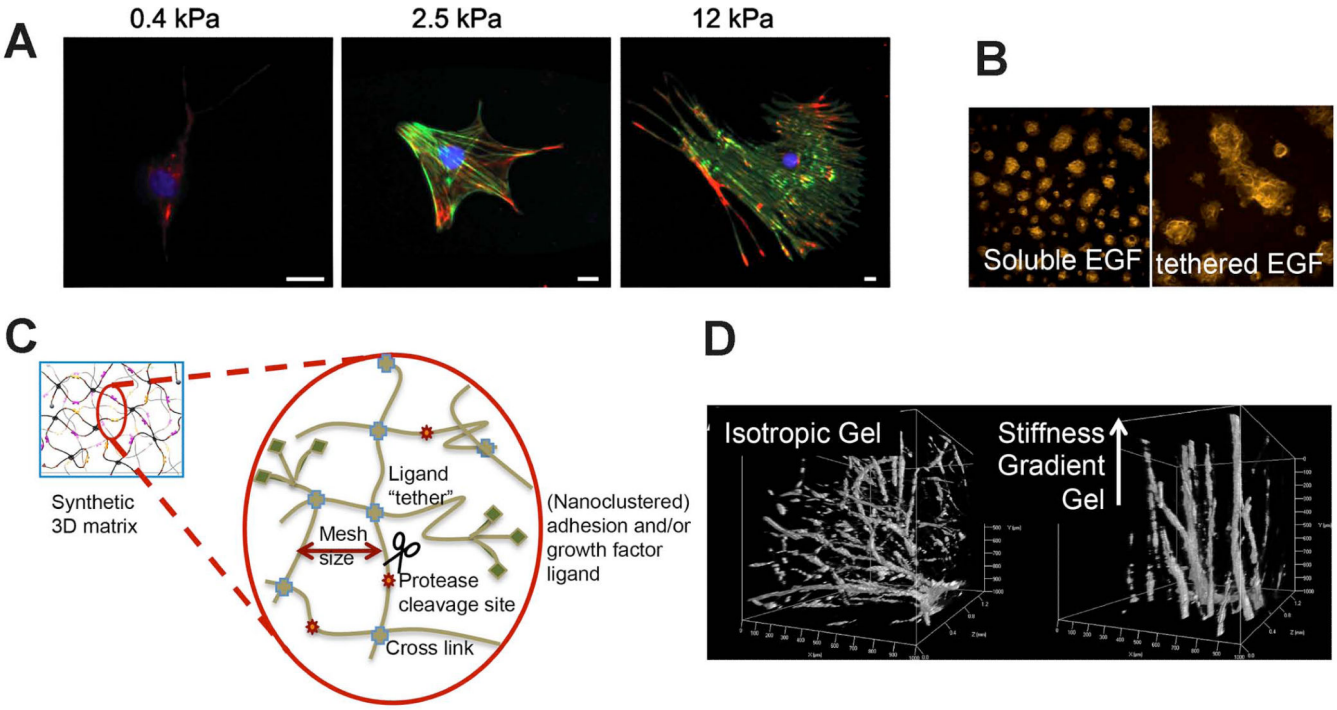

Figure 1.

Designer Synthetic Extracellular Matrix (ECM) Materials Allow Control of Matrix Stiffness, Permeability, and Selective Adhesion and Growth Factor Interactions. (A) ECM-modified polyacrylamide gels tuned to soft, moderately stiff, and stiff bulk elastic moduli reveal the role of ECM mechanical properties on activation of hepatic stellate cells [from (22)]; (B) Matrix-tethered EGF, compared to soluble EGF, dramatically alters phenotypic responses of hepatocytes on self-assembling peptide hydrogels with tuned bulk elastic moduli [from (23)]; (C) Schematic illustrating modular design of synthetic 3D hydrogels for cell encapsulation or invasion. Gels comprise a structural water-soluble polymer (such as polyethylene oxide, dextran, hyaluronic acid, etc) crosslinked by Michael addition, photo-polymerization, temperature- or ion-induced phase change, or enzymatically, and with modules including selective protease cleavage sites and tethered adhesion or growth factor ligands or motifs that bind to matrix or growth factors. The crosslink density and choice of polymer influence the permeability (characterized by a "mesh size" typically on the scale of nm) and the bulk mechanical properties, while local mechanical properties sensed by receptors are also influenced by the tether length, stiffness, and ligand orientation or clustering. (D) Example of directed angiogenesis by endothelial cells encapsulated in a synthetic RGD-modified PEG gel. Cells encapsulated in an isotropic gel exhibit isotropic orientation of capillary tubes, while cells encapsulated in a gel with a stiffness gradient show oriented tube formation. [from (27)].

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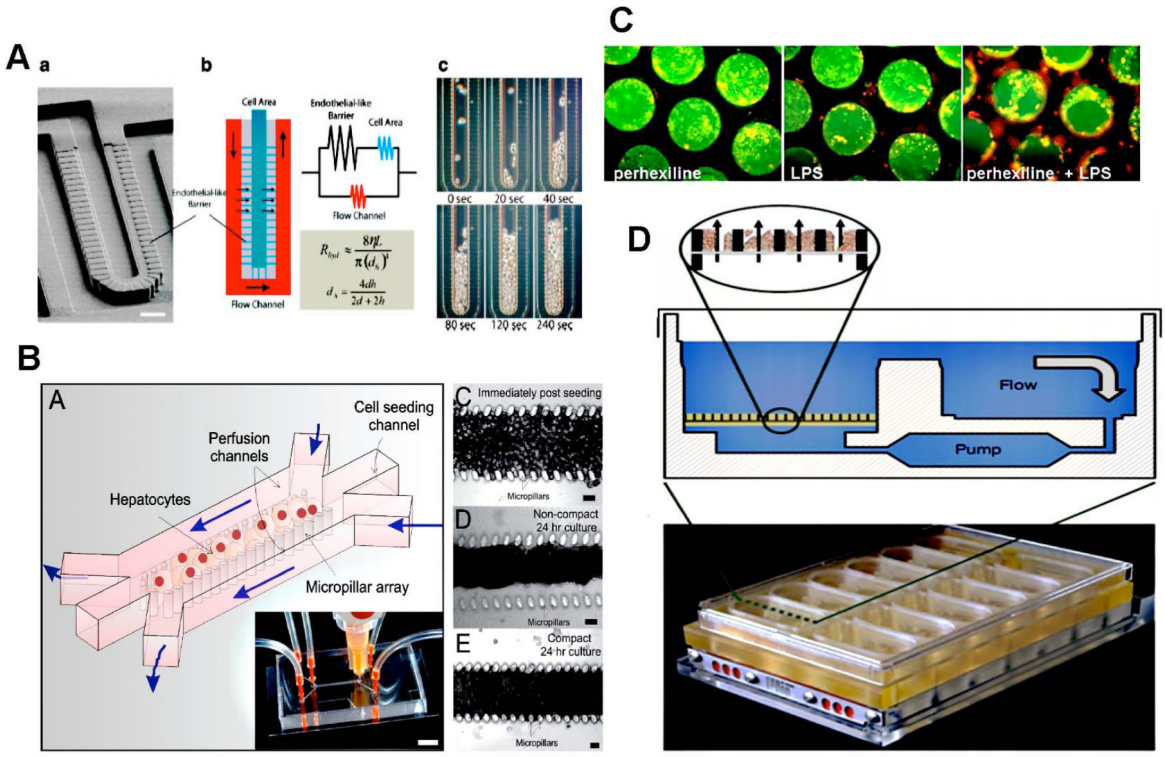

Figure 2.

Microscale bioreactors to control hepatic tissue organization and flow. (A) Microfluidic design to mimic flow and gradients along an hepatic sinusoid, with dual flow networks to enable easy loading with a precise number of hepatocytes [from (33)]. Shown is the research-scale precursor to the commercially available multi-reactor chip version. (B) Microfluidic reactor design for examining how mechanical compaction of cell aggregates during tissue formation influences tissue morphogenesis, including formation of biliary networks [from (35)] (C) Multi-well plate bioreactor system that fosters 3D tissue-like formation in an array of channels of a “chip” scaffold seeded with isolated liver cells, where microscale flow is maintained by a microfluidic pump; images at the top show individual 300 um-diameter channels containing tissues formed from co-cultures of hepatocytes with non-parenchymal cells, and treated with either drug alone (left), inflammatory cue alone (middle) or a combination, then stained with live (green) /dead (red) dyes, illustrating synergy between inflammation and drug metabolism in hepatotoxicity [from (52)].