Extracellular matrix bioengineering and systems biology approaches in liver disease

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Abstract The extracellular matrix (ECM) in the liver as well as in many organs comprises a peripheral network linking numerous macromolecules typically classified into collagens, microfibrillar proteins, proteoglycans, chemokines, growth factors and glycoproteins. In addition to its role as an essential structural and physiological component, it plays a vital role in driving key cellular events such as cell adhesion, migration, proliferation, differentiation and survival. Any structural inherited or acquired defect and/or metabolic or pathologic alteration in the hepatic ECM may cause cellular and organ responses leading to the development or progression of liver disease. Therefore, the ECM molecules are key players in tissue engraftment and in the pathophysiology of liver disease. In this review we provide a snapshot on current efforts for understanding its role in physiological and non-physiological states, by describing how tissue engineering platforms can enhance *in vitro* and *in vivo* models of liver disease, by providing examples where bioengineered ECM can serve as systems biology approaches to study the ECM, and then by evaluating pathological protein regulatory networks in the liver using systems biology tools. These approaches hold great promise for future research.

Keywords Collagen-I · Networks · Scaffolds

Abbreviation

ECM Extracellular matrix

The ECM

The view of ECM has evolved from a merely scaffolding and space filling tissue element to an interface actively controlling cellular activities and tissue function. The protein composition and dynamics of the ECM is of crucial importance for the healthy functioning and physiology of cells. The ECM quantity and quality influence cellular growth, differentiation, morphology, survival and mobility. In fact, ECM remodeling is mediated by a balanced complement of constructive and proteolytic enzymes together with their enhancers and inhibitors. ECM remodeling is an energy costing process regulated by hormones, cytokines, growth factors, free radicals, energy metabolism and mechanical forces. A highly specialized form of ECM is the basement membrane, an ubiquitous sheet-like polymeric structure composed of a set of distinct glycoproteins and proteoglycans.

Cell biologists and biomaterials scientists are searching for materials and methods that mimic the 3D-microenvironment of the hepatic ECM. Such technologies are greatly needed to study vital processes such as tumor development, hepatic metastasis and cell transplant for therapeutic use. Advances in tissue engineering have traditionally led to designing scaffold or matrix-based culture systems to better reflect the biological, physical and biochemical environment of the physiological hepatic ECM. Although their

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clinical applications in regenerative medicine tend to receive most of the attention, it is obvious that other areas of biomedical research could benefit from the powerful tools that have already been developed in tissue engineering.

Understanding physiological and pathological cell behavior within the liver requires systematically studying cell behavior in the context of the specific model ECM (Schmeichel and Bissell 2003; Griffith and Swartz 2006; Yamada and Cukierman 2007). These models will allow breaking down the physiological complexity, while at the same time mimicking some aspects of the *in vivo* micro-environment. For the last few decades, cell and ECM biologists, including our own laboratories, have utilized synthetic models to shed light on the role of ECM in controlling cell fate, signaling pathways and ultimately liver function. Well-established ECM models comprise purified or recombinant ECM components, such as collagens, which are coated on the surface of a tissue culture plate or membrane, or more complex, multi-component 3D-matrices derived from cells, tumors or tissues, such as MatrigelTM. These systems have been particularly useful in studying the activation of liver cells, for example hepatic stellate cells, which play a central role in collagen-I deposition, the key protein involved in the development of liver fibrosis, a common feature of chronic liver diseases.

More recently, fruitful bioengineering efforts have resulted in the development of completely artificial ECM with biochemical and biophysical properties that begin to resemble those of naturally derived ECM model systems, while overcoming some of their complexities associated with purification (and the resulting batch-to-batch variability), comparatively poor control over matrix properties and therefore a limited cell- and tissue-specificity (Lutolf and Hubbell 2005; Tibbitt and Anseth 2009; Lutolf 2009).

These efforts have been primarily driven by a need for scaffold materials for regenerative medicine, cell transplant and tissue engineering (Langer and Tirrell 2004). A key question to address is: Is regenerative medicine aiming to help natural healing processes to advance faster and better by transplanting cells or creating a microenvironment in which absent or damaged tissue that would not ordinarily have re-grown, could regenerate? An ongoing strategy under intense development includes using scaffold materials that emit biochemical or biophysical signals to spur cells into action and promote cell proliferation and survival. The structural features of tissue engineering scaffolds affect cell response and should be engineered to control cell fate; thus, supporting cell adhesion, proliferation, engraftment and differentiation. A challenge is that the scaffold should act as a temporary entity that cells interact with prior to forming their own tissue-specific ECM and new tissue. The test is in that good cell scaffolds,

in analogy with *in vivo* mammalian ECM, should recapitulate both natural biophysical, structural and biochemical features. On the one hand, they should be multi-component, fibrillar, microporous and ought to have viscoelastic properties matching those of the targeted tissue. On the other hand, how can they have biochemical functions, including cell signaling ability and susceptibility to proteolytic degradation, matching those of the ECM or basement membrane of the desired tissue? A few helpful concepts and approaches to address these questions and challenges are recapitulated below.

How can biomaterial model surfaces control cell fate *in vitro*?

Engineered biomaterials surfaces can serve as versatile model systems to deconstruct the complexity of natural cell-ECM interactions in the liver and in other organs. These models, although valid for understanding signaling mechanisms in the liver (Nieto 2006; Nieto et al. 1999, 2002), and in other organs, differ from the extracellular *milieu* of the body, where cells are connected with anchored or adhesion molecules, growth factors cytokines and receptors existing in close proximity to nearby cell surfaces and contained within a 3D-ECM that creates a soft and hydrated matrix. Nevertheless, important knowledge has been gained from studies that utilized such model substrates.

An area that has extensively built on 2D-biomaterials model systems is integrin-mediated cell adhesion, rather critical in invasion, metastasis and hepatic cell signaling (Tang et al. 2010). The ECM provides physically tethered multifunctional proteins, including fibronectin, vitronectin, osteopontin and laminin that bind to transmembrane cell receptors such as integrins to provide cell adhesion but which also generate active cell signaling cues. The activity of many of these ECM proteins can be recapitulated by small peptide sequences such as RGD since RGD residues in proteins bind integrins at the cell surface (Ruoslahti 1996). The well-controlled anchoring of these peptides to substrates (Hersel et al. 2003) has allowed probing, in a quantitative fashion, the role of ligand type, concentration and spatial distribution (e.g., such as in gradients) on cell fate (Palecek et al. 1997; Maheshwari et al. 2000). For these studies, the provision of a passive ('non-fouling') substrate onto which a ligand of interest can be grafted has been crucial; however, a challenge is always the steric hindrance that these molecules may pose for the binding to really occur.

The complexity in molecular signal composition (and dose) of the liver ECM, including crosslinked components such as collagens as well as soluble cues such as growth factors, cytokines and free radicals, necessitates experimental designs with higher throughput than in conventional

in vitro assays in which the role of one signal at a time can be tested. This is the ideal situation, although a true challenge. Researchers have therefore adapted microarray platforms from oligonucleotides and cDNAs to proteins in order to screen hundreds of putative cellular microenvironments (Flaim et al. 2005; Soen et al. 2006; LaBarge et al. 2009). Such microarrays consist of robotically spotted combinations of cell signaling proteins and mediators on cell-repellent substrates. Due to the restricted cell movement, changes in cell behavior can be readily attributed to a particular spotted ECM composition. Flaim and Bhatia et al., who pioneered this approach, fabricated an ECM microarray consisting of 32 combinations of collagens I, III and IV, fibronectin and laminin (Flaim et al. 2005). These microarrays would certainly be useful for connective tissue diseases in many organs as well as in the liver. By screening for liver-specific differentiation in embryonic stem cell colonies, they found a 140-fold difference between the least and the most efficient protein combinations.

Biomaterials engineering in combination with micro-patterning technologies facilitates the development of powerful tools to investigate the spatial role of the ECM organization on hepatic cell fate (Khademhosseini et al. 2006; Falconnet et al. 2006). For example, micro-contact printing, now a classical surface engineering technique (Kane et al. 1999), can be utilized to restrict cell adhesion to geometrically controlled cell-adhesive ‘islands’. Fukuda et al. (2006) have developed a microarray substrate with collagen-modified cavities. Primary hepatocytes spontaneously formed spheroids with a uniform diameter at the center of each cavity on the chip. The spheroids had a controlled cuboidal cell shape, similar to hepatocytes in vivo and stably maintained the liver-specific phenotype, such as liver-enriched transcription factors, albumin secretion, urea cycle enzymes and intercellular adhesion molecules (Fukuda et al. 2006). This strategy holds significant promise for the future.

Topographically, physiologically and biochemically patterned cell culture substrates can also be fabricated and used to study liver cells or any other cell type in the body at the single cell level or to induce the assembly of microscale ‘tissue’ models (Charnley et al. 2009). Single cell resolution may be particularly relevant for studies on cell populations that are highly heterogeneous such as primary liver cells or stem/progenitor cells. That is, conventional in vitro experiments are performed on cell ensembles and therefore miss the behavior of individual cell clones. This setback can be solved by the introduction of microwell arrays that permit the analysis of a large number of single, spatially confined cells. Microwell arrays are topographically structured surfaces with hundreds or thousands of miniature cavities, arrayed into a regular grid. Cells are trapped by gravitational sedimentation and hence the number of

cells per microwell can be controlled by a careful cell seeding density and the microwell diameter. Due to the stochastic capturing process, the number of single cells per trap follows a Poisson distribution. In addition, the same platforms can be used to form arrays of homogeneous clusters or aggregates of cells, as it occurs in the liver parenchyma, with potential applications in many liver diseases as well as in multiple biomedical areas not solely confined to the liver. In fact, Gallego-Pérez et al. (2010) tested such a platform for the culture and co-culture of human hepatocytes, distributed as controlled clusters, with other cell types.

An increasing number of studies highlight a role of biophysical ECM factors in regulating cell fate (Discher et al. 2005, 2009). However, this aspect is not trivial to investigate due to the interconnectivity of biochemical and biophysical cell fate determinants in the common ECM model systems. For example, modifying the biopolymer architecture (e.g., collagen gel) to change matrix stiffness alters the adhesion ligand density, the mode of presentation and the matrix porosity and thus could affect also the behavior of liver cells. To parse out these variables, ‘tunable’ synthetic biomaterial substrates in which matrix stiffness and ligand density can be independently controlled have emerged (Lutolf et al. 2009), as described below, and should also be of interest to study the role of ECM compliance in liver biology and in diseased states such as in hepatic fibrogenesis, where significant ECM deposition occurs.

Taken together, deconstructing complex 3D-microenvironments into 2D-model systems is a powerful strategy for identifying new regulatory mechanisms governing cell biology.

Can we move towards higher complexity by designing materials to regulate 3D-cell fate in vitro?

In order to elucidate in a near-physiological context the intricate molecular cell-ECM crosstalk that regulates liver tissue development, function, restoration and pathology, realistic in vitro 3D-ECM models are really needed. These challenging models could bypass the current gap between 2D-cell culture and animal models of disease, and particularly of liver disease (Schmeichel and Bissell 2003; Griffith and Swartz 2006; Yamada and Cukierman 2007).

The rapid increase in the molecular understanding of the regulatory role of the 3D-cell-ECM crosstalk in recent years has opened the door for the design of novel families of biomaterials, which allow recapitulating key functions of natural ECM, and thus permit manipulating the cell behavior in 3D and in a well-controllable manner (Lutolf and Hubbell 2005; Lutolf 2009). Owing to their structural

and physicochemical similarities to natural ECM, synthetic hydrogels have shown to be particularly promising candidates as scaffolds for cell biology and tissue engineering (Tibbitt and Anseth 2009).

A first important criterion for the design and selection of synthetic hydrogels engineered to perform in biological contexts is a suitable chemical strategy to enable gel cross-linking as well as gel modification with biomolecules under physiological conditions, such as to enable gelation in the presence of biomolecules, messengers, free radicals or specific liver cells. Great progress has been made in the development of such physiological crosslinking reactions (Hennink and van Nostrum 2002; Kopecek and Yang 2007).

An additional considerable challenge in creating functional artificial ECM has been the design of strategies to render synthetic matrices permissive for cell spreading, migration, proliferation, signaling and ultimately morphogenetic multicellular ‘processes’ as it occurs in the liver (Lutolf 2009). In 3D, the extracellular microenvironment may pose a physical barrier to the growth and motility of entrapped cells (Rowe and Weiss 2009). In contrast to biopolymer hydrogels such as collagen or MatrigelTM that have porosities approximately of micrometers, most chemically crosslinked hydrogels are essentially non-porous. They rather have molecular ‘mesh’ sizes of tens of nanometers. Cells in these networks remain trapped for prolonged periods. In most cases, long-term cell entrapment in the absence of cell movement may impair multicellular organization and can lead to cell death or apoptosis. However, hydrogel engineering strategies have been devised to allow cells to overcome their biophysical entrapment. For example, artificial ECM has been designed that either contain microscopic pores that facilitate 3D-cell motility or that are locally degradable by cell-secreted proteases, which are abundant in the liver. The latter strategy allows bi-directional cell-ECM communication, hence permitting cell-autonomous processes to occur within a synthetic hydrogel matrix.

As an example, the Hubbell group has designed hybrid polyethylene glycol-based hydrogels formed via a mild chemical reaction to include integrin-binding ligands as pendant gel moieties, in combination with matrix metalloprotease-sensitive oligopeptides in the gel backbone (Lutolf et al. 2003; Raeber et al. 2005, 2007). These gels not only degrade macroscopically when exposed to soluble proteases, but also locally by proteases present on or near the surface of many migrating cells in tissues, a factor rather relevant for studying liver disease and perhaps metastasis.

In native 3D-ECM, single cells could also migrate via pre-existing matrix pores or ‘defects’ (Friedl 2004), in addition to mechanically deforming the adjacent *milieu* as

it occurs in a highly metastatic environment such as in hepatocellular carcinoma. This feature of matrix biology has also been effectively targeted using artificial ECM engineering because it has been feasible to introduce microscopic porosity in these networks, for instance via selective dissolution (‘leaching’) of a second interconnected phase in the material. Alternatively, artificial ECM has been designed with micro- and nanofibrillar architecture to contain pore sizes that allow cells to migrate inside them (Cui et al. 2010). They have been rendered responsive to the action of proteases and have also been successfully decorated with biologically active mediators to promote cell migration or initiate cells to acquire specific phenotypes both in the liver and in other organs.

Apart from the biophysical and biochemical matrix characteristics, the spatial and temporal presentation of cell regulatory molecules is also an important aspect that drives the liver dynamics. Progress is being made in patterning biomolecules or cells in 3D-matrices. For example, the Bhatia’s group performed 3D-patterning of mammalian cells within hydrogels using dielectrophoretic forces (Albrecht et al. 2006). Other efforts have employed microfluidic and biomaterials technology to fabricate 3D-cell co-culture systems. For example, Sudo et al. have cultured primary liver and vascular endothelial cells on each side-wall of a collagen gel between two microfluidic channels (Sudo et al. 2009). Morphogenesis of 3D-hepatic cultures was found to depend on fluid flow across the nascent tissues. Vascular cells formed 3D-capillary-like structures that extended across the gel to the hepatocyte tissue-like structures. Microfluidic technology can also be explored to create precise biomolecule gradients which play an important role in many dynamic tissue processes (Cosson et al. 2009). The advantages of combining biomaterials engineering with microfluidics for cell applications offers the potential for arrays of individually addressable cell-culture chambers in which artificial microenvironments are exposed to spatially and temporally controlled biomolecule gradients. This strategy holds great possibilities for the future.

Bioengineering ECM as systems biology approaches to study the ECM

Systems biology refers to how collective properties of components generate higher-level behaviors. A central theme of systems biology is the use of computational modeling to reconstruct complex systems from a wealth of reductionist molecular data such as gene, mRNA and/or protein expression, signal transduction pathways and metabolic activity. A number of deterministic, probabilistic and statistical learning models are typically used to

understand sophisticated cellular behaviors such as protein expression during cell differentiation and the activity of signaling networks. In addition, multiway modeling techniques (also known as tensor models) can analyze multi-modal data, which capture more information and provide a broader vision of complex behaviors. These methods of analysis are applicable to a wide-range of cellular and tissue models, and are obviously not restricted to the hepatic context, although the main focus of this review is liver injury (Yener et al. 2008).

Many bioengineering ECM strategies discussed above can also be considered as systems biology approaches to study the ECM. In this section, we compile a few recent examples where both strategies blend, followed by a more detailed example in the next section where multiple disciplines can converge to address a clinical condition (liver fibrosis) affecting many patients with chronic liver disease.

Based on major progress in biotechnology, regenerative medicine is now evolving as a new discipline that could significantly change current therapeutic approaches. Indeed, stem cell biology, tissue bioengineering, nanotechnology, genomics, materials engineering, as well as systems biology and process engineering interrelate hoping to answer key aspects of disease. Thus, the long-term goal is to create a profound knowledge of individualized therapy originating from non-biological organ replacement, through hybridized systems, to fully biological implants and finally to human regenerative systems *in vivo*. Models such as liver tissue are new *in vivo* and *in vitro* regeneration technologies. To this effect, the ECM regulates liver differentiation including the biochemical composition of the matrix constituents, the 3D-organization or architecture and via mechanical forces directed towards the cells by the matrix. The *in vivo* ECM constitutes a biopolymer, which potentially plays a permissive role for liver differentiation (Bader 2002).

Since the liver is the major site for xenobiotic metabolism and detoxification, primary culture of viable and actively metabolic hepatocytes is essential for the development of new drugs; however, their utility is delayed by the rapid loss of their phenotype. Rowe et al. (2010) demonstrated that hepatocytes cultured in a sandwich of ECM maintain better function compared with cells cultured as a monolayer. This same group performed a wide-range proteomics study under these culture conditions using iTRAQ proteomics and systems biology network analysis of the identified and significantly regulated proteins. Both culture systems displayed altered expression of many common proteins and network analysis showed that the primary functions of these proteins were in metabolic pathways, immune responses and cytoskeleton remodeling. Monolayer cultures uniquely regulated proteins mapping to pathways of oxidative stress and cell migration, whereas

sandwich culture affected translation regulation and apoptosis pathways. These experiments provide a detailed proteomics data set to direct further work into maintaining hepatic phenotype using cultured primary hepatocytes and stem cell derived hepatocyte-like cells.

Improvement of O₂ supply in bioartificial liver devices remains a critical challenge in maintaining hepatocyte viability and function (Lee et al. 2006). Recent studies have aimed at investigating whether enhanced O₂ transport through collagen ECM can produce a more stable antioxidant defense under different O₂ tensions during prolonged incubation times. The results suggest that the O₂ enhanced collagen ECM preserves the antioxidant defense when compared to normal collagen ECM, apparently via increased micropathways for O₂ transport to the hepatocytes.

In addition to the above, it is becoming clearer that nanotechnology as a multidisciplinary field has achieved breakthroughs in bioengineering, molecular biology, diagnostics and therapeutics (Nahar et al. 2006). A recent advance in nanotechnology is the development of a functional nanosystem by incorporation, adsorption, or covalent coupling of polymers, carbohydrates, endogenous substances/ligands, peptides, proteins, nucleic acids and polysaccharides to the surface of nanoparticles. Functionalization confers a wide array of interesting properties such as stealth characteristics, a bioadhesive property, and it prevents aggregation of nanoparticles, imparts biostability and solubility, reduces toxicity and provides site-specific delivery. This makes the nanosystem an intelligent tool for diagnostics, prognostics, and controlled and sustained delivery of proteins, peptides, pDNAs, and other therapeutic agents to specific targets. However, high tissue accumulation of non biodegradable nanoparticles has caused toxicity problems and rendered them as not-so-popular therapeutic and diagnostic systems. The toxicity and safety of non biodegradable nanoparticles are subject to future challenging research.

Current developments in the technological fields of liver tissue engineering, bioengineering, biomechanics, microfabrication and microfluidics have lead to highly complex and pertinent new tools called ‘cell biochips’ for *in vitro* toxicology (Baudoin et al. 2007). The purpose of these biochips is to mimic organ tissues *in vitro* in order to partially reduce the amount of *in vivo* testing. The biochips consist of microchambers containing engineered tissue and living cell cultures interconnected by a microfluidic network, which allows the control of microfluidic flows for dynamic cultures, by continuous feeding of nutrients to cultured cells and waste removal. Cell biochips also allow the control of physiological contact times of diluted molecules with the tissues and cells, for rapid testing of sample preparations. Cell biochips can be situated between *in vitro* and *in vivo* testing. These systems can enhance functionality of cells by mimicking the tissue architecture complexities

when compared to in vitro analysis but at the same time present a more rapid and simple process when compared to in vivo testing procedures. Baudoin et al. (2007) have generated cell biochips that allow liver chronic toxicity analysis in vitro on engineered tissues. An extension of the cell biochip idea has also allowed ‘organ interactions on chip’, which can be considered as a first step towards the replacement of animal testing using a combined liver and lung organ model (Baudoin et al. 2007).

Lastly, several extracorporeal bioartificial liver devices are currently being evaluated as an alternative or adjunct therapy for liver disease (Allen and Bhatia 2002). While these hybrid systems show promise, in order to become a clinical reality, bioartificial liver devices must clearly demonstrate efficacy in improving patient outcomes. Allen et al. (Allen and Bhatia 2002), examined the development of human hepatocyte cell lines, strategies to stabilize the hepatocyte phenotype in vitro and emphasize the importance of the cellular microenvironment in bioreactor design. All these key components of bioartificial liver systems will greatly improve the next generation devices.

Liver fibrosis, a complex ECM disease

Under physiological conditions, the *space of Disse* in the liver contains a non electron-dense basement membrane-like matrix, which is essential for maintaining cellular function (Friedman 2000). Upon the onset of the fibrogenic response, ECM deposition increases and becomes rich in interstitial fibril-forming collagens, mostly collagens I and III (Friedman 2000). Scarring is the most remarkable feature, where ECM appears highly organized, connective tissue fibers retract and most of the cellular infiltrates disappear (Gressner 1991). At this stage, when the liver becomes cirrhotic, there is little remodeling and matrix metalloproteinase activity is greatly diminished (Watanabe et al. 2000).

Thus, hepatic fibrosis is the liver’s wound-healing response to persistent liver injury involving a wide variety of cell types and mediators and it is a common pathway in chronic liver disease. There are pro-fibrogenic cells of diverse origin but they all undergo a common process of trans-differentiation and acquisition of a classical myofibroblast-like phenotype. It remains an unresolved dilemma which of these three general sources of pro-fibrogenic cells within the liver plays the most critical role: either resident myofibroblast-like cells, mainly represented by hepatic stellate cells, portal fibroblasts and vascular smooth muscle cells (De Minicis et al. 2007; Tuchweber et al. 1996; Knittel et al. 1999; Hinz 2007; Rajkumar et al. 2005; Desmouliere et al. 2003); or bone marrow-derived myofibroblasts (Forbes et al. 2004) or recruited fibrocytes (Kisseleva et al. 2006; Moore et al. 2005). Recently, some controversy has also

arisen in regards to epithelial-to-mesenchymal transition (Polavarapu et al. 1998) reported in hepatocytes and biliary epithelial cells, which appear to reprogram their gene expression and trans-differentiate into myofibroblasts (Zavadil and Bottinger 2005; Lee et al. 2006; Peifer and McEwen 2002). Although the relative importance of each cell type to the fibrogenic response depends on the origin and extent of the hepatic insult; yet, hepatic stellate cells and portal fibroblasts remain considered the main ECM-producing cells within the injured liver (Gabele et al. 2003).

Liver fibrosis is reversible, whereas cirrhosis is mostly irreversible. Thus, efforts to identify the mechanisms driving fibrosis center mainly on pathways leading to the early accumulation of scar (e.g., collagen-I and III) hoping to identify potential targets to slow its progression or help its resolution (Friedman 2000).

Dissecting the collagen-I hub in liver fibrosis

There is still limited information on how protein-interaction networks, activated during liver injury, may alter collagen-I expression, thus contributing to liver fibrosis in particular and to liver disease in general. Our laboratory has achieved progress in understanding the mechanisms underlying collagen-I regulation by studying the crosstalk between hepatocytes and hepatic stellate cells (Nieto et al. 2002; Nieto and Cederbaum 2003; Nieto et al. 2002) and between Kupffer cells and hepatic stellate cells (Nieto 2006; Cubero and Nieto 2008; Urtasun et al. 2009; Nieto 2007) as well as their impact on the fibrogenic response to liver injury, using both co-cultures, ECM substrates and molecular biology tools. Yet, the field of liver fibrosis is now moving towards more holistic approaches. Thus, available systems biology tools and interdisciplinary approaches will likely answer in the near future, many unresolved questions (the cell types involved, epithelial-to-mesenchymal transitions and how to resolve scar) in dissecting the networks regulating pathological collagen-I deposition. In this section, we provide one example of current ongoing efforts to determine the protein regulatory network that regulates pathological collagen-I deposition under chronic alcohol consumption.

Factors participating in collagen-I induction in hepatic stellate cells following chronic ethanol consumption, as well as the protein regulatory network and the signaling cascades involved, have not been systematically characterized and thus a database of this relevant and biologically active sub-proteome is missing. Access to such database would be of relevance for pharmacological design of drugs that can prevent or resolve scarring.

To establish an in vivo model for building the systems biology database for collagen-I induction under chronic

ethanol consumption, our laboratory has used the Lieber-DeCarli feeding model for early alcohol-induced liver injury (Lieber and DeCarli 1989). After confirming that hepatic stellate cells from chronic ethanol-fed rats were activated, pro-fibrogenic and generated more reactive oxygen species than those from control rats (Cubero and Nieto 2008), we analyzed the differentially expressed proteins by using isotope-coded affinity tags (Gygi et al. 1999), proteolytic digestion of the protein mixture into shorter peptide fragments, sample fractionation and mass spectrometry-based quantitative proteomics analysis.

The ability to globally profile changes in protein abundance is essential for elucidating the events that occur during cellular processes. With the acquired data from the global comparative quantitative protein profiling analysis from hepatic stellate cells isolated from control and chronic ethanol-fed rats, we can then evaluate the identified and quantified proteins in the context of biological pathways and protein interaction networks related to fibrillar collagen-I, the downstream target.

A major goal in protein network modeling is really the ability to model and predict downstream effects on the collagen-I network in hepatic stellate cells when one node (representing one protein or one protein group) in the network is perturbed (e.g., as it may occur after chronic ethanol consumption), ultimately increasing the likelihood for improved therapeutic targeting. Therefore, if we can model or predict the effect of such a perturbation, we can evaluate the virtue of a potential target benefited from the effect, when the entire system is considered. In other words, if we wish to elicit certain long-term behavior from a protein network, what proteins would make the best candidates for intervention to increase the likelihood of this behavior? That is, if the network is operating in a certain ‘undesirable’ state (e.g., increased collagen-I deposition) and we wish to transition it into a ‘desirable’ state (e.g., collagen-I down-regulation) by perturbing some proteins. For practical reasons, it would be better to intervene with as few proteins as possible in order to achieve the goal. Such approach can expedite the systematic search and the identification of potential drug targets for therapy.

Ultimately, predictions will need to be tested in the experimental system using a biochemical and/or genetic approach, whereby knocking down the expression of a ‘critical’ protein (e.g., with a neutralizing antibody, a specific chemical inhibitor, RNA interference, or using cells isolated from null mice), it could be validated whether the predicted protein and/or signaling pathway is really ‘critical’. Systems biology analysis would add clarity as it would facilitate the identification of pathways worth targeting and at the same time reflect the impact of that target on the overall process. We expect that proteomics expression profiling in combination with studies using

human liver tissue by laser capture microdissection and/or further state of the art technologies would help to address key questions in the near future such as the cell types involved, epithelial-to-mesenchymal transitions and how to resolve scarring.

Thus, we provide here an example where multidisciplinary approaches involving an *in vivo* model, proteomics, theoretical and computational analysis and engineered systems merge with challenging experiments using molecular and cellular biology techniques to tackle an unanswered question (e.g., the pathways leading to collagen-I regulation under chronic alcohol consumption). There are still unresolved issues, which are associated with the fibrogenic response in alcoholic liver disease such as how to reverse the fibrogenic response, which may eventually be addressed using tools that lie within the newly developed encouraging field of systems biology.

An imminent shift: targeted quantitative proteomics analysis of liver injury using multiple reaction monitoring

A key element in the field of systems biology, is that identical protein sets are quantified in multiple samples such as those representing differentially perturbed cell or organ states (i.e., progression of liver disease) (Lange et al. 2008). Recent advances in technology for mass spectrometry-based targeted protein quantification have opened new avenues for a broad range of proteomics applications in basic science and clinical research. The major breakthroughs are highlighted by the capacity of using a universal approach to perform quantitative assays for a wide spectrum of proteins with minimum restrictions and the ease of assembling multiplex detections in a single measurement. Pre-determined protein sets need first to be identified and subsequently quantified at high sensitivity and reliably in multiple samples and states.

Multiple reaction monitoring is thus emerging as a technology, which complements the discovery capabilities of shotgun strategies, by its potential for reliable quantitative analysis strictly targeting a predetermined set of peptides and depends on specific multiple reaction monitoring ‘transitions’ for each targeted peptide. Previous information is required to define these ‘transitions’. First, the proteins that constitute the targeted protein set have to be selected. Second, for each targeted protein, those peptides that present good mass spectrometry responses or proteolytic peptides and identify the targeted protein, or a specific isoform thereof, have to be identified (Mallick et al. 2007). Third, for each proteolytic peptide, those fragment ions that provide optimal signal intensity and discriminate the targeted peptide from other species present

in the sample have to be identified. The final step in the targeted proteomics workflow is the ability to obtain absolute quantification of the target peptide and hence of the protein of interest. The quantitative approach relies in the use of synthetic stable isotope labeled peptides or proteins, which mimic their endogenous counterparts and act as internal standards to quantify the corresponding candidate proteins.

Reliable, accurate, disease-specific, non-invasive biomarkers of fibrogenesis in order to prevent or minimize the impact of the chronic liver disease progression are a critical need. Thus, the above-mentioned approach has significant potential for biomarker discovery since the technology allows for building a multiple reaction monitoring-based proteomics analysis platform specifically designed to identify and validate modified proteins involved in any liver disease. Hence, these platforms could be used to analyze samples from patients to determine biomarkers, disease progression and outcome.

Towards the future

The behavior of cells cultured within 3D-structures rather than onto 2D-culture plastic more closely reflects their *in vivo* responses. Consequently, 3D culture systems are becoming crucial scientific tools in liver research and in cancer cell research in general. The efforts have been primarily driven by a need for scaffold materials for regenerative medicine or for tissue engineering (Langer and Tirrell 2004). The aim is to help natural healing processes to advance faster and better, transplanting cells or creating an environment in which absent or damaged tissue, that would not ordinarily have re-grown, could regenerate.

Several challenges lie ahead in the field of ECM bioengineering. First, single cell resolution may be particularly relevant for studies on cell populations that are highly heterogeneous such as primary hepatic stem cells. To avoid missing the behavior of individual clones such as those from progenitor cells, the introduction of microwell arrays will permit the analysis of a large number of single and spatially confined cells. Second, how to use synthetic hydrogels as candidate scaffolds for cell biology, cell transplat and tissue engineering remains an area that needs substantial research, and a background question that remains is: Can they recapitulate key functions of the physiological ECM? Lastly, additional efforts to develop microfluidic and biomaterials technology to fabricate 3D-cell co-culture systems are needed to further advance the field.

Regarding pathological ECM deposition in liver disease, the heterogeneity of hepatic pro-fibrogenic cells raises the question as to whether there is more than one target cell type for designing efficient therapies to prevent or slow down liver

fibrosis. Do we need to target only hepatic stellate cells or should we also target portal fibroblasts, bone marrow derived cells and fibrocytes? The integration of systems biology data would help to decode the commonalities underlying the multiple origins of myofibroblasts. Future studies should identify potential markers to identify the specific cells participating in the fibrogenic response and dissect whether bone marrow-derived cells and fibrocytes need to be considered as well. This remains unresolved and it is a subject of much debate in the liver field. In addition, the relative contribution of each cellular source varies with disease progression and etiology making the scenario more complex.

Moreover, although still rather controversial, importance is now given to epithelial-to-mesenchymal transitions in which cell–cell and cell–ECM interactions are altered to release epithelial cells from the basement membrane and surrounding tissue, the cytoskeleton is re-organized to confer the ability to move through a 3D-dimensional ECM, and a new transcriptional program is induced to maintain the mesenchymal phenotype (Omenetti et al. 2008; Radisky 2005). In addition, there are local mesenchymal stem cells in a niche together with epithelial stem cells close to the canals of Hering. Additional work is required to dissect whether this mechanism really plays a role in liver fibrosis.

Serial liver biopsy specimens from patients offer the opportunity to study liver disease from different etiologies. However, the low protein and RNA yield associated with these small clinical specimens requires the use of ultra-sensitive tools and platforms working at the nanoscale level. The combination of high resolution Fourier transform ion cyclotron resonance mass spectrometry, along with an accurate mass and time-tag strategy, has already been useful in offering the ultra high-sensitivity necessary for analysis of biopsy specimens of post-transplantation livers from HCV-infected patients (Jacobs et al. 2005). The identification of disease biomarkers or signatures has the potential to improve patient diagnosis, treatment, quality of life and outcome. In this regard, the use of targeted proteomics may advance the field significantly by minimizing sampling and increasing accuracy (Lange et al. 2008).

Integrated system-wide approaches, including ECM bioengineering combined with mathematical modeling and computational biology, have great potential for understanding the complexity of liver disease and translate it into preventive and personalized medicine to avoid liver disease. A key goal of many of these studies is to utilize the right pipelines to integrate and analyze high-throughput datasets. A critical point is to be able to predict disease progression. By collecting information from multiple sources, we can increase the likelihood of identifying biomarkers of the broad spectrum of liver diseases, uncovering mechanistic pathways and perhaps predicting outcome. The wealth of knowledge already acquired from quantitative and

functional ‘omics’ studies in the liver will provide vital assistance in our efforts to identify circulating markers possibly resulting from leakage, secretion or shedding of proteins from diseased liver (Diamond et al. 2006). Ultimately, the data generated will be very useful when viewed in the context of a more integrated picture with the idea of helping to slow down or prevent disease progression.

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