5.513. Systems Biology in Biomaterials and Tissue Engineering

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Abbreviations

AFM	Atomic force microscopy
EB	Embryoid bodies
ECM	Extracellular matrix
ES cells	Embryonic stem cells
FT-IR	Fourier transform-infrared spectrometry
hMSC	Human mesenchymal stem cells
HSC	Hepatic stellate cells
iPS	Induced pluripotent stem cells
MALDI-TOF	Matrix-assisted laser desorption ionization time of flight spectrometry
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
NMR	Nuclear magnetic resonance spectroscopy
PAI-1	Plasminogen activator inhibitor -1
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
PET	Polyethylene terepthalate
PLGA	Poly(lactic-*co*-glycolic acid)
R&D	Research and development
Tg	Glass transition temperature
TGFβ1	Transforming growth factor beta -1
TSP-1	Thrombospondin -1
uPA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor

5.513.1. Introduction

Biomaterials research for tissue engineering revolves around various permutations of four fundamental components: biomaterial scaffolds, cells, *in vitro* tissue constructs, and *in vivo* integration into living hosts. Some implantable biomaterials aim at recruiting the native mature or stem cells to the lesion for repair or regeneration; or simply serving as inert prosthesis to replace the functions of the injured or diseased tissues or organs. The choices of biomaterials, cell sources, or design parameters of the tissue constructs have been *ad hoc*, either due to component availability (for certain biomaterials or cell types) or due to the unique constraints of a specific application. This approach has worked well for simpler tissue constructs where the functions are not tightly coupled to the structure, such as extracorporeal devices, prostheses that are self-sufficient to replace the damaged tissues functionally, or cell transplantation that depend entirely on regeneration from the host *in vivo*. In such cases, simply taking available biomaterials and cells, and making a construct that roughly looks like the real tissue, can be reasonably successful. The first section of this chapter describes the discrete approach of working on one material at a time or one cell type at a time, and the discrete approach seems sufficient for these simpler applications. As the field progresses toward repairing and regenerating more complex tissues, the functional demands increase and the structure–function relationships become more tightly coupled. These challenges create a greater need to progress from the discrete approach to a higher throughput, systems-level approach, which typically involves large screens, combinatorial synthesis, or experiments on multiple components. The second section of the chapter summarizes several high-throughput or -omic level projects in tissue engineering, with particular focus on -omics technologies for characterizing cellular responses, and quantitative analytical tools for monitoring tissue integration *in vivo*. Researchers already adopting such systems-level approaches have successfully designed new biomaterials for optimization of complex tissue functions. The second section includes a summary of this progress, followed by a discussion of the limitations of the high-throughput approaches that are still primarily based on individual components (cells, biomaterials, constructs) rather than based on the functional process of interactions and relationships between the components. Quantitative relationships between *in vitro* constructs and *in vivo* responses must be established in order to guide rational biomaterials and tissue engineering efforts to achieve the desired results. High-throughput experiments on individual components may be capable of optimizing a set of parameters, but sometimes this success only highlights the gap of understanding between the optimized parameters and final biomedical goal with integration into a living host. Thus for improving function in tissue engineering, a process-centered approach, described in our third section, will become increasingly important (Figure 1). We have recently explored a process-centered approach to study and facilitate regeneration in a healing defect, liver fibrosis. Our approach, including innovative computational modeling, might be further adopted in the systematic development of biomaterials and tissue engineering applications of the future.

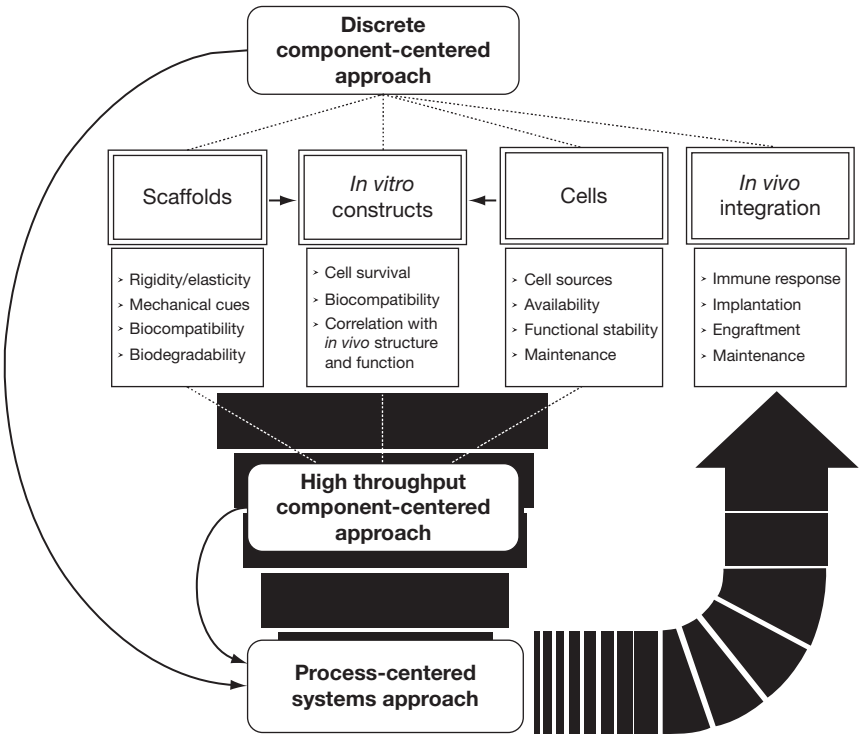

Figure 1 Diagram of the relationship of the four core components and the three tissue engineering approaches.

5.513.2. Discrete Component-Centered Approach

Tissue engineering is an integration of myriad fields, with the ultimate aim of reconstructing a functional tissue or organ for regenerative medicine. In the past decade, engineering tissues with *in vivo* fidelity has found applicability and broad impact in a wide array of additional fields. The chief hindrance to the progress of this field is the complexity of the human body, exemplified by the diversity of cell types; the interplay between the chemical, physical, and topographical factors that regulate cell function; the morphology of tissues; and the astoundingly precise development of organs.[1]

Various approaches have reconstructed living and functional tissue constructs *in vitro*, but over the past two decades, methods based on biomaterial scaffolds have occupied center stage.[2] These methods generally entail seeding cells on scaffolds that serve as centers of tissue regeneration. The scaffolds are tuned specifically for their chemical and biological parameters (e.g., solute diffusivity, porosity, elasticity, and biocompatibility) but are very rarely tuned for their ultimate biological effects or function. Working on one biomaterial or cell type at a time has yielded useful and satisfactory results for reconstructing certain tissues with straightforward structure–function linkage such as bone and ligament,[3] but has failed in areas where there is an intricate interplay between the structure and function such as liver and kidney.[1]

Another context where the focus on specific biomaterials has been successful is in regenerative medicine, where the primary role of the scaffold is simply to help nature take its course in mending lost or damaged tissues, and where control of the scaffold properties occurs on the scale of tens to hundredths of microns.[4,5] The remaining bottleneck in tissue engineering is to develop large *in vitro* models of complex tissues with *in vivo* fidelity, with the facilitation of angiogenesis.[6,7] This phase of research has been arduous, primarily due to poor understanding of the architectural and compositional requirements for creating a large functional tissue, and due to the disparate natures of the fields of biomaterials and biology. Early biomaterial scaffolds did not foster any form of tissue hierarchy and were not tailored for specific *in vitro* purposes, yet the success of these scaffolds *in vivo* fuelled the development of methods that hoped to induce complex tissue architectures.

Although the discrete component approach has been effective in so many different situations,[1–5] it still largely suffers from the narrow focus on either the development of improvised materials to render only a single function or using a construct with a single cell type to mimic multicellular complex tissue functions. A classic example would be the field of skin substitute development. All the commercially available replacements definitely serve the main purpose of rapid wound closure[8] amongst others.[9] But they invariably fall short in creating a complete functional skin (e.g., functions associated with wrinkle and hair follicle) despite the promising developments of new materials[8] and therapeutics for full-thickness skin injuries.[10]

In the perspective of cellular components used in constructs, there is again a growing concern that a single cell type would not be sufficient to emulate the multifaceted functions of any complex tissue. Though there are a few applications where only one type of cells would be deemed sufficient, like in cartilage reconstruction where a construct with chondrocytes and the right substrate[11] would facilitate cartilage repair, tissue engineering with multiple cells types is becoming more relevant with better functions. Interestingly, in urinary bladder replacements there has been a gradual shift from the orthotropic neobladder reconstruction techniques[12] (due to the imminent side effects) to the *ex vivo* engineered bladder constructs,[13,14] in which urothelial cells and smooth muscle cells were both successfully applied on the scaffold. There are other demanding scenarios that require the construct to orchestrate the formation of complex multifunctional structures like in blood vessel generation which can be done only with the appropriate recruitment and organization of different cells in the presence of the right proportions of growth factors.[15] Thus with a more thorough understanding of the inherent mechanisms involved with vascular growth,[16,17] the recent configurations with hydrogel matrices and PLGA scaffolds coupled with VEGF delivery[18,19] have been successful to a great extent in recapitulating various stages of this complex process.

A few methods are drawing attention for tissue engineering. (a) Microfabrication technology – with importance exemplified by its use for maintaining the differentiated phenotypes of primary cells (which had previously proven very difficult to maintain *in vitro*), and its use for probing homotypic and heterotypic cell interactions.[20,21] (b) Microscale flow – fluidic flows in microfluidic channels mimic the interstitial flow in vascularized tissues, and play important roles in mechanical and chemical signaling. The validity of microscale flow models has been demonstrated by microscale fluidic flow bioreactors.[1] (c) Synthetic microenvironments – this field has focused on mimicking the biochemical composition, fibrillar structure, and viscoelastic gel-like character of natural tissue matrices. A good early work has used PEG (polyethylene glycol) scaffolds, which are capable not only of remodeling, but can also be chemically customized to incorporate biological molecules that facilitate signaling, to and from the microenvironment.[22,23]

The explosion in the number of devices that can be implanted into the body includes tissue-engineered constructs, or biomedical implants such as glucose sensors and stents. The implantation of devices *in vivo* can lead to major complications in the body, or other detrimental effects reducing its functionality and longevity in the body. The fact that no single material is biocompatible under all conditions only creates further challenges in designing better biomaterials or tissue-engineered constructs.[24]

Although the diversity of factors governing inflammation and foreign body responses is well-documented,[25] the complexity of the response of the body to different biomaterials is poorly understood. The degree of the biological response is also governed by the (a) morphology and porosity, (b) shape, (c) size, (d) surface chemistry and roughness, (e) design, (f) composition, and (g) degradation.

One disease application where this plays a very important role is the cardiovascular system where stents have long been used with reasonable success, and where longevity has been improved via the use of drug eluting stents.[26,27] Even with technological advances, there has been limited understanding of device/host interactions, which hinders the development of better constructs for *in vivo* applications.[27] One source of the problem is the study and testing of particular constructs in isolation without considering the systemic effects of

biomaterial response and without considering regions beyond the immediate area of the implant.[26] Multiple dimensions of biological response governed by a large number of factors create a process of enormous complexity.[28,29]

5.513.3. High-Throughput Component-Centered Approach

To efficiently search a broad variety of possible cell culture conditions, scaffolds, or other tissue engineering parameters, new experimental platforms offer the possibility of massively parallel experiments, robotic execution of repetitive steps, high-content imaging with automated computational analysis, miniaturization and microfluidics, or lab-on-a-chip platforms that achieve dramatic improvements in experimental throughput. High-throughput measurements can give noisy results, often causing many type I (false positive) errors. Utilization of results from high-throughput experiments is inseparable from computational methods of statistical data analysis and data mining.[30] The availability of free[31,32] and economical general-purpose data analysis software allow data mining needs to be met without customized programming for the tissue engineering context. Meanwhile, a host of experimental tools specific to tissue engineering are necessary to create and assess large-scale multivariate quantitative systems.[33] Microarray and microfluidic technologies, including high-throughput live cell assays, have been developed for performing highly parallelized experimental perturbations, and measurements of the resulting cellular responses.

5.513.3.1. Manufacturing Substrates

In order to increase assay throughput and reduce reagent consumption, and reduce the number of cells required, the substrates being manufactured must be miniaturized. Emerging technologies may ultimately lead to array formats. Current tools include rapid prototyping, the setup of polymer gradients, and spotted microarrays, microwells, as well as microfluidics. Parameters of interest for control include porosity, volume fraction, mechanical properties, oxygen diffusion, degradation rate, tortuosity, permeability, homogeneity of composition, fiber orientation, surface chemistry, biomaterial composition, and protein adsorption.

Nanoliter volumes of different chemicals, biochemicals, and cells, as well as combinatorial combinations of these, can be dispensed in a spatially addressable pattern using automated robotic spotting technologies.[34,35] For example, a library of over 1700 photopolymerizable acrylate-based biomaterials was synthesized and the mechanical properties were measured with nanoindentation.[36] Other applications include toxicology assays,[37] compound screening,[38] gene function screening,[39] cell adhesion assays,[40] and probing embryonic stem cell[41] fate.[42,43] In the work of Flaim *et al.*[42,43], various extracellular matrix (ECM) compounds such as collagen-I, -III, -IV, laminin, and fibronectin were immobilized on hydrogel surfaces. A combination of ECM compounds was identified that most synergistically induced differentiation of ESCs to a hepatic fate. Also some interesting findings were made with regards to the crosstalk between various ECM proteins which culminated in the development of the right substrate for hepatocytes that lead to high levels of albumin synthesis *in vitro*. Combinatorial methods can also be used on the macroscale in well plates, but the miniaturization of such systems would aid in increasing throughput.[44]

For large-scale screening of cell–biomaterial interactions, a continuous polymer gradient can be generated, instead of discrete spots with varying compositions.[11,45] Both 2D and 3D gradients can be generated for exhibiting a gradient of some property along a biomaterial film, hydrogel, or other surfaces. The gradient as presented to cells and cellular response is observed in monolayer (2D)[46] or on cells seeded within porous scaffolds (3D).[47] This way, the optimal point of interaction along a gradually changing surface can be identified.[48,49] Besides being used as a screening tool, scaffolds with a gradation in properties could also serve as a template for the generation of a graded tissue.

Arrays of microwells with defined dimensions can be fabricated using soft lithography, which affords control over the substrate size and cellular aggregates.[34,50] Microwells are attractive because they allow control of parameters such as size, shape, and homogeneity of cellular aggregates, for instance in the expansion of human ES cells. As an example, 3D embryoid bodies (EBs) have been formed within poly(ethylene glycol)[17] microwells.[35,51] Microengineered hydrogel microwells have also been used to regulate ES cell fate in an aggregate size-dependent manner.[52] Similar template-based assembly of cells could also be used to form aggregated tissue sections that contain multiple cell types organized into specific geometries relative to each other.[50] Reproducibility of homogenous cultures has been identified as a potential problem in recreating exactly similar conditions across the board for valid experimental repeats, as in the current difficulty of controlling the homogeneity of EB formation within microwells.[53] Improved reproducibility may occur in the future with improvements in microwell material composition, cell seeding procedures, and handling of aggregates during retrieval.

Microfluidics also presents the opportunity for conducting high-throughput analysis of signals within an array system.[33,34] Microscale channels are formed by casting a polymer, polydimethylsiloxide (PDMS), on a prefabricated mold. Advantages include application and control of laminar flow, shear stress, spatial positioning of soluble factors including concentration gradient generation, and ease of imaging.[35] Systems that can be set up include platforms for drug toxicity studies and multiphenotype cell arrays.[50] By combining microwells and reversibly sealed microfluidic channels, Khademhosseini *et al.* were able to localize multiple cell phenotypes. This work gives an example of the ability to pattern tissues within microfluidics channels in a high-throughput way.[50]

For manufacturing of substrates on the larger scale, rapid prototyping technologies allow fast fabrication of porous biomaterial scaffolds with tunable micro- and macroscale structures.[54] This term generally refers to a computer-controlled manufacturing technique that allows layer-by-layer deposition and processing of materials, resulting in a totally controllable structure.[2,55] Some examples include fused deposition modeling, stereolithography, electron beam melting, and 2D printing.[56] Others include high-throughput laser printing,[57] electropatterning,[58] and solid free-form fabrication.[2]

5.513.3.2. Measuring Substrate Properties

Combinatorial chemistry has made it possible to develop vast assortments of polymers and biomaterials (see **Chapter 4.412, Rational and Combinatorial Methods to Create Designer Protein Interfaces**);[59] however this advance creates a pressing need for equally efficient methods to characterize the different polymers.[60] Because the surface chemistry, topology, and mechanical properties of biomaterials have profound effects on cell behaviors, these properties are crucially important to measure when evaluating their implications for tissue engineering.[61] The current trend in tissue engineering toward developing more customized and tailored biomaterials for specific applications, has amplified the need for fast and easy methods to characterize biomaterials.[62] The following sections describe methods for characterizing certain categories of material properties, with efficient methods highlighted.

5.513.3.2.1. Surface chemistry and techniques

An efficient and scalable method for characterizing the surface chemistry of biomaterials was developed by Kohn *et al.*, who established a correlation between glass transition temperature, hydrophobicity, mechanical properties, and polymer structure. To measure the underlying parameters for these correlation trends, they used differential scanning calorimetry (glass transition temperature, Tg), gel permeation chromatography (molecular weight), thermogravimetric analysis, goniometry, and the sessile drop method (water contact angle).[63] The results of these tests give insight into the structure–property relationship for large quantities of materials developed.

Another important development has been the use of MALDI-TOF (matrix-assisted laser desorption ionization time of flight) mass spectrometry for analysis of macromolecules. Inkjet printing of polymers has been shown to greatly improve the throughput of MALDI-based analysis.[64] Among the characterizations that have been most successfully implemented using high-throughput MALDI platforms are the measurement of molecular weights combined with high-speed columns, and parallelization combined with flow injection analysis.[33] These automation tools provide good predictive power and they vastly improve the speed of material detection, without much loss in resolution.

For high-throughput characterization with less technical complexity than MALDI-TOF methods, new optical methods have been developed, including measurements of absorbance and fluorescence, including the far and near infrared regions.[65] Light scattering and viscometry have also been shown to greatly improve the automated batch characterization of polymer solutions while continuous monitoring of the polymerization of the monomers is done using high-throughput optical techniques like FT-IR (Fourier transform infrared) spectroscopy.[66]

5.513.3.2.2. Mechanical characterization

The mechanical properties of biomaterials (such as tensile properties, modes of deformation and stress–strain behavior) are also of utmost importance for engineering functional constructs. Various studies have shown differential responses of cells when grown on substrates with differing mechanical properties.[67] For example, the mechanical stiffness of a substrate can direct stem cell differentiation into various cell lineages.[67] The mechanical properties are often measured by techniques like nanoindentation, which measures displacement at the nanometer scale. Strain-induced elastomer buckling can also be used for measuring mechanical properties of thin films. Measurement of the surface toughness and pattern formation is performed in a rapid manner using AFM (atomic force microscopy), with the field moving toward nanorobotics to characterize biomaterials with reduced human error (see **Chapter 3.302, Atomic Force Microscopy**).[41] The chemical and mechanical properties of biomaterials have a tremendous impact on cell–material interactions and on tissue engineering outcomes, thus emphasizing the need to characterize materials extensively before they are used for tissue engineering applications. The understanding of the interdependency between chemical and mechanical properties for cells and biomaterials will also play an important role in developing designer biomaterials for future tissue engineering applications.

5.513.3.3. Measuring Cellular Response

Systems-level approaches have afforded great advances at characterizing the responses of cells to different types of biomaterials. The -omics revolution has contributed tremendously to the understanding of biological responses to biomaterials, which aids in understanding which biomaterials and soluble factors achieve phenotypes closest to those observed *in vivo*. Contributions from genomics, proteomics, and metabolomics are helping to elucidate functional and structural aspects of subcellular systems, ultimately helping to engineer better tissues. This section describes how genomics, proteomics, and metabolomics have contributed to tissue engineering.

5.513.3.3.1. Genomics

Genomics, including functional genomics (gene expression analysis), has provided extensive evidence about the diversity of factors responsible for maintaining the function of highly specialized cells, and for determining the levels of mimicry of *in vitro* constructs to *in vivo* tissues.[68] The development of the microarray platform has provided broad insight into understanding the gene expression of *in vitro* constructs (see **Chapter 3.321, Microarrays in Biomaterials Research**). It has led to the large-scale (whole genome) screening of engineered tissue constructs and even to greater understanding of the responses of implants to hosts. Microarray technology has had tremendous impact on the fields of comparative and functional genomics and has improved the understanding of pathways that were perturbed or differentially regulated in culture versus *in vivo*. Gene expression analysis has been used for deriving how various culture configurations modulate pathways required for cell behavior to meet the requirements of bioartificial reactors and drug testing platforms.[69,70] Genomics has also provided insights and a mechanistic understanding on goals such as understanding the divergence of phenotypes in 3D and 2D cultured cardiac cells, and uncovering pathways that contribute to *in vitro* tissue genesis.[71] Culturing cells in a 3D environment has long been known to provide advantages for mimicking the *in vivo* phenotype, compared with 2D environments. 3D culture conditions are more conducive to the formation of multicellular organization, similar to that found *in vivo*.

Primary cells, such as cardiomyocytes for cardiovascular tissue engineering, and hepatocytes for liver tissue engineering,

are the cells of choice for studying tissue genesis, regeneration, and drug-induced toxicity.[72,73] For hepatocytes to maintain proper function, the flow of blood through the liver sinusoids has been hypothesized to induce necessary signals mediated by shear stress. The liver chip developed at MIT[69] demonstrated this by measuring gene expression levels for various important drug metabolism enzymes, comparing them with enzyme activity levels *in vitro*, and with gene expression levels *in vivo*, to confirm the relationship between the culture conditions and the *in vivo* mimicry.[69] This valuable information is obtained in a high-throughput manner, and the consistency of the data obtained makes it a powerful tool to study cell–biomaterial interactions and to determine the phenotypes of various tissue-engineered constructs.

5.513.3.3.2. Proteomics

Proteins are regulated by various mechanisms such as phosphorylation, glycosylation, and other posttranslational modifications (targeted cleavage and degradation) that occur downstream of mRNA that cannot be observed with functional genomics. The field of proteomics involves system-wide analyses at protein levels, sometimes including observations of posttranslational modifications, and often emphasizing differentially expressed proteins. Cellular responses traditionally have been characterized at the proteomic level using methods such as 2D gel electrophoresis and MALDI-TOF mass spectrometry.[74] Additional methods of high-throughput protein analysis such as protein microarrays and antibody microarray[75] are currently being developed. Traditional low-throughput methods of studying proteins have used, for example, antibody methods to detect the release of growth signals, cytokines, or proteins. For tissue engineering, techniques like 2D gel electrophoresis and MALDI-TOF are used to characterize the surface adsorption of proteins to biomaterials and to determine the relationship between the surface and the intracellular signaling pathways affecting cells.[76,77] Used synergistically, they have also helped identify proteins which are secreted into the medium and intracellular proteins which are present in cells cultured on polystyrene and titanium surfaces.[78,79] If similar methods can be pursued with a higher level of spatio-temporal resolution, they will be useful for characterizing cells in their environments *in vitro* or *in vivo*, since the complexity and the lack of reproducibility of this technique has made it difficult to apply it to study all cellular responses.[80,81]

There has been some progress made in utilizing this technology in studying *in vitro* constructs and in measuring protein expression during differentiation and in various cardiovascular diseases, but very little progress has been made in tissue engineering *per se*. Hepatocytes, when cultured *in vitro*, undergo a change in their proteome and many proteins are differentially regulated when compared to cells *in vivo*.[82] A coculture of hepatocytes with nonparenchymal cells like fibroblasts have been shown to increase hepatocyte differentiated functions.[83] In a quest to find out which of the factors is responsible for the improvement in hepatocyte function, we have carried out a proteomic analysis of the culture media of the cells cultured in the coculture configuration. We found that transforming growth factor-beta 1 (TGF-β1) was an important cytokine upregulated in the culture and the substitution of the TGF-β1 in the culture media replaced the need to coculture cells,[84] by maintaining higher order differentiated function of the cells.

Thus the understanding of how cells react when cultured with various cell types and identifying in detail the components responsible for the differential cellular responses could help us identify better biomaterials, supplements to improve and develop better cellular constructs.

5.513.3.3.3. Metabolomics

Another field of great interest today is metabolomics, which involves the measurement of metabolites, in order to create a metabolic map of different tissues or microorganisms. Understanding metabolite flux at a systems level helps provide a direct point of access for normal physiological systems. Metabolomic methods have recently helped identifying markers for cardiovascular diseases,[85] assessing drug toxicity,[86] and characterizing anti-inflammatory compounds.[87] Metabolomics has not been utilized extensively in the field of tissue engineering, but the combination of metabolomics and proteomics could provide a profoundly powerful characterization of *in vitro* constructs.[88]

For example, metabolomics are intricately informative about liver cell function, which meets the demand for robust methods to assess the functionality of liver cells for bioartificial liver devices.[89] Recent work has characterized metabolite profiles using nuclear magnetic resonance (NMR) spectroscopy, leading to the discovery that cells cultured in 2D and 3D bioreactors showed differential consumption of glutamine, lactate, and alanine.[90] The above examples illustrate how the integration of genomics, proteomics, and/or metabolomics can aid in defining culture conditions, media, supplements, or 3D configurations, thus furthering the goals of tissue engineering by allowing us to create constructs that better resemble natural tissues *in vivo*.

5.513.3.4. *In Vivo* Integration into Living Hosts

Over the years, technological advances have provided a wealth of information about how an introduction of foreign stimuli influences various individual molecular species.[91,92] These discoveries, although vitally important for biological progress, have not yet been successfully translated into clinical applications or other success stories *in vivo*.[93,94] For example, synthetic hip prostheses are one of the most widely used implants in the clinic. However, complications often emerge after implantation, which are mainly caused by mechanical failure of the implant material or by the coeffects of chronic inflammation. Revision surgery is often necessarily due to subsequent bone loosening and fracture.[95] Another example is the coronary artery stent, which is widely used in the established therapy of percutaneous coronary intervention for coronary heart diseases. The stent is placed inside the narrowed branch of a coronary artery to keep it open. However, the site with stent bracing up will inevitably become narrowed again gradually after the operation.[96] One of the major reasons is that the exogenous materials of the stent can trigger immune stimulation *in vivo* that creates chronic inflammation *in situ*. At the same time the stent damages the vascular wall by the force it creates inside the artery that will slowly recover by forming tightly

contracted scar. There are various kinds of adjuvant therapeutics to prevent clotting forming again, but still the patient needs another stent intervention when the renarrowing happens.

Observation of *in vivo* response is necessary for the R&D of implantable biomaterials. A typical biomaterials project with *in vivo* implantation might have the following pipeline: after design and fabrication of a construct, researchers will implant it into animal model; then systemic samples like blood are collected at different time points for toxicity analysis; function may be tested to check the phenotypic improvement; and finally the implanted construct is removed again for histological analysis. Interpretation of the above observations, directly or indirectly yields the conclusion about the *in vivo* responses. No single project can measure everything, but too often neglected are the detailed reactions *in situ*, the system-level effects in the body, and a breadth of observations that could identify the underlying mechanisms for positive or negative outcomes.

The *in vivo* integration challenges that remain will require a paradigm shift in the way we identify and associate molecules with specific phenotypes. The multifactorial nature of host/biomaterial interaction makes it crucially important to develop tools and detection methods for studying the entire system simultaneously.[94] This is a divergence from the traditional reductionist approach that is based on studying individual factors associated with complex interactions.[97] Along these lines, the identification of molecules or markers associated with the presence and response of host to biomaterials should rely on a better understanding of the biology at a systems level. To construct a holistic view of systems biology, multiple and different types of endpoints must therefore be combined, including the molecular scale (gene expression, proteomics, lipidomics, metabolomics,), cellular scale, and tissue/organ scale.[98–100] Clinical measurements (e.g., pathology) must also be incorporated when possible. *In vivo* imaging, particularly because of its scalability, is an ideal tool for deeper investigation and integrated pursuit of relationships across components and across scales, at a systems level in the living host.

5.513.3.4.1. Image informatics: Transcending scales with improved speed and resolution

Pursuing a project from molecular manipulations through to clinical endpoints creates difficult problems for integrating information across scales: from nanometers to centimeters, from microseconds to months, and from molecules to tissues. Changes at any scale can affect others, particularly the next higher level of scale.[101] There are no universal recipes for navigating such complexity, but computational modeling can be useful for integrating information derived from traditional bottom up data driven approaches, with top down knowledge, to achieve better predictions in areas of systems medicine and in prognosis of certain conditions (see **Chapter 3.313, Histological Analysis**; **Chapter 3.316, Immunohistochemistry**; **Chapter 3.317, Fluorescence Imaging of CellBiomaterial Interactions** and **Chapter 3.318, Molecular Imaging**).[102]

Imaging techniques, developed over the last century, have greatly advanced our understanding of biological systems and have played an important role in functional understanding of complex organs such as the brain. A great advantage of imaging is that many of the imaging tools are noninvasive and the information obtained is rich in the spatial and temporal dimensions.[103] The need for staining and the possibility of artifacts remain to be disadvantages of imaging techniques.[104] Current imaging systems also suffer from the inherent problems of low throughput of the techniques, which generate images at a high resolution. This has led to development of imaging tools, which can image at higher resolutions with higher throughput.

The working principle of high-throughput imaging-based screening systems is the automation in the imaging of microtiter plates, often in multiples of 96-wells, with images produced via detection of fluorescence.[66] Combined with robotic handling, high-throughput imaging methods result in a large amount of image data and statistically significant information,[105] that can be useful for systems biology analysis. One prominent application of automated microscopic techniques is the use of cell-based assays for screening small-molecule drugs from combinatorial synthesis.[4] Whole body imaging techniques have been developed using epiplanar illumination mechanism and have been useful in neurological detection of cathepsin activity in glioma tumors using MRI coregistered fluorescence tomography.[106] Imaging with infrared light has also improved penetrative power and has enabled the capture of images with higher resolution.[107] The development of new technologies like multifocal multiphoton microscopy hold great promise in imaging 3D tissues with high resolution and improved speed achieved by parallelized illumination.[108,109] Very often, the information obtained in a single measurement is the result of several different mechanisms acting simultaneously, which makes the interpretation challenging. Therefore, a major issue in cell-based imaging is to seek biological relevance from a large pool of collected data and subject them to rigorous statistical analysis.

Integrating, modeling, and understanding the data obtained from different techniques of image acquisition pose a challenge. Sartori *et al.*[110] have combined the use of multi-scale imaging by correlative light microscopy with cryo-electron tomography, to compare images obtained by both of these methods in 2D. Such comparison methods can also be used to correlate the structural information obtained using cryotomogram and the functional information obtained using fluorescent methods.[111] There have also been a few reports of imaging techniques that have been used to study response to a biomaterial. For example, Murata *et al.*[112] studied neointimal coverage after stent implantation, and they found a good correlation between optical coherence tomography and histology-based approaches, for the purposes of observing neointimal area, thickness and luminal area. Thus, by combining the various approaches available by transcending scales and linking data driven approaches with new technologies which provide information at a larger scale we will be able to get a better understanding of life processes and thus predict better integration of engineered constructs with *in vivo* systems.

High-throughput experimental platforms have provided valuable results for diverse applications, and tissue engineering has made significant strides at adapting high-throughput technologies for specific needs. As familiarity and facility with these methods improves, another phase of progress is beginning, where high-throughput or -omic experiments are starting to be used for discovering knowledge, constructing models, and inferring relationships that form the foundation of a system-level understanding and a process-centered approach.

5.513.4. Process-Centered Systems Approach

The transition toward high-throughput platforms may increase efficiency for screening and experimentation, but it does not change the fundamental orientation of most tissue engineering research toward optimizing particular components. In other words, the four core foci; biomaterials, cells, culture configurations *in vitro*, and integration *in vivo*; are most often considered individually and optimized, whether using low-throughput or high-throughput methods. Although we acknowledge that the components are interdependent, we often address them separately for lack of established quantitative relationships between the experimental variables *in vitro* and their ultimate effects *in vivo*. Even for *in vivo* tissue engineering, the choice of biomaterials, cells, and microenvironments must rely heavily on the unpredictable behaviors of cells to proliferate, migrate, and remodel their environments. Therefore, much of what we have accomplished has relied on an empirical, trial-and-error approach.

Future research in tissue engineering and biomaterials has the potential to achieve greater success particularly for functionally ambitious projects, by taking a process-centered approach. The study of components and processes can occur through the use of system-level computational models. With a better understanding of the way cells interact to form tissues in different contexts (e.g., embryonic development or tissue repair); we can identify subsets of parameters that should be covaried simultaneously in subsequent experiments. We can also choose to vary parameters that are mathematically predicted to have highest probability of having the desired impact on the outcome. Achieving fully functional tissues for regenerative medicine involves a huge number of variable dimensions, and performing an exhaustive screen of all simultaneous combinations of all variable parameters is not possible, even with high-throughput platforms. Instead we must understand the larger processes and system-level trends that determine the clinical outcome, and design focused screens that actively manage and quantitatively navigate the landscape of interdependent parameters. In this section on process-centered systems approaches, we discuss example projects that bridge the gap between component-centered approaches and a more holistic quantitative prediction of *in vivo* processes, often through the use of computational modeling and biophysics.

Classical biophysical models use measured parameters in construction of a model that better facilitates the understanding of how certain biomaterials affect or are affected by biological processes. Examples can be seen in the work of Shreiber *et al.*[113] who have modeled the effect of fibroblast cell migration on the compaction of ECM. Other examples of classical biophysical modeling include proliferation of liver cells as a function of the metabolic load on the cells[114] and bone regeneration on scaffolds as a function of substrate porosity.[115] These models are better known for their accuracy and complexity of construction, than for their ability to mimic broad physiological outcomes. These physical models are applicable to biological phenomena but their outlooks are oriented more toward understanding mechanistic issues in tissue engineering, rather than toward simulating clinical outcomes.

For predicting the interplay between complex and simultaneous effects, mathematical modeling has obvious usefulness, but knowledge of the effects is required before a network of multiple effects can be simulated. Initial models have most often been built for extremely well-studied systems, but in general filling this information gap will entail the creation of new knowledge. Better instrumentation is increasing the availability of information, and more biophysical parameters are becoming available for purposes like modeling.[116] A contrasting avenue for assembling information in the future may involve the reuse of high-throughput measurements, due to the following innovation: component-centered measurements become useful for process-centered work when assembled *en masse*, because automated data mining algorithms are increasingly capable of using observed correlations to infer relationships and to reconstruct networks of effects.[117–120] High-throughput studies are widely recognized for having a close interface with statistical data analysis methods, but in addition to data analysis is an opportunity for automated knowledge discovery, to elucidate system-level and process-oriented knowledge from experiments that may have been designed for narrower purposes.

A landmark study[121] reconstructed a genome-wide transcriptional network for mesenchymal transformation of gliomas. Network reconstruction was also used in a study of adipocyte differentiation[82] where correlations between gene expression patterns and transcription factor phosphorylation patterns from large-scale proteomics and phosphoproteomics experiments were sufficient to infer relationships between transcription factors and target genes. Complete biological pathways are too numerous to be fully elucidated by humans and they will increasingly often be pieced together by computational methods. As systems-level approaches become more common in fields such as molecular cell biology, the resulting accumulation of pathway knowledge, regulatory mechanisms, computational models, and other fruits of the research will become available to help advance process-centered system-level research in tissue engineering and regenerative medicine. Few examples of process-centered systems research have been published for biomaterials or regenerative medicine thus far, but among them are projects summarized below on *ex vivo* implants, pathways of cell regulation, and regulation of liver fibrosis.

5.513.4.1. Modeling of Systems in Tissue Constructs

Bridging the gap between more biophysical models and regenerative medicine is the still developing field of *ex vivo* implants.[122] In such implants, cells are seeded on biodegradable scaffolds and after culture for a period of time, are transferred to a region of defect inside the body. An exciting field of tissue engineering research emerges from the possibility of transplanting adult stem cells, such as induced pluripotent stem cells,[97] through engineered constructs. In such systems there is a fusion between basic bioengineering principles and the underlying biology of cells, to create a model that is useful if not exactly accurate for understanding the role of bioengineered substances in tissue engineering. Mathematical models using ordinary differential equations have been constructed by Lemon[123,124] to study the proliferation and adhesion of cells on *ex vivo* implants. The authors used human mesenchymal stem cells (hMSCs) cultured on fibrous PET

(polyethylene-terephthalate) scaffolds, and studied the effect of hypoxia on growth. There are also some detailed models of stem cell attachment on scaffolds which emphasize metabolite diffusion and cell growth kinetics.[125]

Successful survival of an *ex vivo* implant within the host tissue is facilitated by the development of vascular tissue, which can transfer essential nutrients to the stem cells via the capillaries. Lemon *et al.*[123] constructed a highly simplified model of angiogenesis on scaffolds for human embryonic stem cells, and used the chick chorioallantoic membrane[46] as an experimental assay for estimating parameters and validating predictions. Their model predicts that the rate of cell infiltration through the pores of the scaffold to form blood vessels is a limiting factor of angiogenesis. Angiogenesis is increased in VEGF pretreated scaffolds when compared to untreated ones, both in the model as well as the experiments, thereby increasing the success of *ex vivo* transplants. As angiogenesis is a determinant of the success of scaffold integration within tissue, there have been other studies of blood capillary formation in the presence of specific scaffolds.[126] The presence of implants stimulates an inflammatory response by the body. Chang *et al.*[127] have modeled the chain of events from substrate presence to inflammatory stimulation, including increased monocyte adhesion and conversion into foreign body giant cells. These modeling projects use very simplistic representations of how biomaterials affect system processes and might have poor accuracy because they neglect spatio-temporal knowledge. Also these models are coarse-grained and do not contain specific pathway knowledge. In some cases the effects of biomaterials on a system can be better understood when they are studied in relation to more specific pathways rather than the effect on general biological processes.

5.513.4.2. Pathway Modeling for Process-Centered Approaches

With recent advances in systems biology, well-defined and quantitatively calibrated pathway models are now available for apoptosis,[128] the cell cycle,[129] and various growth factor-induced signaling pathways.[130] Sorensen *et al.*[131] *built a* model of platelet activation incorporating the interactions between thrombin, prothrombin, and its inhibitor. Their model predicts the effects on platelet activation from the shear stress of blood flow, and adhesion to biomaterials, which are used as cardiovascular implants. In their subsequent publication[132] the model was validated using a collagen type-I substrate and a parallel-plate flow to show experimental results that agreed with the model predictions. Diverse types of information, about interaction pathways, cell-specific interactions, biophysical characteristics, and substrate–cell interactions, were integrated through modeling to provide guidance for the development of substrates with improved outcome in a physiological context.

5.513.4.3. Pathway Modeling for Activation of TGF-β1 in Liver Fibrosis

Our team undertook a process-centered approach aimed at regenerative therapy for liver fibrosis (Figure 2). Liver fibrosis is driven by high levels of the cytokine TGF-β1 and by the accumulation of fibrotic proteins secreted from activated hepatic stellate cells (HSCs). There are many causal influences in liver fibrosis, but we do not seek simply to perturb the most important cause driving the disease because the mechanisms important to fibrosis are also likely to be important for beneficial wound healing, repair and regeneration. Indeed, elevated TGF-β1 and HSC activation occur normally when the liver regenerates after an injury, and we do not want to impair the pathways of natural regeneration. Rather than considering alternative perturbations individually, we studied the system of TGF-β1 and closely related pathways, seeking to understand the differences in how a healthy system avoids excessive levels of TGF-β1 after a single injury repair event, versus how TGF-β1 activation remains pathologically elevated in the case of chronic injury and fibrosis.

TGF-β1 is secreted in latent form and its bioavailability is regulated by extracellular activation. We studied the regulation of TGF-β1 activation by measuring levels of TGF-β1 activating

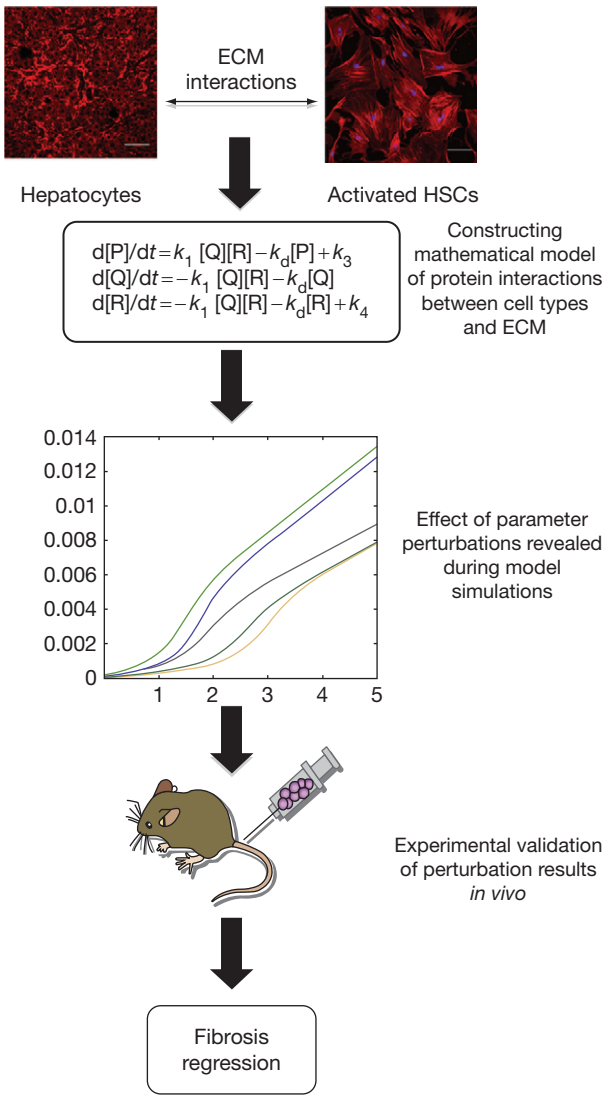

Figure 2 Workflow of systems biology-oriented therapy for liver fibrosis.

proteins in normal liver and at stages of fibrosis progression in rat models of liver fibrosis.[134] Thrombospondin-1, a known activator of TGF-β1, is strongly upregulated during liver fibrosis and has already been implicated as a driver of fibrosis.[133] Plasmin, another activator of TGF-β1, was found at significant levels normally and at dramatically lower levels during liver fibrosis progression.[133] This suggests that plasmin-mediated activation of TGF-β1 occurs in normal liver, and suggests that the thrombospondin-1 pathway becomes a more prominent mode of activation during fibrosis. Toward understanding why normal and fibrotic liver would utilize different pathways of TGF-β1 activation, one notes that TGF-β1 increases expression of plasminogen activator inhibitor-1 (PAI-1), an antagonist of plasmin activation.[135]

The decreased plasmin signaling during fibrosis thus has an explanatory mechanism, but why is the thrombospondin-1 pathway not more active in normal liver, except during injury response? A combination of results *in vitro*, *in silico*, and *in libris* (from data in the published literature) allowed us to establish a system-level model in which plasmin is acting both as a low-potency activator of TGF-β1, and a high-potency antagonist of the thrombspondin-1 pathway. Through its direct cleavage of thrombospondin-1, plasmin antagonizes the thrombospondin-1 pathway of TGF-β1 activation and reduces net TGF-β1 activation.[134] Therefore, plasmin has different downstream effects depending on the level at which it is studied. At a molecular level and in isolation, plasmin functions directly to activate TGF-β1. Meanwhile in a system of extracellular proteins, plasmin functions indirectly to decrease TGF-β1 activation. A process-centered mindset for approaching system-level issues facilitated our discovery of these dual roles of plasmin in the regulation of TGF-β 1 activation.

We next studied how this TGF-β1 activation machinery could be exploited for the therapeutic goal of triggering regeneration. A previous study of plasmin regulation and its upstream activation by uPA (urokinase-type plasminogen activator) revealed that plasmin activation occurs through a bistable switch-like feedback mechanism.[136] Combining this bistability with the previous model of TGF-β1 activation by plasmin and thrombospondin-1 yielded the hypothesis that, changes in the balance between plasmin and thrombospondin-1 might cause a distinct switch-like transition in the regulation of TGF-β1. Using rat models of liver fibrosis, sources of plasmin were added *in vitro* to the culture medium or *in vivo* by transplanting plasmin-secreting hepatocytes into the host.

The results in both experiments showed that TGF-β1 levels decreased and fibrotic disease markers returned to near-normal levels.[134,137] Elevating plasmin as a method to antagonize TGF-β1 is counterintuitive, but it proved to be remarkably effective. Moreover, it mimics the correlations found in the physiological system *in vivo*, and it may prove to be part of the physiological mechanism by which healthy liver tissue naturally switches away from fibrotic pathways during a normal injury response. From an engineering viewpoint, plasmin may also prove to be a relatively safe, robust way to downregulate TGF-β1 because its dual nature allows any overdose of its strong, indirect antagonistic function to be self-corrected via its mild but direct activating function.

Taking a process-centered approach helped us in multiple ways: to integrate knowledge about the various observed effects, to gain mechanistic insight into the switch-like regulation of the system, and to design an effective intervention for regenerative medicine in a rat model of liver fibrosis. However, the system-level nature of the project was only made possible by unusually extensive previous work that elucidated the detailed effects of the individual participants, at the molecular level and the cellular level. For general problems without such resources available, future research will be forced to establish the interactions and molecular relationships explicitly, perhaps one day simply from data mining and knowledge discovery in databases, or for the near term via additional biological experiments *ab initio*.

5.513.5. Future Outlook

Future goals for biomaterials and tissue engineering will require multiple interdependent components (biomaterials, cells, constructs), each with multifaceted optimization and variable parameters. Instead of the discrete or component based approaches studying particular biological outcomes of tissue engineering (**Table 1**) focus should be directed toward a systems-level perspective or process-centered approach.

Biochemical responses need to be assessed, possibly including functional genomics, proteomics, metabolomics, or other

Table 1 Broader perspectives of process-centered implications and approaches to study functional outcomes of tissue engineering

	Tissue engineering approaches	*Biological effects studied using tissue-engineered constructs*	*References*
Process-centered tissue engineering approaches	High-throughput approaches with process-centered implications	Mesenchymal stem cells gene expression	39
		Cell adhesion studies	40
		Embryonic stem cell fate	42
		Osteoblast adhesion, spreading and proliferation on biomaterial	49
	Classical biophysical models	Effect of cells on substrate/ECM compaction	113
		Bone regeneration on scaffolds	115
	Network reconstruction	Cancer gene prediction from B cell lymphoma interactome	118
		Mesenchymal transformation	121
	Systems modeling in tissue constructs	Stem cell attachment and spreading on *ex-vivo* implants	124
		Angiogenesis of human embryonic stem cell on scaffolds	123
	Pathway modeling for process-centered approach	Modeling the effect of vascular implants on the platelet aggregation	131
		Modeling activation of TGF-β1 in liver fibrosis	134

system-wide '-omic' responses. In addition, physical responses such as adhesion, migration, and morphological change need to be assessed. The chemical and physical properties of biomaterials would strongly influence cellular response and these parameters depend on both the underlying biomaterial as well as adsorbed proteins. On top of this are the biochemical and biophysical cues from the culture environment and the host microenvironment. Given this multilayered complexity, the most efficient and useful heuristics for correlating key experimental parameters with *in vivo* outcomes are to focus on biological processes and to define intermediate goals that have systematic and quantitative relationships with respect to the ultimate *in vivo* goal. Meanwhile, high-throughput methods will provide fast, parallel fabrication and measurement of cellular and tissue constructs,[33] and combinatorial synthesis approaches will be increasingly useful for biomaterials development.

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