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2011 Biofabrication 3 030202

(http://iopscience.iop.org/1758-5090/3/3/030202)

Biofabrication **3** (2011) 030202 (8pp) doi:10.1088/1758-5082/3/3/030202

EDITORIAL

Engineering a 3D, biological construct: representative research in the South Carolina Project for Organ Biofabrication

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The SC Project is an alliance of 10 colleges and universities working together to achieve the goal of engineering a functional, 3D, bioengineered construct. Scientific progress includes computational modeling of vascular trees and experimental testing of natural and engineered constructs. Future directions of the science focus on overcoming challenges such as scalability, sustainability of biofabricated constructs, and identification of chemical or physiological factors that can accelerate the differentiation and maturation of biofabricated vascular tissues (maturogens). Studies include those of hemodynamic forces or growth factors that can promote expression and assembly of collagen and elastin fibers.

Introduction

The relatively small scientific community inherent to an emerging technology such as biofabrication necessitates extensive networking, collaboration and communication for rapid development. The research of the South Carolina Project for Organ Biofabrication (SC Project) is guided by five scientific 'thrusts' and supports collaborative initiatives among stem cell biology, cell and developmental biology, molecular biology, tissue engineering, extracellular matrix biology, biomathematics, and chemical and mechanical engineering. The thrusts of the SC Project align with the top strategic priorities in the field of tissue engineering: angiogenic control and regulation; stem cell science; systems biology; cell sourcing and cell/tissue characterization; and manufacturing/scale-up [1]. Thrust I focuses on development of the necessary tools, including mathematical models, to support testing of vascular constructs and needed software for bioprinting. Thrust II focuses on directed differentiation of adult stem cells into monomer units of vascular cell types. For thrust III, the structure-function and biomechanical properties of engineered and naturally occurring branched vascular trees are being investigated. Thrust IV is concerned with the actual assembly and integration (or biofabrication) of vascular spheroids into linear or branched 3D, hollow tubular segments of different diameters. Ongoing studies include assembling vascular spheroids of different or mixed stem cell lineages, some with a commitment to smooth muscle, others with potential to form endothelium or both phenotypes (mixed lineages). Thrust V focuses on accelerated tissue maturation and endothelialization of biofabricated, branched vascular tubes.

Research

Scientific results of the SC Project, which began in July 2009, have appeared in 52 peer-reviewed journal articles and 41 conference proceedings. Select results highlighting the research activities of the project are presented here.

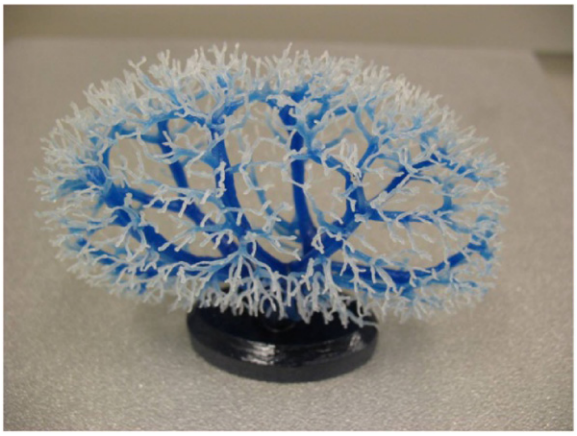

Figure 1. A kidney vascular tree prototype printed with a ProJet rapid prototyping system using HR200 plastic material showing a range of 0.5 mm to 1 mm diameter resolution of branching. (Image: William Wayne Beaver, Director, Advanced Manufacturing, York Technical College).

Computational/modeling efforts

Branching patterns are organ specific and have both symmetric and asymmetric characteristics. The biofabrication of a vascular tree requires detailed knowledge of anatomical geometry, branching characteristics, and structure-function and biomechanical properties of naturally occurring vessels. Comprehensive integrated datasets are still largely lacking. Analyses of branching geometry are based on 3D images prepared by different methods such as corrosion casting or injection of contrast agents followed by x-ray, MRI or micro-CT imaging, or by computer-aided reconstruction of serial histological sections. To advance thrust I, SC Project investigators have demonstrated that micro-CT provides the most rapid method with sufficient resolution for analysis of any selected organ having a branched vascular tree [2]. SC Project investigators are using CAD technologies to create a blueprint for engineering a seamless branching vascular tree. Data inputs for *in silico* simulations are being acquired from 3D images of naturally occurring vascular trees (e.g. kidney, lung, heart). SC Project investigators have printed an intraorgan branched vascular tree for a kidney using a ProJet rapid prototyping system and HR200 plastic material. The prototype shown in figure 1 is based on a computer-aided design provided by the University of Oxford (UK) using expertise and facilities at 3D Systems University/York Technical College in Rock Hill, SC. This initial success and preliminary data strongly suggest that existing rapid prototyping technology using layer by layer addition of building blocks has sufficient resolution for bioprinting a complex branched vascular tree.

The technology of biofabrication provides a plethora of challenges and opens up a wide range of uncharted courses for biomathematicians to derive plausible computational models [3]. The SC Project has established a PhD track in applied mathematics at the University of South Carolina and resources are being used to hire core faculty having expertise in mathematical biology and cellular dynamics, computational biology and multiscale modeling of biological materials. These new faculty and students are developing mathematical models to track the evolution of the multiple material interfaces and tissue development as well as transport of the internal micro/meso-structures coupled with the spatial-temporal dynamics of cell signaling. SC Project biomathematicians are working closely with experimentalists to tackle two major challenges that are significantly advancing the aim of thrust IV, i.e. biofabrication of 3D hollow 'Y' or 'T' vascular units. In one, they have developed a mechanistic model and a state-of-the-art computational tool to study the focusing process in the microfluidic device during biofabrication; in the other, they have developed a mechano-chemical model to address the issue of maturation, fusion, and lumen formation of multicelluar spheroids by incorporating the cellular response to environmental signals by extracellular signaling molecules. Specific technical details of this approach are provided elsewhere [4]. For instance, the transport equation for the fluid mixture system of the

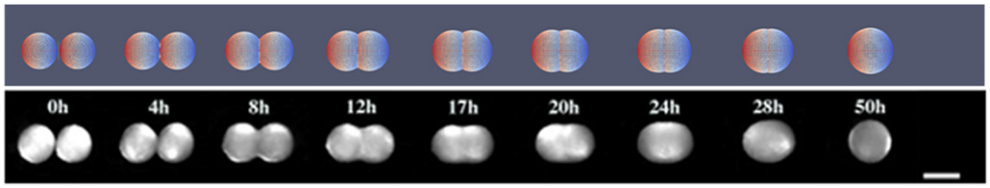

Figure 2. Theoretical (upper) and experimental (lower) tissue spheroid fusion. Theoretical results depict front tracking simulations of the fusion of two tissue spheroids. Experimental results depict spheroids of adipose derived stem cells, formed using the hanging drop method, and the fusion process was followed for two days. The scale bar on the experimental result is 500 μm.

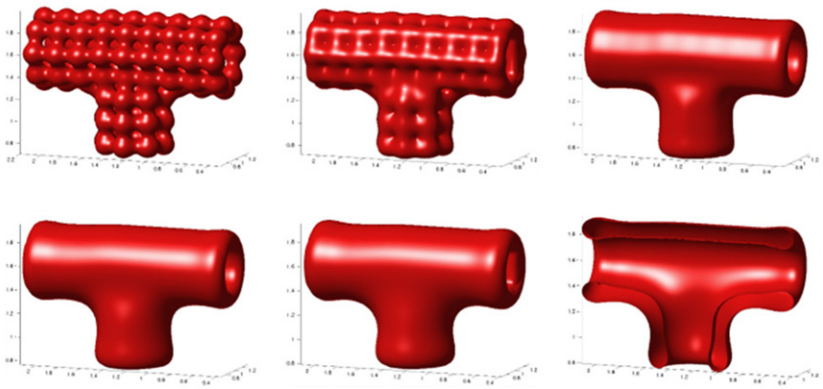

Figure 3. T-shaped branching vascular construct simulated for a layer-by-layer deposition. Authentic branching vascular trees are made up of select fundamental shapes such as T- or Y-shaped junctions. This simulation shows that computational models are capable of capturing the essential structures of a naturally occurring vascular tree.

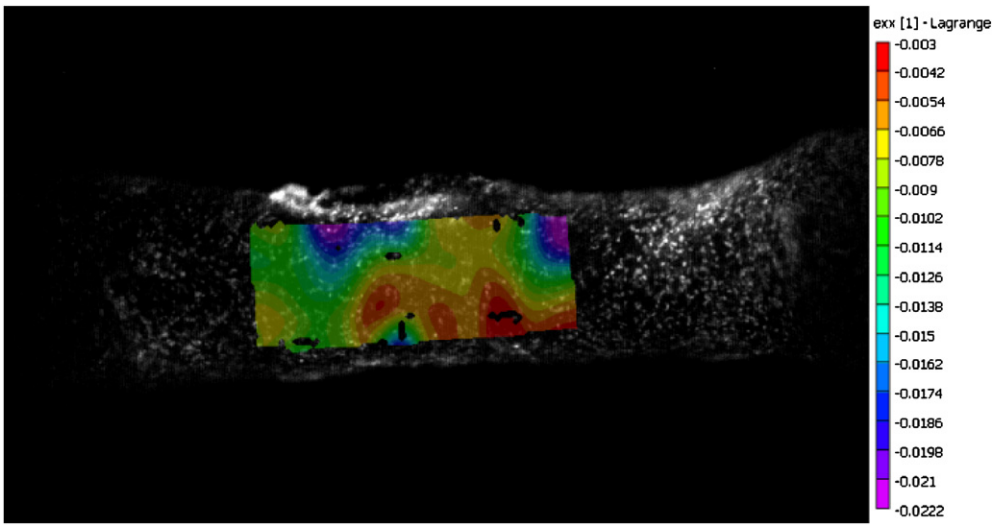

Figure 4. Color-coded local strain (ε_{xx}) on mouse carotid artery during pressurization, measured by 3D digital image correlation.

inside and outside of multicellular spheroids is given by

$$\nabla \cdot v = 0$$
$$\rho \frac{\mathrm{d}v}{\mathrm{d}t} = \nabla \cdot (\phi\tau_1 + (1-\phi)\tau_2) - [\nabla p + \gamma_1 kT \nabla \cdot (\nabla\phi)]$$
$$\frac{\partial \phi}{\partial t} + \nabla \cdot (v\phi) = \nabla \cdot \lambda \left(\nabla \frac{\delta f}{\delta \phi} \right)$$

where v is the averaged velocity, ϕ is the phase variable to label each fluid, $\rho = \phi\rho_1 + (1-\phi)\rho_2$ is the effective density for the binary fluid, p is the pressure, ρ_i and t_i are the density and the extra stress tensor for viscous fluid $i = 1, 2$, T is the temperature, k is the Boltzman constant, λ is the mobility coefficient, a function of the volume fraction ϕ and the

biology of multicellular spheroids, γ_1 is a parameter measuring the strength of the conformation entropy and γ_2 is the strength of the bulk mixing free energy defined by $f = \frac{\gamma_1}{2} kT \|\nabla \phi\|^2 + \gamma_2 KT\phi^2 (1-\phi)^2$.

When developing mathematical models and simulation tools for the focusing process, SC Project investigator Liu and co-workers have implemented and combined the models using the powerful front-tracking package [5], and a host of in-house phase field programs [4, 6]. The multicelluar material is modeled as a multiphase complex fluid with live cells, extracellular matrix, solvent and additional dendrimers/$CaCl_2$ as separate phases. The simulation is done within the geometry of the microfluidic device. The ambient fluid, made up of mineral oil, is treated as a viscous fluid. This numerical tool is enabling the investigation of the effect of concentration of dendrimer and/or $CaCl_2$ solution, the channel size, and the focusing solution input rate as well as the mixing mechanism on the size distribution of cellular spheroids and the production speed. These computational simulations can facilitate optimization of a biofabrication device design in lieu of the experimental trial and error approach. The upper and lower images of figure 2 depict the simulated and experimental results, respectively, for the fusion of two cellular spheroids. Figure 3 depicts the morphological evolution of a biofabricated T-shaped junction using multicellular spheroids. The models used in these simulations are simple mechanistic models for two phase viscous fluids [6].

Experimental studies

The research aims for thrust III of the SC Project include (1) comparison of tissue engineered vascular structures with natural ones to prove functionality and maturity of the engineered constructs; and (2) detailed functional and biomechanical characterization for validation of biofabricated tissue engineered constructs. Appropriate datasets for biomechanical properties of naturally occurring vascular trees will enhance *in silico* modeling of the requirements to engineer vascular segments. Datasets are being generated that include characterizations at multiple levels from nano to cellular to whole tissue. At the tissue level, SC Project engineers Sutton and Lessner were the first to demonstrate the feasibility of using 3D digital image correlation (DIC) to measure strain fields on a micrometer length scale on soft tissues using mouse carotid artery specimens (figure 4) [7]. They have shown the advantages of using this noncontacting method for strain field measurements on fragile soft tissues which may undergo large out-of-plane deformations (e.g. during pressurization). They have also developed a method to label soft tissues with fluorescent microspheres to create a high-contrast pattern for 3D DIC analysis [8]. Using this protocol, the fluorescent microspheres are covalently bound to proteins on the tissue surface. This method can be used to create a speckle pattern which remains intact when the specimen is submerged. In addition, they have validated the application of 3D DIC for strain field measurements on soft tissues by demonstrating good agreement between global strain values measured in the Bose ELF mechanical test bed and averaged local strain fields measured simultaneously using a two-camera stereo-microscope system [9].

Recently, Sutton and Lessner have shown that surface strains acquired by 3D DIC can be used as input to an inverse finite element (FE) problem to estimate material parameters in a structure-dependent, soft tissue constitutive model [10]. They used ABAQUS, a commercially available FE software package, for simulation of boundary value problems using this constitutive formulation. They demonstrated that the orthotropic, hyperelastic constitutive model could be incorporated into the ABAQUS framework in a FE model of the normal mouse artery. After identifying the model parameters from surface strain data acquired during vessel pressurization and tensile loading, they used the model to predict through-thickness stresses and strains under arbitrary loading conditions. Their approach is diagrammed in figure 5.

The first step is to identify the material parameters for a given constitutive model using an inverse method and 3D DIC data from pressurization and tensile loading tests. They have tested this approach using several different constitutive models, and found a very good match to experimental pressure–strain data and tensile stress–strain data from the normal mouse carotid artery using an orthotropic, hyperelastic model [10]. Mathematical details of the

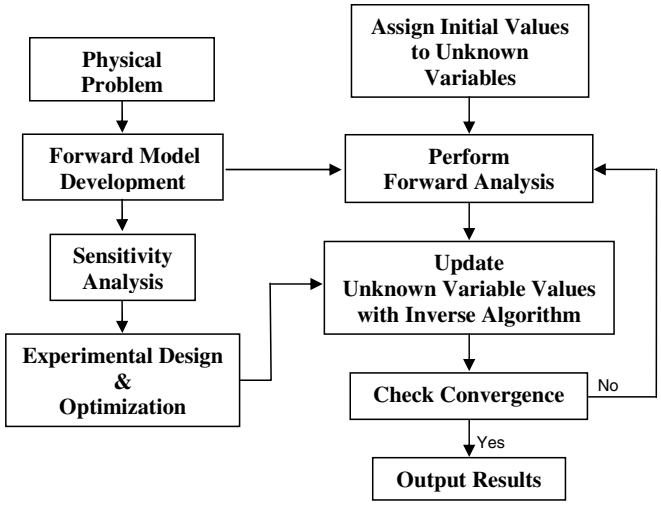

Figure 5. Schematic of inverse analysis.

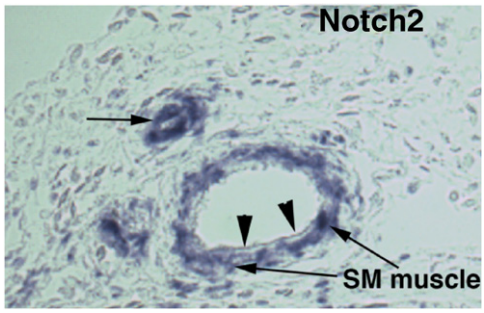

Figure 6. Intense Notch2 expression is seen exclusively in the tunica media of the forming coronary vessels (arrows). Note: endothelial cells do not express Notch2 (arrowheads).

4-parameter constitutive model, originally formulated by Bischoff and colleagues, have previously been published [11]. By determining the constitutive parameters that describe the behavior of the native artery, they have established a benchmark to measure the ability to produce engineered vessels having equivalent mechanical behavior.

The basic developmental biology research described by thrusts II and V of the SC Project are being driven by an editorial in *Tissue Engineering* [1] noting that angiogenic control, stem cell direction/differentiation and molecular/systems biology were the top three dominant concepts to advance tissue engineering to clinical application in the short term. Three-dimensional vascular tree construction is the top priority for today's tissue engineering technologies, including biofabrication. Many studies involving vascular biology and engineering have focused on differentiation of vascular endothelial cells and their network formation; however, few studies have addressed directed differentiation of vascular smooth muscle (SM) cells to form 3D vascular trees. To establish the foundation for engineering 3D vascular trees, SC Project investigator Sugi is studying the role of a defined factor, Notch2, for directed differentiation of vascular SM cells.

Notch is a highly conserved signaling pathway involved in many aspects of development and congenital cardiovascular diseases [12]. Notch pathway components, Notch receptors (Notches 1–4) and ligands (delta-like 1, 3, 4 and Jagged 1, 2) are implicated in angiogenic vascular remodeling and artery/vein specification [13–15]; however, Notch2-null mice die at embryonic day ED 9.5–10.5 [16] before vascular SM cell differentiation, which hampered elucidation of the role of Notch2 in SM cell formation and differentiation.

As a first step to study the role of Notch2 in vascular SM cell formation/differentiation, Sugi and colleagues localized Notch2 during embryogenesis and found intense Notch2 mRNA expression exclusively in SM cells in the tunica media of the developing vasculature (figure 6). Importantly, endothelial cells do not express Notch2. These data provide a

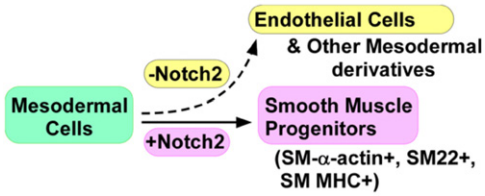

Figure 7. A schematic illustration shows segregation of vascular smooth muscle lineage from mesodermal cells.

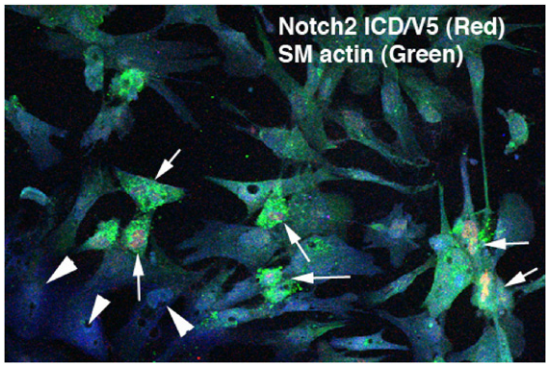

Figure 8. Posterior mesodermal cells from H-H stage 5 chick embryos were infected with Ad-Notch2$_{ICD}$. Notch2$_{ICD}$-positive cells (red nuclei, arrows) express SM α-actin (green). All nuclei were stained with Topro3 (blue). Arrowheads indicate SM α-actin negative cells.

foundation to test the hypothesis that Notch2 regulates initial formation of vascular SM cells by segregating SM cell lineage from naive mesodermal cells as illustrated in figure 7.

To test their hypothesis that Notch2 regulates vascular smooth muscle cell formation/differentiation, investigators used cell cultures from Hamburger and Hamilton stage-5 [17] chick posterior mesoderm which does not express SM markers. Notch2 activation was performed by treating the cultured mesodermal cells with adenovirus encoding the Notch2 intracellular domain (Ad-N2$_{ICD}$, kindly provided by M Mercola, Burnham Medical Research Institute). As seen in figure 8, N2$_{ICD}$-positive cells express a SM marker, SM α-actin, suggesting that Notch2 activation has potential to induce SM cell formation from the naive mesodermal cells.

These studies to elucidate embryonic vascular SM formation are allowing SC Project investigators to identify the optimal conditions for directed differentiation of stem cells into functional SM cells, which is essential to develop novel approaches to construct functional 3D vascular trees.

Other ongoing research within the concept priorities of angiogenesis and stem cell direction/differentiation involves the study of hypoxia as a potential environmental factor contributing to accelerated tissue maturation, a major focus of thrust V. Cell response to reduced oxygen tension is primarily regulated by hypoxia-inducible factors (HIFs) [18, 19]. HIFs are transcription factors which belong to the bHLH-PAS (basic Helix-Loop-Helix-PER-ARNT-SIM) family. HIF-1 is a heterodimer composed of HIF-1α and HIF-1β. HIF-1β, also referred to as ARNT (aryl hydrocarbon receptor nuclear translocator), is stable regardless of local oxygen tension whereas HIF-1α is only stable under hypoxic conditions. The heterodimer can only form under hypoxic conditions (<5% oxygen) leading to transcription of genes which contain hypoxia-responsive elements (HREs) in their promoters, introns and/or 3′ enhancers. HIF-1 interacts with HRE leading to transcription of oxygen-regulated genes like vascular endothelial growth factor (VEGF) and certain stress-response genes. Oxygen-sensitive regulation of transcription allows adaptation to changing oxygen tensions and survival in environments where hypoxic conditions persist.

SC Project investigator Blanchette and co-workers have used a responsive, fluorescent marker to identify regions of cell aggregates where HIF-1 is stable. The marker is a recombinant adenovirus, whose construction has been detailed previously [20]. Three-dimensional aggregates of cells could display varied activity of hypoxic signaling. Hypoxic signaling can influence stem cell differentiation and angiogenesis which makes it an

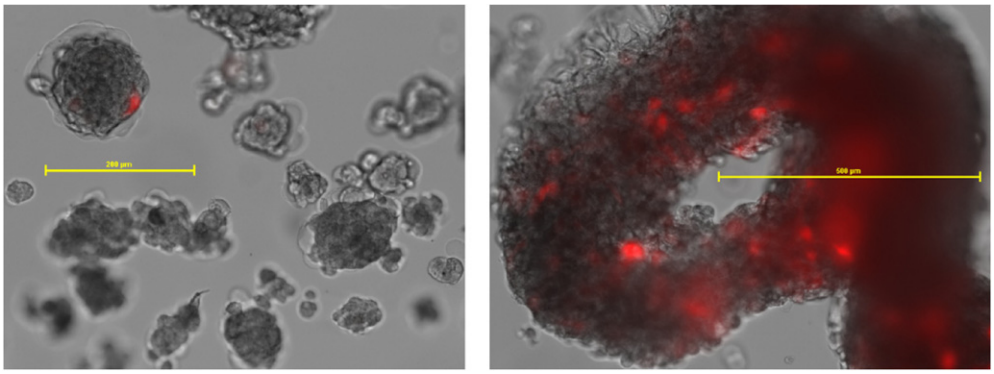

Figure 9. Aggregates of ADSCs infected with a fluorescent hypoxia detection marker imaged after 48 h of culture under 20% oxygen conditions. The scale bars are 200 (left) and 500 (right) μm.

important pathway to monitor in adipose-derived stem cell (ADSC) aggregates. The location of a cell within an aggregate and the local oxygen tension would be examples of parameters which would affect this phenomenon. Figure 9 shows aggregates of ADSCs cultured in 20% oxygen for 48 h following aggregation. Hypoxic signaling is indicated by the presence of a red fluorescent protein whose transcription is regulated by an HRE trimer. The diameter of the aggregates impacts the presence of hypoxic signaling. Central regions of large aggregates, which fused into a ring-like structure, display the fluorescent signal at this early time point, whereas peripheral cells do not.

Each research thrust of the SC Project has been designed with the intent to support priority concepts that will advance the translation of biofabrication technologies to the clinical setting. The design requires the expansion of intellectual capital, physical resources and collaboration to create small scale prototypes of vascular tissue that can be ramped up to functional biofabricated constructs. The choice of adipose cells is based on their availability in large numbers (including commercial sources), human derivation, and tendency to differentiate into vascular tissue phenotypes. However, SC Project investigators are exploring other options, including bone marrow-derived cells and induced pluripotent cells. Funding priorities span those studies that identify genes and proteins that control 3D tissue formation and functional complexity; accelerate tissue, spheroid and cell aggregation; and lead to computer controlled robotic nozzle design for patterned deposition of biologics.

Future directions

Research

The five research thrusts of the SC Project are considered a guide to advance the field of biofabrication and more specifically the technology of bioprinting. However, research priorities of the SC Project in order of importance are angiogenic control and regulation, stem cell science, systems biology, cell sourcing and cell/tissue characterization, and manufacturing/scale-up. Significant progress has been made in all five thrusts, especially stem cell biology and angiogenic control, and the overall feasibility of bioprinting as a technology has been demonstrated *in silico*. Several major challenges remain to be addressed for bioprinting. First and foremost is scalability. To biofabricate a functional 3D tissue construct, it will be necessary to generate millions of tissue spheroids and develop scalable technology for spheroid biofabrication. For bioprinting to become a viable means of biofabrication, a new generation of robotic bioprinters specifically designed for organ printing technology is needed. Engineered tubules created by the fusion of vascular spheroids currently take months to mature. A major challenge is to sustain the viability of printed constructs during maturation, and even accelerate maturation. Two extracellular structural proteins, collagen and elastin, are responsible for 95% of the biomechanical properties of vascular walls in a natural vascular tree. Collagen and elastin take time to be secreted, assembled into fibrils and cross-linked resulting in the need to advance perfusion

reactor technology. Project resources are targeted for investment in seed grants focused on the identification of maturogens for accelerated vascular tissue maturation. Development of high throughput *in vitro* assays for screening maturogenic factors and designing maturogenic cocktails of growth factors is also a priority.

Additional emphasis has been placed on producing computer simulations of vascular tissue spheroid fusion in hydrogels and bioreactors, and the development of a computer-aided design for intraorgan branched vascular trees using functional representation software. This task will require not only development of new software, mathematical models and computer simulations, but also the utilization of large quantities of data storage and a well-developed cyberinfrastructure.

Acknowledgments

The authors would like to acknowledge support by the National Science Foundation under grant no EPS-0903795, EPS-0919440 and EPS-1006833, the State of South Carolina, technical support provided by William Beaver of York Technical College, and editorial support provided by the staff of the International Society for Biofabrication.

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