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Future Approaches to Organ Regeneration: Microscale Environments, Stem Cell Engineering, and Self-assembly of Living Tissues

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Abstract

A promising means to address the limited supply of donor tissue is through the generation of artificial organs consisting of cells and materials. Progress towards this goal is limited by three main obstacles namely the generation of a sufficient number of cells specific to the organ, the arrangement of these cells in a functional tissue architecture and the delivery of nutrients and removal of waste from the tissue mass. This chapter describes the emerging approaches that may be achieved by the control of stem cell differentiation, control of the local tissue environment on the microscale, and the generation of complex structures containing multiple cell types.

1. Introduction

Transplantation has emerged as a viable approach to regenerate tissue and restore lost function however, the demand for available organs exceeds supply[1]. As of April 20 2009, 101,693 people were waiting for an organ transplant[2]. For those that do receive transplants, the constant threat of attack and rejection of the donated organ by their immune system necessitates a lifetime of immunosuppressive therapy[3]. This is undesirable since suppression of the immune system presents unwanted complications such as elevated risk of cancer and other organ failure[4-6]. Transplants may also succumb to long term rejection, failing after many years of asymptomatic function[7]. Thus, a promising solution

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is the generation of a new organs or replacement tissues to avoid waiting lists and immunosuppressive related issues.

1.1 Organ structure and Requirements

Each organ contains billions upon billions of cells that are exquisitely arranged. A large portion of these cells are organized into functional units (groups of cells or structures) that perform specific biological processes. Examples of organs with functional units are the liver (comprised of lobules), the kidney (comprised of nephrons) and the pancreas (comprised of islets). Other cells make up the vascular, neural, lymph and other specialized tissue structures (e.g. ducts, sinusoids) that support the function of the organ.

At the microscale, organs possess unique microenvironments believed to be important for cell function. Organs consist of specialized cells performing specialized functions in close proximity to one another. Cell-cell proximity and communication result in the formation of specialized microenvironments. In many tissues this cell-cell signaling is important for cell differentiation and maintenance of cell phenotype. Thus recreating the microarchitecture and the local cellular environment is also critical to organ function. The purposeful design and engineering of cell microenvironments within the tissue structure and architecture is a promising means to address some of the limitations and obstacles to organ regeneration.

To support the metabolic demands and physiologic function of these cells, organs are highly perfused, often possessing intricate vascular networks. After the lungs, the liver is one of the most highly perfused organs. The perfusion rate of the liver in milliliters per minute averages twice the tissue mass (200 mL/100 g tissue)[8]. Re-establishing these high perfusion rates is a challenge for tissue engineers as they strive to develop organized and vascularized tissues that replace organ structure and function.

1.2 Practical obstacles to organ regeneration

1.2.1 Cell number and differentiation

The number of cells in a mass of tissue such as an organ presents significant hurdles to organ regeneration. The logistics involved with the maintenance and expansion of cells suitable for the population of an organ is a burdensome task. In addition, the delivery of such a large number of cells to a tissue engineered scaffold and the maintenance of their viability presents significant difficulties. This is further complicated by the fact that most organs contain multiple cell types in close proximity organized with microscale resolution. Thus, one of the formidable goals of large, scaffold based tissue engineering is to generate scaffolds that can direct and coordinate cell growth and organization from a more manageable number of precursor cells. Ideally these precursor cells would be derived from the patient so as to avoid any interactions with the immune system.

1.2.2 Arrangement of cells into a functional architecture

Organs are generally arranged into functional units. These units perform the “work” of the organ and possess features with microscale spatial resolution. The organ is supported by a network of vascular, connective tissue and other cell types also possessing features on the micron scale. For example, the tubules in the nephrons of the kidney are approximately 50µm in diameter, islets in the pancreas average 400 µm in diameter and liver lobules are

about 1000µm in diameter with central vein being about 200 µm in diameter[9]. This presents another layer of fabrication complexity whereby large, macroscale structures musts be generated that possess micron scale resolution (figure 1).

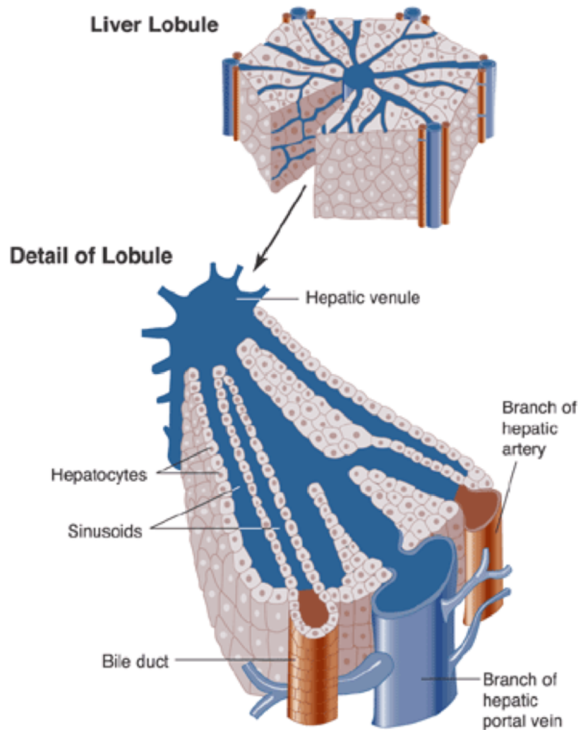

Figure 1. Top. Diagram of the liver lobule, a hexagonal structure with central and peripheral ducts. Bottom. Lobules contain hepatocytes arranged in complex formations along sinusoids. From Cunningham *et al.,* 2003

1.2.3 Nutrient delivery and waste removal

Vascularization is a key hurdle to overcome for the maintenance of cells in large cell laden constructs. In the body, cells are generally located within 200 µm of vessels, suggesting a minimum spatial resolution for blood carrying conduits. Work with cell laden hydrogels suggests that channel spacing of 200 µm or less may be optimal for the maintenance of cell viability within perfused hydrogels *in vitro* [10]. In addition, conduits for the guidance and development of nerve and lymph systems are required. Perfusion of the tissue must also be maintained without long-term interruption from initiation to implantation. This suggests that a vascular system should be in place prior to cell seeding and that this system must be able to connect to the vasculature and withstand physiologic blood pressures in excess of 120 mm/hg[8].

A potential means to address the issues of donor organ shortage and organ rejection is the generation of artificial organs through tissue engineering. Progress towards this goal

is limited by three main obstacles namely the generation of a sufficient number of healthy cells specific to the organ, the delivery of nutrients and removal of waste from the tissue mass and the arrangement or organization of these cells into a functional architecture. This paper describes emerging approaches for organ regeneration that may be achieved by control of stem cell differentiation, control of the local tissue environment at the microscale, and the generation of complex structures containing multiple cell types.

2. Cell Sources For Organ Regeneration

Organs contain large numbers of specialized cells, the generation of which presents a significant challenge for organ regeneration. In this regard, stem cells are promising candidates as a cell source, because of their capacity for self-renewal and their ability to produce many different cell types.

While stem cells can be harvested from different tissues, a few types are of particular interest for organ regeneration. Embryonic stem cells (ESCs) are derived from the inner mass of the blastocyst, a cellular structure formed early in embryonic development. ESCs are pluripotent stem cells since they can form all cell types in the body. Adult stem cells (ASCs) are cells found in a developed organism that have a capacity for self-renewal and can differentiate into a more specialized cell type. Induced pluripotent stem cells (iPSCs), are an artificial class of stem cells that have recently been developed. iPSCs are derived from a non-pluripotent cell by the inclusion of certain genes[11, 12].

2.1 Strategies and Techniques for Understanding and Directing Stem Cell Behavior

Realizing the therapeutic potential of both ASC's and ESC's presents a number of challenges. For example, the pluripotent natue of ESCs and their relative ease of isolation makes expansion as promising as it is problematic. The generation of differentiated cells with a high degree of specificity remains problematic for clinical applications since the transplantation of undifferentiated cells with the inherent ability to generate undesired tissue may result in a tumor [13-16]. While the problem of uncontrolled differentiation is reduced by using ASCs, they have been difficult to isolate and expand. However, since ASCs for therapeutic applications would likely be derived from the patient they have the added advantage of immunogenic compatibility.

A potential means to address these issues is to exert control over stem cell behavior during expansion and differentiation. Control of stem cell behavior can be achieved by manipulating environmental cues, such as intra and extra-cellular signals, interaction with the ECM and various other stimuli such as mechanical and electrical stimulation. Not surprisingly, research in this area has focused on the regulation of the stem cell microenvironment[17, 18] and a number of technologies and techniques have been developed to modulate and explore stem cell behavior *in vitro* as outlined below.

2.1.1 Two dimensional (2D) cultures

Coculture patterning is a useful method to study *in vitro* cell–cell interactions to control ESC differentiation and to generate tissue engineered constructs. Coculture techniques are used to discover the interaction of ESC with other cellular patterning. To organize different regions on a surface with different chemical properties which can boost selective attachment of specific cell types, co-culture cellular patterning has been used [19-21].

2.1.2 Three dimensional (3D) cultures

Mono-layer cell cultures have been successfully developed to study cell proliferation, differentiation, cell-cell and cell-ECM interactions, however 3D functional engineered tissues are much more complex due to the hierarchical arrangements of multiple cell lines with complex vascular structure to maintain nutrient transport to and waste removal from the tissue [22-24]. Enriched culture conditions based on specific lineage of precursors can be generated to control the *in vitro* differentiation pathways. *In vitro*, ESC differentiation can be directed through different strategies and techniques that involve the use of chemical signals and molecular cues [25-34]. Stem cells are commonly differentiated within a suspension culture of cell aggregates called embryoid bodies (EBs) which contain derivatives of all three embryonic germ layers [35, 36] and mimic some features of normal embryonic development. To mimic functional cues present in the ECM a variety of different methods to stimulate stems cells have been studied. These methods can be divided in two major groups including chemical cues such as the presence of different proteins, and physical means which can be classified in mechanical forces, electrical stimuli, nanometer scale topography of ECM, and heat stimuli.

Cell attachment, proliferation, and differentiation are supported by the natural structure of nano-sized fibers found in the ECM. Scaffolds offer physical support but can also facilitate cell–cell and cell–material interactions found in native tissues. As a result, scaffolds have been designed to mimic a number of features of the ECM[37-41]. Chemical, mechanical, electrical, and thermal cues presented in the scaffold can direct ESC differentiation. 3D scaffolds have been used for stem cell cultures to enhance ESC differentiation by mimicking the *in vivo* 3D micro-environment. In this regard, cell–cell interactions, cell–matrix interactions, and cell signaling have been enhanced by 3D scaffolds[37-41]. Scaffold based studies have been particularly influential in understanding embryoid body (EB) formation. Culture and differentiation of ESCs in 3D polymer scaffolds have been reported leading to the formation of neural, hepatic, and mesenchymal tissues. Complex 3D *in vitro* arrangements have been used to generate tissues with specific embryonic features. Using this approach, ESCs have undergone directed differentiation in poly (lactic-*co*-glycolic acid)_poly(L-lactic acid) (PLGA) polymer scaffolds[41, 42].

Increased attention has been paid to the use of scaffolds as one of the major techniques for stem cell engineered tissues. One of the major breakthroughs in this field has been recently made by Macchiarini *et al.*[43]. They successfully transplanted a tissue engineered airway in a clinical trial. A de-cellularised donor tracheal scaffold was used as a natural 3D airway scaffold without detecting signs of rejection. In this process, cells and MHC antigens were first removed from the human donor trachea. Then autologous mesenchymal and epithelial cells derived from recipient's stem cells were seeded on the airway scaffold, cultured in a bioreactor, and eventually used to replace the left bronchus of the recipient. The implanted airway demonstrated normal form, functionality, and mechanical properties. In contrast with allografted and xenografted tracheal constructs, there was no immunological response to the engineered trachea obviating the requirement for immunosuppressive drugs. They suggested that patients with serious clinical disorders might be treated using a combination of autologous cells and appropriate biomaterials.

2.1.3 Chemical Cues

The influence of intra and extracellular signaling on stem cell differentiation has been extensively studied. Chemical messengers that are known to regulate stem cell

differentiation are growth factors, cytokines, synthetic materials, and biomolecules such as TGF-b1, BMP-2, and BMP-4, Insulin growth factors I, Fibroblast growth factor, Oxytocin, and Erythropoietin[25-34]. Heng *et al.*[34] reported the influence of different growth factors, cytokines, and synthetic chemicals including TGF-b1, BMP-2, BMP-4, Insulin-like growth factor I, fibroblast growth factor (FGF), oxytocin, Erythropoietin, 5-azacytidine, ascorbic acid, retinoic acid (RA), dimethyl sulfoxide, and dynorphin B on the cardiomyogenic stem cell differentiation[34]. For example TGF-b1 family can induce cardiomyogenic differentiation on the human ESCs, carcinoma cells, fetal stem cells, and progenitor cells. Oxytocin, insulin and fibroblast growth factors can also promote cardiomyogenic differentiation of ESCs and carcinoma cells[25, 26]. Schuldiner *et al.* [29] studied the influence of different growth factors on the ESC differentiation. In this study, eight growth factors including NGF, bFGF, activin-A, TGF-b1, HGF, EGF, BMP4, and RA were investigated to analyze their effects on ESC differentiation in culture. The influence of these growth factors was traced on the ECS differentiation to eleven different tissues. In the absence of growth factors ESC were differentiated to several different cell lineages while in the presence of growth factors, just a couple of homogenous lineages with defined morphology such as neuronal cells were produced. ESCs that were treated with bFGF in culture were differentiated to large populations of small fibroblast cells while those ESCs that were treated with RA in culture pronounced neuronal-like cells. They concluded that treating the ESCs by specific growth factors may direct the ESC lineage differentiation.

Synthetic chemical compounds with long active half lives such as dimethyl sulfoxide and ascorbic acid can prolong *in vitro* cell culture over several days and have been used to promote *in vitro* cardiomyogenic differentiation [34, 44-47]. The advantage of synthetic chemical compounds in comparison with natural proteins is that they can be controlled more easily, do not need to be synthesized in living organisms, and have a defined structure. In comparison with growth factors and cytokines, synthetic biomaterials including 5-azacytidine, ascorbic acid, retinoic acid, dimethyl sulfoxide, and dynorphin B, may also enhance cardiomyogenic differentiation of ESCs and carcinoma cells [34].

2.1.4 Mechanical Stimuli

Mechanical stimuli can be used to control stem cell fate and behavior by inducing proliferation or differentiation[48-52]. Mechanical forces in combination with biochemical signals can be used to control differentiation of ESCs for therapeutic applications, however the mechanisms of mechanical induction on ESC fate are not known. Mechanical stimulation can be applied in the forms of contractile, load stretch, cyclic stretch, and shear stress while ECM elasticity and stiffness play a significant role for mechanical stimulation. Huang *et al.* reported that ESCs can be differentiated to vascular cells under *in vitro* pulsatile flow loading[53]. Kada *et al.* [48] showed that cyclic stretch stimulation can change the orientation of induced cardiocyte cells. Ruwhof *et al.* [49] investigated the influence of cyclic stretch on cultured neonatal cardiomyocytes and cardiac fibroblasts to induce the release of growth promoting factors. Saha *et al.* [50] used mechanical strain to regulate stem cell differentiation. They measured the differentiation rate of ESC in the absence and presence of biaxial cyclic strain and concluded that mechanical forces in combination of chemical cues could regulate ESC proliferation and differentiation. Adult cardiomyocytes have been stimulated by contractile mechanical stretching for protein synthesis [51] while passive mechanical stimulation can potentially regulate cardiomyocyte organization into parallel arrays of rod-shaped cells [52].

In addition, differentiation of stem cells can be controlled by mechanical properties of scaffold or substrate. Cells sense and react to the stiffness of the scaffold or substrate

and this phenomenon can be used to direct stem cell differentiation. Engler *et al* demonstrated that mesenchymal stem cell (MSC) differentiation can be guided by extracellular matrix elasticity. MSC's cultured on stiffer substrates resulted in the generation of osteoblast cells while those cultured on softer substrates resulted in the generation of nerve and skeletal muscle cells[54].

2.1.5 Electrical Stimuli

Electrical stimulation in the form of electrical pulses with different frequencies has been used to control stem cell differentiation. Primary cardiomyocytes cultures have been stimulated *in vitro* using electrical stimulation [55-58]. Electrical pulses are reported to enhance ESC cardiomyogenic differentiation within embryoid bodies [55]. Yamada et. al. [56] have also used pulse trains consisting of five pulses with 950 ms inter-pulse time with several different voltage amplitudes including 0,5,10, and 20 volts and examined the influence of inter-cellular and intra-cellular ion balances on stem cell differentiation. They concluded that electrical stimulation somewhat induces EBs to differentiate into neuronal cells. Schwartz *et al*. demonstrated that pulsed electromagnetic fields can enhance BMP-2 dependent osteoblastic differentiation of human mesenchymal stem cells [58].

The Influence of EB Size on ESC Differentiation: The first step for differentiation of ESCs is creation of EBs. ESCs differentiate into three embryonic germ layers: mesoderm, endoderm, and ectoderm through formation of EBs [35, 36, 59-61]. The most important methods for differentiation of EBs are: supplementation with differentiation inducing factors or growth factors, plating EBs on tissue culture plates coated with gelatin, and EB formation from suspension culture [35, 61].

The lineage commitment and ESC fate may be influenced by the size of EBs. EBs with different sizes might differentiate along one particular germ cell lineage compared to others due to differences in nutrient gradient across EBs, mechanical stimulation, and oxygen tension. Reproducing EB size is often difficult and results in heterogeneous differentiation [59]. EB size may directly or indirectly be influenced by micro-environmental stimuli including cell–cell, and cell–ECM interactions and physicochemical factors including oxygen availability, temperature, and pH [62, 63].

A microwell is a convenient microsystem for the study of EBs in high throughput experiments. A microwell is a fabricated device with defined shape and size (approx 100-500 μm diameter) which can be used to culture stem cells and/or to deposit combinations of different ECM materials such as collagen, laminin, and fibronectin. Microwells can be used in a combinatorial fashion to study the effects of different parameters that may promote ESC cluster formation, regulate stem/adult cell proliferation, and control stem cell differentiation. We can also control microwell parameters such as microwell shape and size that shape the cultured EBs and may indirectly influence the morphology of EBs. Therefore microwells may be used to precisely control the microenvironment and in turn to uniformly regulate the differentiation of ESCs. Microfabricated microwell platforms of poly(ethylene glycol)(PEG) have been developed to control the size, shape and homogeneity of EB populations [59]. Arrays of microwells, as shown in figure 2**,** have also been used either individually or in combination with microfluidic systems for high throughput analysis, monitoring, and long term screening of stem cell fate [64, 65].

2.1.6 High throughput Analysis

ESC differentiation can be directed by different combinations of factors that influence the microenvironment. Spatiotemporal microenvironment signals may be influenced by the

size of EBs, co-culture of different cell lineages, small molecules in the microenvironment, ECM materials and some other unknown parameters. To study the combination of all influential stem cell differentiation factors is a huge combinatorial problem that will be extremely difficult to analyze which will likely be facilitated by high throughput analysis, screening, and imaging are essential.

Cells respond to multiple complex signals which are passed on through the ECM microenvironment, hence ESC differentiation can be regulated and controlled by the microenvironment. There are several factors that can influence microenvironment which can be combined in different ways. Because of significant number of possible combinations, high throughput approaches[62, 66-69] can be used to test them and to discover important parameters in ESC differentiation. There is multiple microenvironment parameters such as ECM proteins[69], small molecules[70], and biomaterials[23] that influence the direction of ESC differentiation. To improve our understanding about ESC. differentiation, study the influence of different parameters that may regulate stem cell expansion and specialization, high throughput analysis, screening, and imaging may prove beneficial.

In addition to growth factors and cell-secreted morphogenetic factors, synthetic small molecules can direct ESC differentiation toward particular cell types[70, 71]. Small cell permeable molecules such as vitamin C, sodium pyruvate, dexamethasone, thyroid hormones, and retinoic acid have been used to regulate stem cell fate. New heterocyclic small biomolecules have been studied to induce stem cell differentiation as factors that can alter stem cell fate[65]. Ding *et al.*[71] reported ESC differentiation into variety of specific cell types employing small molecules. Synthetic molecules can be used to selectively control and regulate stem cell differentiation and proliferation by adjusting the protein activities. For example small molecules have been used for nerogenesis and cardiomyogenesis induction in murine ESCs, osteogenesis induction in mesenchymal SCs, and skeletal muscle cell differentiation[71].

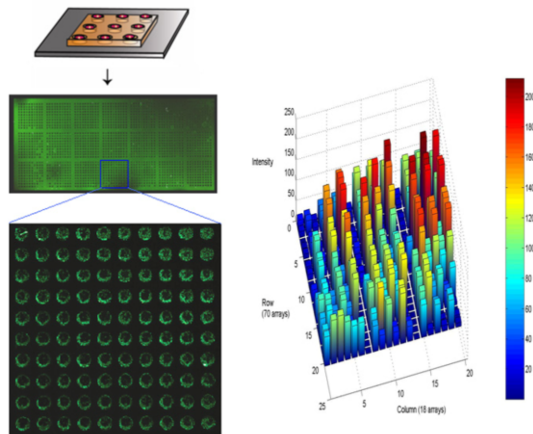

Figure 2. A microarray was used to culture stem cells on combinations of different ECM materials to optimize ESC cluster formation, stem cell proliferation, and stem cell differentiation. Master silicon wafer molds are used to make PDMS stamps (top left) which in turn the latter will be used to fabricate 3D PEG microarrays (mid left). Cells seeded in a microarray can be imaged (bottom left) to quantify cell viability using high throughput image analysis systems (right)

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Scaffold composition can also influence stem cell behavior and direct the ESC differentiation. Anderson *et al.*[66] tested the effects of a library of 576 different materials on stem cell behavior in a biomaterial array format. Combinations of different materials were mixed in 384-well plates and were robotically printed on coated glass slides. After printing the slides were exposed to long wave UV, dried, sterilized with UV, and washed with PBS and medium. EBs were seeded onto the slides and the influence of the printed materials on ESC differentiation was observed. Based on the observations, they concluded that ESC differentiation may be induced toward epithelial cells by specific materials.

3. Self-assembly for the generation large, organ-like constructs

When considering organ complexity and structure, it is of great interest to generate a variety of components that may contain different cells, present different geometries and possess different functional properties. In terms of organ fabrication, self-assembly or directed-assembly presents potential benefits for the efficient generation of tissues of clinically relevant size. First, since organs contain billions upon billions of cells, self-assembly presents a means to rapidly generate organized tissue-like structures with little manual manipulation. Second, by nature of their small repeatable units, self-assembly provides a means to generate structures that can be differentiated, validated and matured *in vitro* before assembly and implantation. Finally, self-assembly provides a means to generate structures within structures by the combining micro and macro scale assembly.

In terms of tissue engineering self-assembly has different meanings at different scales which can be defined as the molecular, cell and tissue level. At each level, the potential degree of organization and control of self-assembly can vary widely. For example, larger tissues may simply be formed by assembling aggregates of cells. Alternatively, they may also be formed by the self-assembly of complementary shaped structures each containing different cells and each intended to support a different function of the structure.

3.1 Self-assembly at the molecular level

There are many examples of self-assembly at the molecular level, such as protein aggregation, lipid membrane development and the annealing of complimentary DNA strands[72]. Another example of molecular self-assembly in tissue formation is biomineralization. During bone formation and repair, hydroxyapatite crystals form between collagen fibrils at specific nucleation sites to produce a highly ordered composite structure of organic and inorganic components[73]. A biomimetic approach has been used to generate a nanostructured fibrous scaffold from the self-assembly of a peptide-amphiphile. The subsequent mineralization post assembly presents interesting options for the generation of complex composite scaffolds[74].

3.2 Self-assembly at the cellular level

Cell level self-assembly is a phenomenon whereby cells coalesce to form 3D aggregates[75]. This is desirable since cell aggregates are associated with increased gene expression and enhanced maintenance of cell phenotype when compared to monolayer cultures[76]. Self-assembly at the cellular level has been used to generate cell aggregates for the *in vitro* culture of a variety of cells types in a scaffold-free environment[76]. Aggregates grown under "hanging drop" conditions are generally restricted to the microscale due to limitations in nutrient and gas diffusion. While aggregates may contain more than one cell type, the nature of the technique precludes precise control over cell position.

By growing cells in constrained environments, microtissues comprised of cells and their ECM have been formed. Cells have been grown in microwells to form spheroids made by primary[77] or stem cells[59]. This process enables the formation of microtissues with specific shapes and geometry suitable for post- assembly. In a similar fashion, the generation of structures with defined shapes and mixtures of cell populations has also been described. Dean *et al*. generated microtissues shaped like rods, tori and honeycombs by seeding cells onto non-adhesive agarose gels[78]. Upon removal from the gel after 24-48 hours, tori and honeycomb shaped microtissues that contained patent lumens remained so for up to 2 days. By combining two cell types during the microtissue casting process, Dean *et al*. were able to modulate stability of the structures and demonstrate cell sorting within the microtissues. Rat Hepatoma cells demonstrated a distinct preference for the surface of the structures while human fibroblasts filled the bulk (Figure 3).

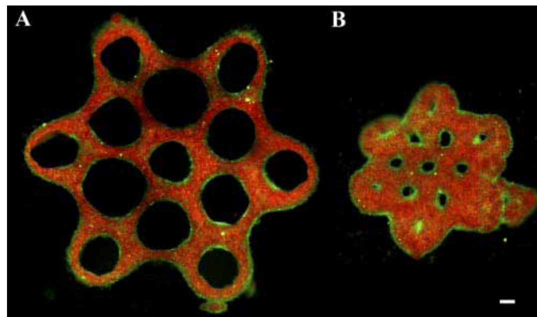

Figure 3. Honeycomb structures created by casting a mix of labeled normal human fibroblasts (NHF) (red) and rat hepatoma cells (H35) (green) onto non adherent gels with honeycomb features. The resulting structures were viewed by fluorescent microscopy after 24 h (*A*, *B*) of directed self-assembly. The hybrid structures still in mold (*A*) and out of mold and relaxed for 4–6 h (*B*) are shown. Scale bar 200 µm. From Dean *et al.*[78]

This approach is not ideal for cells that produce limited amounts of ECM or require numerous cell-cell contacts. However, in avascular and acellular tissues such as cartilage, where the ECM is the primary material of interest, self-assembly of chondrocytes has been used to generate ECM with compositional similarities to that of cartilage[79]. Ofek, *et al.* placed chondrocytes in high density in a non-adherent agarose mold. The chondrocytes coalesced and formed cadherin expressing aggregates at day 1 and were secreting Collagen type IV and chondroitin 6-sulfate at 1 week. At 4 weeks histological changes indicated a relatively mature tissue matrix.

3.3 Self-assembly at the tissue level

Two approaches commonly employed to create organized, cell-laden and structured materials from hydrogels are the top-down and bottom-up approach[80-82]. From the macro to the microscale, the "top-down" approach controls the many features of relatively large scaffolds as is common with bio-printing techniques. Jakab *et al*. used a modified inkjet printing system to deposit a variety of cell types onto a collagen-based scaffold[83]. In one experiment, cells derived from embryonic chick heart tissue were printed in discrete spots and fused after 70h incubation to form a thick asynchronously beating tissue graft. In another experiment, ring-like structures comprised of small spots of Chinese hamster ovary cells were printed onto a collagen-based scaffold. Repetitive printing of the same shape in the same location resulted in the generation of a 3D structure similar to a tube.

In contrast to the "top down" approach, a "bottom-up" approach to tissue fabrication may be achieved by the assembly of repeating subunits in a predictable and directed manner to form larger functional and organized structures. In this regard, self-assembly may be defined as the preferential organization and linkage of micron scale structures by interactions based upon shape, chemical moieties or interfacial energy.

Bottom-up assembly has been achieved by using self-assembled precursors to generate larger, composite cell laden structures. These precursors can be created in a number of ways, such as self-assembled aggregation, microfabrication of cell laden hydrogels, creation of cell sheets or direct tissue printing. Once formed, these precursors can be assembled into larger units resembling tissues by random assembly, directed assembly or stacking of layers. By creating modular tissues with microarchitectural features resembling native physiology, bottom-up tissue engineering aims to provide more guidance on the cellular level to direct tissue morphogenesis.

Such structures may arise from the relatively simple molding or self-assembly of cell aggregates. Kelm *et al*. demonstrated viability and vascularity of assembled tissues based upon the secondary assembly of self-assembled cell aggregates in molds[84]. Similarly, rod-like structures consisting of connected aggregates of proepicardia have been formed by secondary self-assembly[85].

In one example demonstrating a bottom-up approach to tissue engineering, Harrington *et al*. generated a bladder-like tissue comprised of urothelial and smooth muscle cells in two distinct layers[81]. The cell-laden scaffold was formed by partially submerging a poly(glycolic acid) fiber spun scaffold in media containing smooth muscle cells and a peptide-amphiphile. The peptide-amphiphile was triggered to gel around the fiber spun scaffold, entrapping the cells and forming the first cell laden layer. The composite structure was inverted and the process was repeated by submerging the unexposed portion of the fiber spun scaffold in in media containing urothelial cells and a peptide-amphiphile. After 3 weeks *in vivo*, samples demonstrated a bilayer appearance and with both urothelial and smooth muscle cells identified by immunostaining (Figure 4).

While promising, the controlled or directed self-assembly of microscale tissue components such as microgels offers certain advantages. In terms of materials for fabrication, hydrogels are well suited to both tissue engineering and self-assembly. This is a direct result of their biocompatibility, high water content, ability to support the sequestration and diffusion of growth factors and the relatively facile production of cell-laden microgels of well defined shapes and properties by simple and rapid techniques such as casting[80, 82, 86, 87]. Du *et al*. demonstrated that self-assembly of cell laden hydrogels can be achieved by controlling surface tension, shape and assembly conditions[80]. Square (400 µm x 400 µm x 150 µm), cell laden microgels were fabricated by the UV

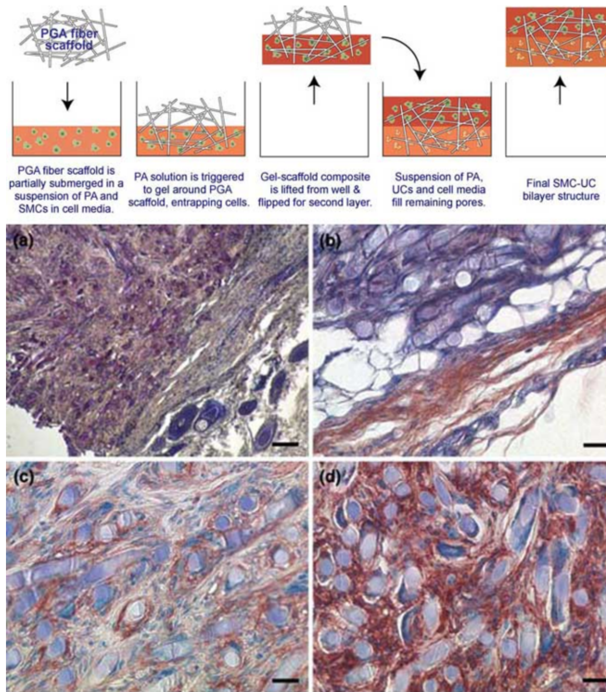

Figure 4. (Top) Schematic of gelation process for making PA-PGA composite scaffolds. (Bottom) Immunostaining of PA-PGA composite scaffolds after 3 weeks *in vivo*. **a** MHC staining, demonstrating the retention of human SMCs (*red*); **b** uroplakin staining, demonstrating the formation of a UC layer (*red*), and the retention of a SMC-UC bilayer; **c** α-SMA staining of SMCs on a plain PGA scaffolds, and **d** cells on a scaffolds with PA and bFGF. More intense α-SMA staining is observed on the sample in **d**. *Scale bars* **a** 100 µm; **b**–**d** 25 µm. From Harrington *et al.*[81]

polymerization of PEG under a photomask. Hydrophilic microgels were placed in a hydrophobic solution and agitated and it was determined that agitation rate, time and surfactant concentration all influenced the extent of self-assembly and organized structures up to 1000µm in length were achieved. The efficiency of self-assembly based upon shape was also demonstrated with lock and key shaped cell-laden microgels (Figure 5). Post-assembly, microgels were further cross linked to maintain their secondary structure.

Viable structures for tissue engineering can also be fabricated from the self-assembly of cells grown *in vitro* in the form cells sheets[88]. L'Heureux generated tissue engineered blood vessels, by culturing human cells[89]. Fibroblasts were extracted from the skin of patients undergoing cardiovascular bypass surgery and cultured in conditions that promoted deposition of ECM which produced cells sheets that were strong enough for manipulation. In a technique referred to as "sheet based tissue engineering" grafts were fabricated by wrapping and maturing sheets of tissue around a rod like Teflon-coated support *in vitro*. Grafts implanted in non-human primates showed complete tissue integration at 8 weeks with a smooth lumen and no signs of stenosis, thrombosis or mechanical failure. Histology demonstrated a confluent endothelium and a smooth muscle layer with in the graft.

Different approaches to self-assembly exist in this active research area. While a preferred methodology does not seem clear, self assembly may be a promising means to efficiently generate of tissues of clinically relevant size.

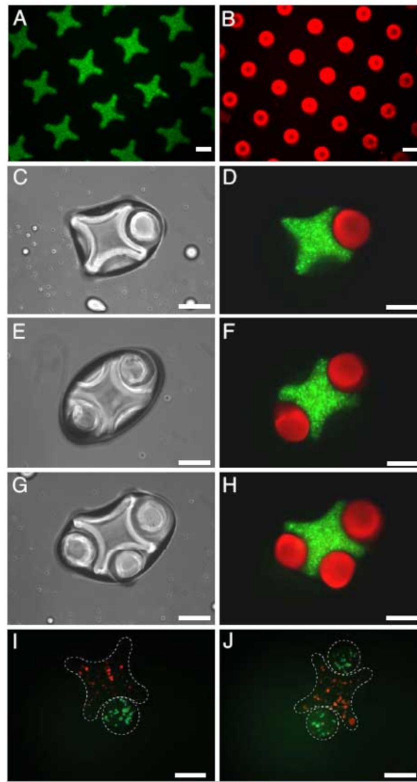

Figure 5. Directed assembly of lock-and-key-shaped microgels. (*A*) Fluorescence images of cross-shaped microgels stained with FITC-dextran. (*B*) Rodshaped microgels stained with Nile red. (*C–H*) Phase-contrast and fluorescence images of lock-and-key assemblies with one to three rods per cross. (*I* and *J*) Fluorescence images of microgel assembly composed of cross-shaped microgels containing red-stained cells, and rod-shaped microgels containing green-stained cells. (Scale bars, 200 µm.) From Du *et al.*[98]

4. Microfluidic tools for organ regeneration

Microscale technologies are potentially powerful tools that can address current challenges in organ regeneration such as vascularization, stem cell differentiation and microenvironmental control[66, 90-92]. Microelectromechanical systems (MEMS) can be used to control features at small scales <1 µm to > 1 cm. These techniques are compatible with cells and are now being integrated with biomaterials to facilitate biofabrication of cell-material composites which can be used for tissue engineering and regenerative medicine. Micropatterning of substrates can be useful for localizing cells into a particular region on the substrate to allow high throughput testing of cell-material interactions or differentiation[66, 92]. In addition, microscale technologies enable control of the cellular microenvironment *in vitro* and the miniaturization of conventional assays for high throughput applications.

4.1 Controlling stem cell microenvironments

Microfluidics provide unique opportunities for detecting stem cell responses to several soluble compounds in an effort to fully understand and control their behavior.[93] Microfluidics can be useful for studying the response of individual stem cells to various microenvironmental signals[66]. For instance, microarrayers enable, thousands of combinatorial soluble cues to be synthesized, so that their effects on the differentiation of ESCs could be evaluated[94]. Specifically, microfluidic systems can integrate engineering, chemistry, and biology for conducting experiments at much smaller scales which deal with small volumes of liquid in a controlled manner. There have been many driving forces to use potential benefits of micro-sized apparatus of fluidic systems relative to conventional systems: (i) reduced consumption of samples and reagents, (ii) shorter analysis time, (iii) greater sensitivity, (iv) portability that allows *in situ* and real-time analysis, and (v) disposability[95]. A promising vision for the field is that micro/nanofluidic chips can implement large-scale automation of biological processes using nanoliter/picoliter volumes. 3D environments that capture the molecular, structural, and physical factors regulating cellular processes and at the same time provide control and monitoring of environmental factors are instrumental toward these goals. Moreover, essentially the same biologically inspired blueprints define the engineering design of each of these systems. In addition, microfluidics is potentially beneficial for studying the development of artificial microvasculatures in microfluidic networks. In addition, this approach can be integrated to investigate the extracellular signals that regulate cell fate, because microfluidics can control temporal and spatial cell-microenvironment interactions in a high throughput and controlled manner. Therefore, microfluidic systems can be useful for understanding basic and applied stem cell biology because they precisely manipulate extracellular microenvironments such as cell-cell, cell-ECM, and cell-soluble factor interactions (Figure 6-HTS microfluidic system for screening stem cell microenvironments, *unpublished results*).

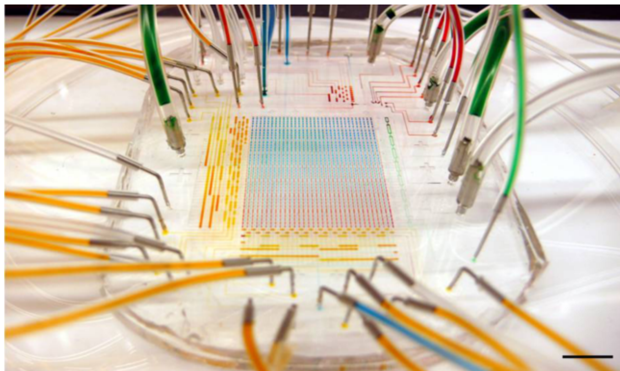

Figure 6. Photograph of HTS microfluidic system for studying stem cell microenvironments. This photograph shows the state-of-art technology that can enable researchers to conduct HTS approach for individually addressing and generating stem cell microenvironments in a controlled manner. Scale bar is 1 cm. (Unpublished data, Courtesy of Dr. Won Gu Lee).

4.1.1 Generating vascularity

The initial designs for the fabrication of microfluidic vascular patterns were performed in 2D systems and more recently have evolved to more biomimetic systems. Here we will

provide an overview of this field beginning with 2D systems of microchannels with non-tissue engineering materials. Microfluidics can be used for generating 2D and 3D vascularization as initial stage by engineering tissue constructs in a controlled manner. Furthermore, a potential role of hydrogels in a microfluidic format is discussed.

- *2D vascularization microfluidics*: Most of the initial work which could generate engineered vascular constructs used microscale technologies to fabricate such patterns with various biomaterials. Specifically, soft lithographic techniques were applied to mold PDMS on a silicon wafer by implementing bifurcated vascular networks[96]. These PDMS patterns were irreversibly bonded to the PDMS layers to create enclosed network channels that were seeded with endothelial cells. To generate highly uniform flow patterns, which mimic both large-scale physiologic properties and small-scale fluid velocity in the capillaries[97], three network designs were fabricated to approximate the fluid dynamics: (i) a stepwise scaling from arteries to capillaries (higher flow resistance), (ii) a high capillary cross section (higher flow rate, but non-uniform flow profile), and (iii) both uniform flow and reduced resistance of the microfluidic network. For example, these scaffolds have been successfully used for seeding endothelial cells in the PDMS channels with dimensions on the order of capillary diameters.
- *3D vascularization microfluidics*: *In vivo*, cells live within 3D environments in close proximity to blood vessels that supply tissues with nutrients and oxygen and remove waste products and carbon dioxide. It has been reported that numerous *in vitro* studies have identified critical features that allow 3D cultures to replicate physiology better than 2D cultures.[98-101]. Thus, building 3D vascularized microfluidic scaffolds is of great importance for the success of tissue engineering applications. For example, the 3D microfabricated vascular tissue constructs have a unique property of providing constant maximum shear stress within each channel of the device, holding a great promise for an artificial vascular tissue engineering scaffold. Specifically, multi-layered microfluidic scaffolds have also been used for liver tissue engineering[102]. The multi-depth channels mimic various physiological flow patterns with lower flow resistances and more gradual changes in the flow velocities across different generations of branching compared to the channels of uniform depth[103].

  Hydrogel microfluidics: Tissue engineering scaffolds made from hydrogels have raised interest in this field[104]. Hydrogels can be used for fabricating networks of hydrophilic polymers, bringing a number of potential advantages compared to other materials such as PDMS, PGS, and PLGA. This is because their physical properties (i.e. mechanical strength and biodegradability) and biological properties (i.e. the biocompatibility and resemblance to the natural ECM) can be tailored to mimic tissues. The merger of microengineered hydrogels and microfabrication techniques for microfluidic transport has been of significant potential to generate 3D tissue constructs. For instance, Stroock. introduced a hydrogel microfluidic system within calcium alginate hydrogel[105]. Specifically, they demonstrated that a higher mass transfer could be achieved within the hydrogel microfluidic system by arraying the channels in appropriate dimensions. These results also showed the feasibility of using an embedded microfluidic system which can control various concentrations of soluble species within

the 3D volume defined by hydrogels. These microfluidic channels enabled an efficient exchange of solutes within the interior of hydrogel scaffolds and provided a quantitative control of the soluble environments for the cells in the 3D environment[106, 107]. This approach is very promising for directing the cells in the scaffolds with spatial and temporal control and growing thick sections of tissue without necrosis. Recently, Ling . built cell-laden microfluidic channels from hydrogels by directly encapsulating the cells within the microfluidic channels[10]. Using standard soft-lithographic techniques, the agarose solution was molded onto a SU-8 patterned silicon wafer to form microfluidic channels. In particular, microfluidic channels of different dimensions were generated, showing that agarose was a suitable material for fabricating 3D hydrogel microfluidic networks. The cells embedded within the microfluidic network were well-distributed and media perfused through the channels, allowing the exchange of nutrients and waste products (Figure 7 - hydrogel microfluidics)[10]. Cell-laden hydrogel microfluidics could also be scaled up by stacking the biomimetic vascular patterns to generate multi-layer vascularization in multiple discrete planes.

For practical applications, however, one limiting factor of microfluidic scaffolds has been given to the choice of material. To address this limitation, a new approach for cell and tissue engineering, known as biodegradable microfluidics has been explored to fabricate potential implantable microfluidic tissue constructs[108]. Although vascularized microfluidic systems are also readily constructed in a 2D format by photolithographic or soft-lithographic techniques, their construction in 3D still remains a challenging problem. For example, building 3D vascular microfluidic structures by stacking 2D layers is a

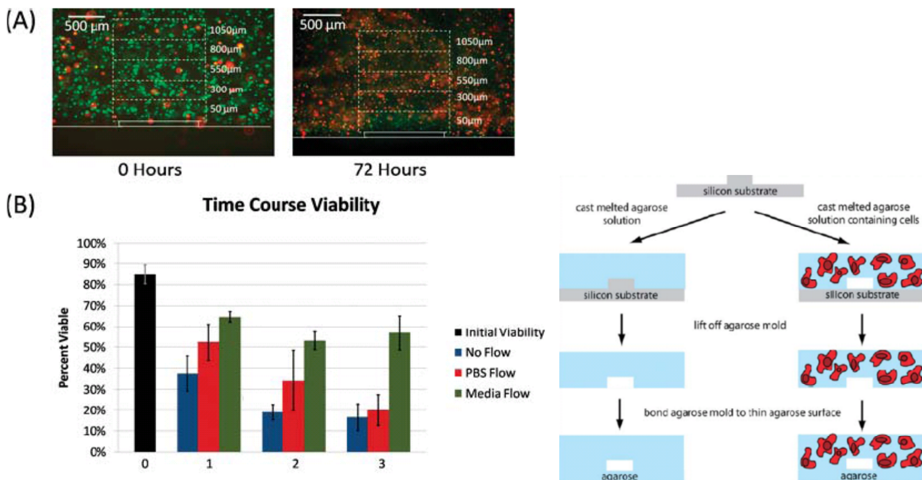

Figure 7. Schematic of the fabrication of agarose microfluidic devices with (right) and without (left) embedded cells. (Right) Quantification of cell viability in the agarose microchannels over time. The images in (A) are representative live/dead staining of AML-12 murine hepatocytes encapsulated in agarose channels after 0 (left) and 3 days (right). Rectangular regions demarcated by dashed white lines correspond to 250 µm thick zones where the labeled height values correspond to the mean distance of each zone above the channel floor. The graph in (B) plots percent viability values for initial (n = 27) as well as for up to three days under 2 control conditions(no flow, PBS flow) and experimental media flow conditions (n = 9 for all three conditions). From Ling *et al.*[10]

cumbersome process requiring multiple fabrications and masking steps that is difficult to scale-up. In contrast, a microfluidic gel approach was also suitable for fabricating scaffolds for 3D cell culture, which can be used to culture human microvascular endothelial cells, e.g., to form rudimentary endothelial networks for potential tissue engineering applications. It is also envisioned that the growth factor–based approaches can be incorporated within the existing microfluidic-based tissue constructs to improve vascularization. Consequently, a number of obstacles need to be overcome to build microfluidic systems for clinical applications in the future. These include: (i) how to achieve full endothelialization of the microfluidic systems with different geometries and materials; (ii) how to precisely fabricate microfluidic vasculature networks with small vascularized structures which can mimic the capillaries in a scalable manner; (iii) how to implement real complexity of the microfluidic tissue constructs by involving extracellular matrices, multiple cells types and controlling cell-matrix and cell-cell interactions; and finally (iv) how to implant the engineered tissue vasculature constructs built *ex vivo* into the human body *in vitro* to compensate for loss of tissue or its function without occlusion of blood.

5. Future Perspectives

Advances in our understanding of stem cell behavior as it applies to organ and tissue development will likely accelerate the pace of discovery in organ regeneration. In this regard, technologies that enhance our understanding and control of stem cell activity in a rapid manner will likely be of great benefit. The continued development of microscale technologies as well as the generation of enhanced 3D environments for stem cell maturation and tissue development *in vitro* hold near term promise.

6. Conclusions

The major challenges for organ regeneration include the generation and differentiation of a suitable number of precursor cells, arrangement of these cells into a functional architecture and nutrient delivery and waste removal. Microscale techniques offer promising approaches to these complex problems by facilitating fabrication of organized self-assembling tissues, by enabling fabricating scaffolds with predetermined conduits suitable for vascularization and by enabling high throughput evaluation of cells in many different micro-environments. As a result, it is anticipated that these microscale technologies will be important tools for advancing the state of organ regeneration.

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