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Tissue Engineering at the Micro-Scale

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Abstract. **The possibility to replace damaged or diseased organs with artificial tissues engineered from a combination of living cells and biocompatible scaffolds is becoming a reality through multi-disciplinary efforts. A number of critical components within this effort are being facilitated by microfabrication and MEMS approaches, including research tools to elucidate mechanisms which control cellular behavior as well as development of methods to manufacture cellular scaffolds at ever higher resolutions. This article reviews recent advances in tissue engineering that have been facilitated by interaction with the microfabrication community. We highlight the potential opportunities for microfabrication to make to the development of mainstream medical therapies for tissue replacement.**

***Abbreviations*. CAD, computer-aided design; ECM, extracellular matrix; PMMA, polymethyl methacrylate; PDMS, polydimethyl siloxane; PLA polylactic acid; PLGA, polylactic (co-glycolic) acid; SFF, solid freeform fabrication; TEMP, tissue engineered medical product.**

Introduction

While a large number of patients succumb to multiple organ failure in their final days, the majority of disease in the current population results from damage, failure, or loss of a single organ or tissue component. For example, in the case of liver failure due to toxin ingestion, the only existing therapeutic options are supportive measures and transplant. Similarly, mortality and morbidity due to large area, full-thickness skin burns is a direct result of our inability to replace the barrier functions of skin quickly enough to prevent infection. While transplant biology has advanced rapidly in the past several decades, the supply of donor organs still remains extremely limited, and the accompanying lifelong immunosuppression that is usually required can itself be a source of disease. One of the most innovative and promising cross-disciplinary approaches to addressing this wide range of diseases involves the development of engineered tissue and organ replacement products (Lanza et al., 1997; Langer and Vacanti et al., 1993). Such products involve the use of biomaterial constructs combined with tissue-specific cells either by combining the two components prior to implantation or by encouraging cells to populate the construct upon implantation in a host. Ultimately, by assembling cells and scaffolds into engineered tissue, it is hoped that these implants can virtually replace the functions of the damaged tissue. Eventally, tissue engineered medical products (TEMPs) may be created that even outperform our natural tissues. In every step of this endeavor, from our fundamental understanding of how to organize cells into tissues to manufacturing highly ordered scaffolds, the opportunity presents itself for the microfabrication community to contribute essential technology and experience. In this review, we hope to highlight the past successes and future challenges in which microfabrication technologies have played a role in forming the vision of engineered biological tissues.

Already, several clinical products exist which have been used to replace human skin that has been damaged by burn or insufficient blood supply (Yannas et al., 1982; Bell et al., 1979; Parenteau, 1999; Eaglstein and Falanga, 1998). The products are similar in that they all contain a highly porous, flexible scaffold consisting of natural extracellular matrix components found in normal skin, yet the precise cellular and scaffold components as well as processing conditions vary significantly between manufacturer. In the pipeline, investigators are also attempting to build bone (Peter et al., 1998; Solchaga et al., 1999; Isogai et al., 1999), liver (Griffith et al., 1997; Rozga and Demetriou, 1995; Yarmush et al., 1992; Nyberg and Misra, 1998), arteries (Black et al., 1998; Niklason et al., 1999), bladder (Oberpenning et al., 1999), pancreas (Colton, 1995; Lanza and Chick, 1997), nerves (Borkenhagen et al., 1998; Chamberlain et al., 1998), cartilage (Binette et al., 1998), heart valves (Carrier et al., 1999; Mayer et al., 1997), and various soft tissues. Despite significant progress in the field, a number of issues have arisen that have forced the industry to take pause. Simply producing a highly porous scaffold and seeding it with the appropriate types of cells in most cases does not recapitulate the desired features of a normal tissue. Tissue structure and function are known to

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highly inter-related. In particular, cells must interact with other cell types, the surrounding scaffold, distributions of different soluble factors, and the presence of non-uniform physical forces (such as shear flows, tensile and compressive loads)- all depend, to some degree on tissue structure. In fact, the ''microenvironmental'' conditions surrounding individual cells not only largely determine how those cells behave, but such relationships are also essential for the emergent properties of the multicellular networks. In Figure 1, three adult tissues are highlighted as examples of the structural complexity seen *in vivo*: skin, liver, and retina. The many layers of the skin depicted in this histological stain of the epidermis allow the skin to offer protection, sensation, thermoregulation, and key metabolic functions simultaneously. In particular, the interdigitation of the epidermis with the underlying dermis occurs with a periodicity of $\sim 500\,\mu m$ and is thought to enhance adhesion of the two layers. Similarly, the fundamental structural unit of the liver, the acinus, has a typical radius of 500 μm. Within this structure at least six cell types interact with one another to coordinate the diversity of liver functions. When the acinar structure is enzymatically disrupted, liver functions are often lost within a few days. Finally, the function of the retina is a direct result of the specific interconnected relationships in its highly convoluted, complex wiring diagram. This spatial relationship of cells and supporting structures allows the eye to convert quanta of light energy into nerve action potentials that can be interpreted by the brain. The full function of these organs clearly can not be expected to be recapitulated without re-building the structure of the tissue itself. To accomplish this, sub-cellular scale structures (i.e., 1–10 μm) need to be constructed to control the cellular environment, cell scale structures (i.e., 10–100 μm) are needed to control cell-cell interrelationships, and supracellular scale structures (i.e., 100–1000 μm) are needed to build the essential functional units of the tissue.

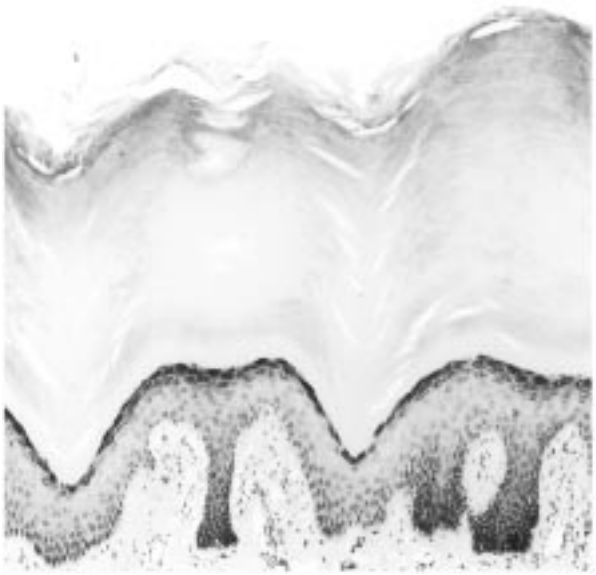
The challenge for the near future in tissue engineering, then, lies in our ability to (1) understand how the cellular microenvironment, at the 1–10 μm scale, dictates cell function, (2) fabricate organ-scale structures with cellular-scale resolution (10–1000 μm), (3) integrate functioning cells into the appropriate multi-cellular architectures for tissue function, and (4) integrate the engineered tissue into a patient such that it is vascularized, immune responses are controlled, and tissue function rises to match the needs of the patient clinically. Microfabrication approaches have advanced to provide many of the tools that may facilitate our efforts towards these goals. The development of photolithography and microcontact printing to pattern biological molecules onto surfaces (Kumar et al., 1994; Kumar and Whitesides, 1993; Singhvi et al., 1994), novel tools to move silicon-based fabrication to polymer-based constructs (Kapur et al., 1999), and the move from 2-D to 3-D fabrication (Chu et al., 1999; Cumpston et al., 1999) each provide essential tools in the development of well-defined *in vitro* systems to study the control of cells through their environment, the production of scaffolds to recapitulate tissue architecture, and the fabrication of living hybrids of cells and scaffolds that demonstrate incremental steps toward reconstruction of tissue function.

Components of Tissue Engineered Constructs: Cells and Scaffolds

TEMPs are unique due to the use of biological and synthetic materials in combination. Typically, biological materials (cells and cellular products) provide the biological function whereas the synthetic material provides the structural support. Ideally, the interaction results in: integration of the product with the host, maintenance of biological function, and control of signaling between TEMP and host.

Cells

Cells in every organ play a central role in building and maintaining specific tissue function, yet when they are removed from their innate environment, the stability of cellular behaviors is uniformly lost. Therefore, a principal aim of tissue engineering has been to develop a fundamental understanding of the factors in the microenvironment directly surrounding cells which can induce and maintain the stability of differentiated functions. Subsequently, these environments are mimicked to encourage isolated cells to recapitulate their *in vivo* function. While a large number of tissue-specific soluble cytokines have been identified to play a role in inducing differentiated functions of parenchymal cells (Moore et al., 1990), recent evidence has indicated the complex binding interactions between cells and: (1) the insoluble extracellular matrix can and (2) other cells, can modulate the response of cells to these soluble factors (Renshaw et al., 1997; Aplin and Juliano, 1999; McNamee et al., 1993). A major contribution that microfabrication technology offers in this arena is to provide model substrates (often glass and Si) with precise spatial control of surface chemistry and architecture to study the effects of the microenvironment on stable cell behavior. This general area, sometimes referred to as ''cellular micropatterning'' is quite expansive and a comprehensive review is beyond the scope of this overview. Here, we specifically highlight areas where



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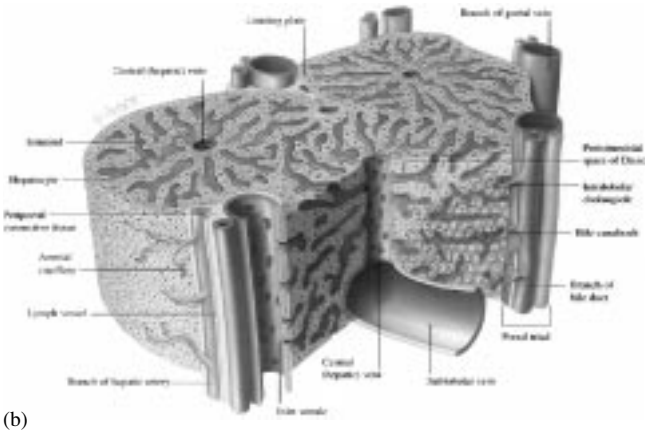

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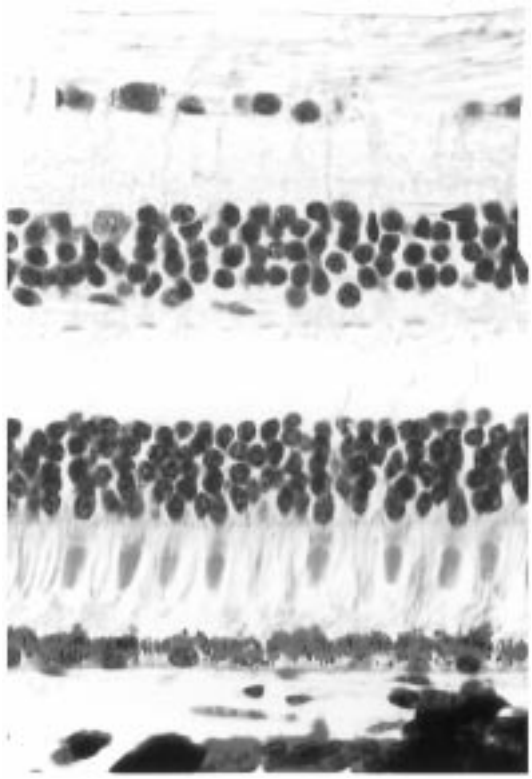

(c)

Fig. 1. *Adult mammalian tissues. Note the complexity of tissue structure and the precisely defined cellular microenvironments. Typical structural features occur on the order of 100 μm.*
(a) Skin. This histological stain of the epidermis from a fingertip demonstrates the general features of skin. Together, these structures allow the skin to provide its four major functions: protection, sensation, thermoregulation, and metabolic functions. Keratinocytes divide in the germinal layer (B) and move progressively to the surface every 25 to 50 days. As they mature they progress through different layers: S is the prickle cell layer containing cells which are growing and in the process of early keratin synthesis, G is the granular layer which contains cells with intracellular granules, L is the stratum lucidum, only present in thick skin (as seen here), and C is the cornified layer. Note the interdigitation of the epidermis with the underlying dermis (below B), the Rete ridges with a periodicity of approximately 500 μm are thought to enhance the adhesion of the epidermis to the dermis. Reproduced here by kind permission of Churchill Livingstone (Burkitt et al., 1993).
(b) Liver. This schematic of the liver acinus demonstrates the complex architecture necessary for efficient mass transport in this highly metabolic organ. Each lobule is fed directly by a oxygenated arterial source to provide needed energy for its many functions: detoxification, metabolism (fat, protein, and carbohydrate), bile production, and serum protein production. Hepatocytes, the functional cell of the liver, are aligned in cords in close proximity to a fenestrated endothelium and blood as it travels along the sinusoids (the capillary of the liver). Numerous other cells (not depicted here) interact with hepatocytes and each other to provide the ability for phagocytosis (Kupffer), fat-storage (stellate), and bile excretion (biliary epithelial). The typical sinusoid is 500 μm in length. Smaller structural features are also vital to normal liver function (i.e. the bile caniliculi form between hepatocytes and bile travels retrograde to collect in the bile duct). Reproduced by permission of the artist, J. Daugherty.
(c) Retina. The retina is responsible for photoreception in the eye. It contains two types of nerve cells (rods and cones, 2–4), an integrating system of neurons (5–9), pigmented epithelial cells (1), and neuron support cells for structural support (Muller cells, not depicted). This highly complex architecture occurs on the order of 100-200 μm in depth. Together, these cell layers act to convert quanta of light energy into nerve action potentials that can be interpreted by the brain. Reproduced here by kind permission of Churchill Livingstone (Burkitt et al., 1993).

microfabrication techniques have been utilized to gain biological insight relevant to tissue engineering that may not have been gleaned by conventional means.

Engineering cellular behavior for tissue reconstruction has focused on the understanding of a number of critical cell functions including: adhesion to matrix and other cells, migration, proliferation, differentiation, and suicide (apoptosis). Adhesion to matrix may be important to localize cells in a pre-defined arrangement an may be required in the case of complex, solid organs, like the liver. In other cases, migration may be a critical feature for a TEMP. For example, nerve guides have been proposed to re-connect severed peripheral nerves-facilitating regrowth of nerve axons through the damaged area. Cell proliferation and apoptosis are key processes that would allow control of the cellularity of an implant. Finally, in order to replace organ function, the cells must reach a stable structural and functional state of differentiation (i.e., keratin production for skin, matrix production for cartilage, etc). Microfabrication has contributed significantly in the fundamental understanding of cells in all these areas.

Several model systems have been developed to understand fundamental aspects of cell adhesion and patterning. The earliest work in microfabricating surfaces for cells has focused on the problem of controlling the pattern of adhesivity on 2-dimensional surfaces. Briefly, the most robust current approaches rely on two principal technologies-surface chemistry to promote or resist cell adhesion, and a method to pattern these chemistries contiguously onto a surface (e.g., photolithography, microcontact printing). The promotion of adhesion turns out to be trivial, because most surfaces are adhesive to proteins and cells (Hubbell, 1995). Because recent advances in cell biology have demonstrated the importance of the specificity of the adhesive ligands for specific integrin receptors, the most robust approaches have developed methods to coat the adhesive regions of the surface with any generic extracellular matrix protein (Folch and Toner, 1998; James et al., 1998) or to covalently link integrin ligands to the surface (Hubbell, 1995; Drumheller and Hubbell, 1995). More challenging has been the development of surface chemistries that resist the adsorption of proteins and cells. Currently, only a few chemistries exist which can truly prevent cell adhesion. These include oligo-ethylene glycols, certain carbohydrate and cellulose derivatives, and fluorinated surfaces (Hubbell, 1995). The physical basis for the ability of these compounds to resist protein adsorption remains a mystery, but is of intense interest to the biomaterials scientists (Harris, 1992). The patterning of adhesive and nonadhesive regions onto a substrate at the resolution of single cells (i.e., sub-micrometer) has relied on three principal techniques: photolithographic patterning of surface chemistries with either covalent linking or chemical adsorption of the protein (Lom et al., 1993; Georger et al., 1992; Healy et al., 1994; Britland et al., 1992; Kleinfeld et al., 1988), microcontact printing of self-assembled monolayers of different chemistries or proteins (Kumar et al., 1994; Kumar and Whitesides, 1993; Singhvi et al., 1994), and solution coating of surfaces that are pre-protected with a removable mask (Folch and Toner, 1998; Bhatia et al., 1998a; Bhatia et al., 1998b; Folch et al., 1999; Flounders et al., 1997). Using these approaches, investigators have not only been able to control the position of cell attachment but also the precise geometry of the cells (Singhvi et al., 1994; Chen et al., 1997; Ireland et al., 1987; Thomas et al., 1999). Experience with many different types of mammalian cells in numerous labs suggest that these patterning technologies are generically applicable, provided that the appropriate ligands for adhesion are used for the specific cell being investigated (Hubbell, 1995). Ultimately, these approaches may provide spatial cues to important to direct tissue assembly from an initial pattern.

If cells are not placed in position on surfaces, they instead can be encouraged to migrate appropriately. Numerous soluble chemotactic factors have been used in controlled-release form to create concentration gradients that attract cells into the device (Park et al., 1998), but being soluble, they lack the spatial resolution that immobilized cues could potentially give to specific cells. A number of microfabrication labs have demonstrated that parallel adhesive lines can direct cell migration along the lines, even when the lines are spaced closely together such that cells are able to spread across multiple lines (Hammarback et al., 1985). Interestingly, the width and spacing of the lines has also been shown to control the speed of cell movement. Furthermore, the geometry of adhesive regions regulate migration, but also the surface topography. For example, etched parallel grooves also control cell spreading and migration in the axis of the lines, even when the depth and spacing of the grooved features are as small as 500 nm, about ten times smaller than the typical length scale of mammalian cells (see reviews for details—(Flemming et al., 1999; Curtis et al., 1990; Brunette and Chehroudi, 1999; Vonrecum and Vankooten, 1995; Curtis and Wilkinson, 1997; Singhvi et al., 1994). These discoveries in such simplified *in vitro* systems have led to the possibility of developing implants that accelerate the invasion of host cells across or through the engineered substrate.

More recent findings have shown that not only cell migration, but a number of fundamental cellular functions can be regulated by the cell-substrate interactions. Cell adhesion is not solely a thermodynamic

interaction between a particle and a surface. In fact, this process mediated by cell surface integrin receptors, comprises the classic binding and clustering of the receptors, the subsequent recruitment of focal adhesion proteins to the sites of adhesion, and mechanical linkage to the actin cytoskeleton, and finally spreading and flattening of the cell against the substrate. The biological activity of extracellular matrix appears to depend on every aspect of the cell adhesion process. This realization has both focused our efforts on fundamentally understanding how different aspects of adhesion modulate cell behavior, and broadened our goals to encompass which key aspects of the adhesive environment can be engineered specifically to produce a desired response.

A central goal in engineering any tissue requires the ability to modulate cells (native or seeded) in their decisions to proliferate, differentiate, or undergo apoptosis (programmed cell suicide). Once cells progress beyond a specific stage in this decision process, it is irreversible for a period, and are considered ''committed'' to that decision or ''terminally differentiated''. While the chemistry of extracellular matrix ligands and the presence of specific soluble cytokines and metabolic factors needed to modulate cellular responses have been identified for many specific cell and tissue lineages using traditional biological and biochemical approaches, microfabrication tools have recently aided in the identification of previously ignored parameters in the cellular microenvironment. For example, evidence had suggested that adhesion to ECM regulates capillary endothelial cells to proliferate or commit suicide by changing cell shape (Chen et al., 1997). To investigate the role of cell spreading in the regulation of cell growth and death, substrates were microfabricated using microcontact printing of alkanethiols on gold such that cells attached and spread to the size and shape of the engineered micrometer-scale islands of ECM (Figure 2A) Progressively restricting capillary cell spreading on ECM-coated islands of decreasing size regulated a transition in cellular commitment from growth to quiescence to apoptosis (Figure 2B). Furthermore, when cell-cell contacts were allowed to form by patterning cells on thin lines of adhesive substrate, these cells formed grossly apparent capillary-like tubes only if cell spreading was held to an intermediate degree where neither cell growth nor apoptosis occur. Interestingly, this shape-dependent regulation of cell behavior appears to exist in a number of different cell types. For example, in keratinocytes (from skin), osteoblasts (from bone), and hepatocytes (from liver), increasing the adhesive island area induces progressively increased proliferation until cells can no longer spread to cover the size of the island (Singhvi et al., 1994; de Beus and Jacobson, 1998; Rezania and Healy, 1999). In these systems, decreasing island size resulted in either active increase in differentiated function in the cases of skin and bone cells, or deceleration of the loss of differentiated function that occurs in hepatocytes cultured *in vitro*. Interestingly, like the response of capillary cells, cell-cell contact formation in hepatocytes not only prevents 'de-differentiation', but improves differentiated functions (N. Koide et al., 1990; Guguen-Guillouzo et al., 1983).

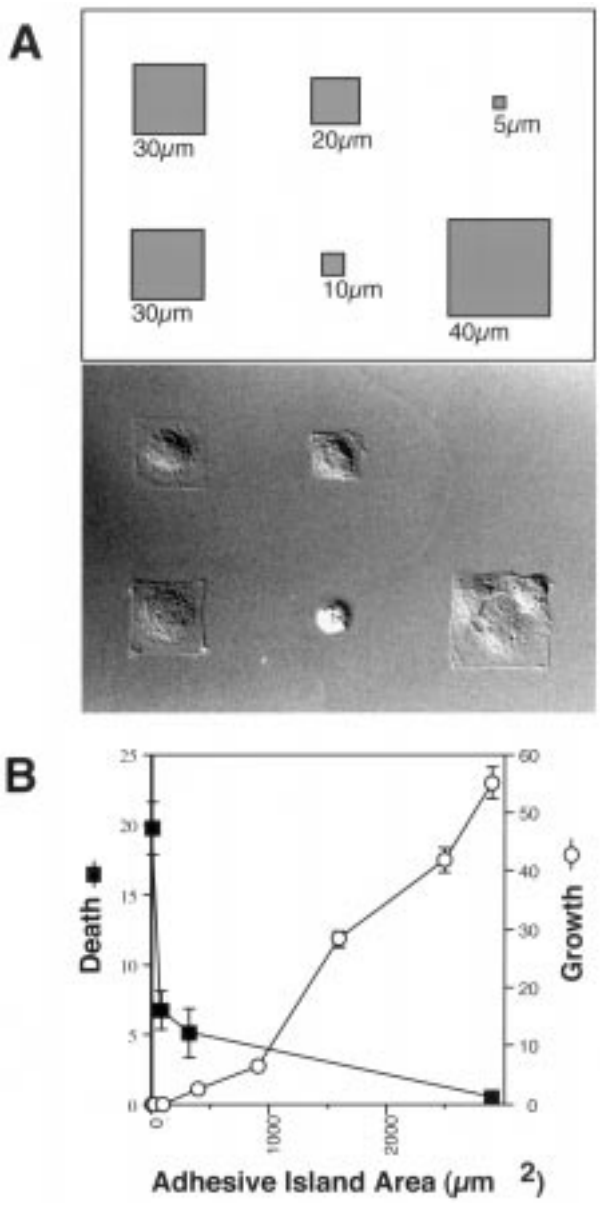

Fig. 2. *Using microfabrication to control cell shape and function. A. Schematic drawing and phase contrast micrograph of capillary endothelial cells cultured on an array of adhesive square islands of different sizes. B. Plot of programmed cell suicide or cell proliferation as a function of area of the adhesive island. Reproduced here by kind permission of the American Association for Advancement in Science (Chen et al., 1997).*

By using microfabrication tools to produce patterns of two different cell types on a single substrate, the spatial map of the interactions between these cells could be quantitatively controlled (Bhatia et al., 1998; Bhatia et al., 1998; Bhatia et al., 1997). This approach uncovered that this cell-cell interface plays a critical role in upregulating and stabilizing hepatocyte (liver cell) function *in vitro* (Figure 3). Indeed, when hepatocytes were co-cultivated with a model mesenchymal cell, increase in hepatocyte/fibroblast interaction achieved through micropatterning, produced higher long-term function of cultures with similar cell populations but different spatial arrangements. Furthermore, cell signaling was localized near the interface between cell populations, offering insight into potential mechanisms by which organ-specific cells communicate with surrounding supportive (stromal) tissue. Thus, basic cell biology has demonstrated that not only soluble growth factors and extracellular matrix composition, but also matrix architecture and cell-cell interactions, can play critical roles in the local modulation of cell proliferation, differentiation and apoptosis (programmed cell death). These cell fate processes are essential to the development and maintenance of tissue pattern, cellularity, and function in TEMPs. The next section therefore describes the opportunities for microfabrication to generate biomaterial scaffolds that could be used to regulate these aspects of cell behavior for tissue engineering.

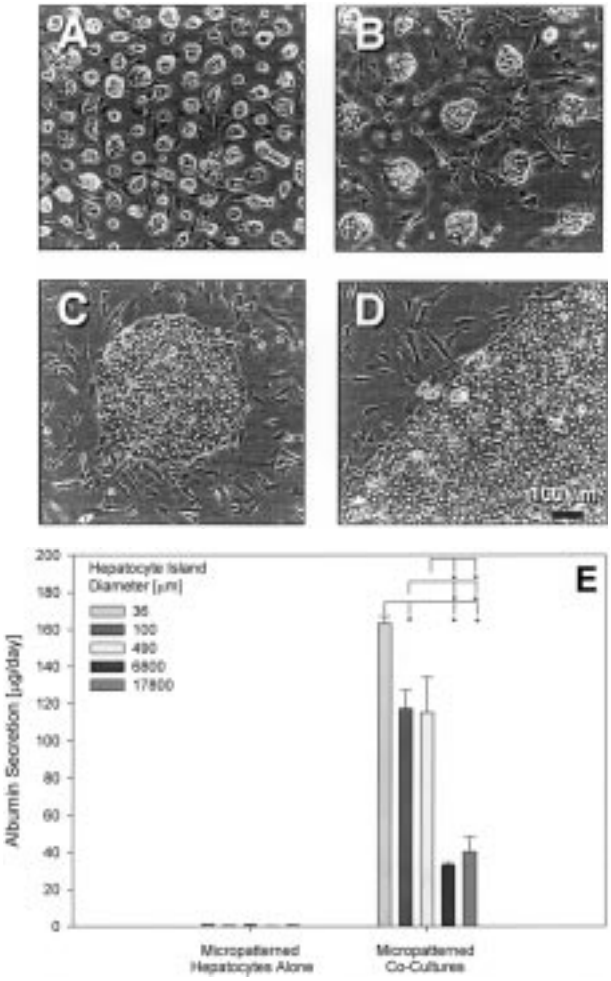

Fig. 3. *Using microfabrication to control cell-cell interactions. A–D. Co-cultures of hepatocytes (liver cells—in islands) and fibroblasts with varying degree of interaction, yet similar cell numbers in each culture. E. Effects of spatial reorganization on long-term liver-specific function (albumin secretion) of co-cultivated cells. Co-cultures with smallest islands of hepatocytes, and therefore the greatest interaction with fibroblasts, functioned maximally. Cultures of micropatterned hepatocytes alone (not depicted) deteriorated without fibroblast interaction (Bhatia et al., 1998).*

Biomaterial scaffolds

Biomaterials have been primarily utilized in tissue engineering as scaffolds for the cellular components of tissue-engineered constructs. These materials vary in composition from entirely synthetic to biologically-derived materials (Alexander, 1996). Synthetic biomaterial scaffolds include inorganic materials (e.g., metals, ceramics) as well as synthetic polymers (polyurethanes, polyesters, polysiloxanes). Biologically-derived materials include proteins and polysaccharides (fibrin, chitosan, hyaluronic acid, collagen). The field is rapidly evolving towards specialized biomaterials that have been engineered to: erode into an naturally-occurring byproduct during implantation (temporary scaffold), incorporate bioactive moieties to direct tissue ingrowth and host response (drug delivery device), block undesirable biological phenomena (barrier), and that alter their material mechanical properties in response to an environmental stimuli (''smart'' materials) (see (Hubbell, 1995; Pachence and Khon, 1997) for reviews). Together, biomaterial scaffolds exert control over cellular interaction with physicochemical signals, provide structural support, dictate matrix presentation for cell signals, and provide sites for attachment, migration, tissue ingrowth, and matrix deposition. Control can be exerted over the biomaterial surface, bulk characteristics, toxicity, degradation, and replacement rates. Increasing awareness of the structure/function relationship of tissues has led to the application of microfabrication techniques (though not always from the realm of conventional microelectronics) to precisely define the structure and chemistry of these scaffolds.

Scaffold architecture

Typically, biomaterial architecture is defined by process parameters. 3-dimensional shapes are macroscopically

defined by traditional manufacturing processes such as extrusion, melt molding, solvent casting and particulate leaching, phase separation, hot embossing, laser and mechanical machining, and polymer atomization. Molding of PLGA (a biodegradable block co-polyester), for example is often done using a tube, or vial, resulting in a cylindrical or disc-shaped construct (Thomson et al., 1997). More complex structures have also been achieved by progressive membrane lamination of thin polymer layers (Mikos et al., 1993), yet these techniques are limited in their resolution, reproducibility, and amenability to manufacturing.

Material microstructure is often dictated by process parameters such as the choice of solvent in phase separation (Lo et al., 1995), doping with leachants (Mooney et al., 1994), controlled ice crystal formation and subsequent freeze-drying to create pores (Yannas et al., 1982), and polishing (Alexander, 1996). Many of these techniques generate a wide distribution of feature sizes that are very sensitive to processing parameters. Fabrication at the micro-scale borrowed from microelectronics, manufacturing, and chemical engineering, have enabled advances in the structural design of tissue engineering scaffolds at both the micro and macro scale.

At the microscale, biological casting of tissue surfaces or molecules has allowed creation of structural replicas at the molecular (nm) and macromolecular/subcellular (μm) scale. PMMA casting of endothelial-stripped blood vessel, removal of tissue, and solution casting of the PMMA mold in a biomedical polyurethane, yielded a 30-dimensional, textured, biomimetic surface with features as small as 50 nm. These substrates promoted rapid spreading of endothelial cells and more native morphology than on flat polyurethane surfaces (Goodman et al., 1996). While this technique offers the ability to effectively mimic biological architecture, it relies on availability of tissues, and is limited by the inability to alter features that may be determined to be important. Similarly, biological casting on a smaller scale, Shi et al. (1999) have used radio-frequency glow-discharge plasma deposition of fluoropolymers on disaccharide-coated proteins to create template-imprinted nanostructured surfaces for protein recognition. These model systems aim to direct adsorption of specific proteins, in their native state, once implanted. Thus, synthetic materials may ultimately be created that selectively recognize particular proteins thereby rendering scaffolds more ''biocompatible'' or ''bioactive''.

Alternatively, traditional injection molding and casting of biocompatible polymers have been combined with microelectronics fabrication to define microscale structure. For example, use of reactive-ion etched silicon or patterned photoresist (EPON SU-8) on a solid substrate have been utilized as templates for creating microtexture on PLGA, low density polyethylene, and polydimethylsiloxane substrates (Kapur et al., 1996). Figure 4 depicts an SEM image of a 3-dimensional LDPE surface which has been texturized by injection molding against a patterned silicon template with spherical projections of ~ 2.5 μm diameter and holes of ~ 1 μm diameter. Furthermore, the authors demonstrated 90 to 95% pattern fidelity in all three planes when a single silicon wafer was used as a template over 60 times.

Advances in microscale fabrication have occurred due to application of solid freeform fabrication techniques-classically utilized for rapid prototyping of automotive parts. This class of additive fabrication techniques has not typically been used in microelectronics manufacturing due to limits in resolution on the 10's of micron scale. However, since cells and tissues vary in structure on the 1–1000 micron scale (Figure 1), they have recently proved useful to offer increased structural definition to implantable biomaterials. Stereolithography, amethod for rapid prototyping from computer-assisted design drawings utilizes an ultraviolet-curable resin and uv light to build up structures layer by layer from a vat of photosensitive polymer. This method of rapid prototyping has been integrated into surgery for quite a few years. CAT scan (computer assisted tomography of X-ray) images are used to generate prototypes for surgical planning (Lambrecht and Brix, 1990). Recently, these prototypes have been utilized as molds for casting biomaterials for facial implants (maxillo-facial regions), thus offering rapid generation of patient-specific implants. However, the limits of resolution of stereolithography have not been adequately exploited for finer tissue architecture that may be useful in various tissue engineering applications (approaching 50–100 μm).

Another form of solid freeform fabrication (ceramic

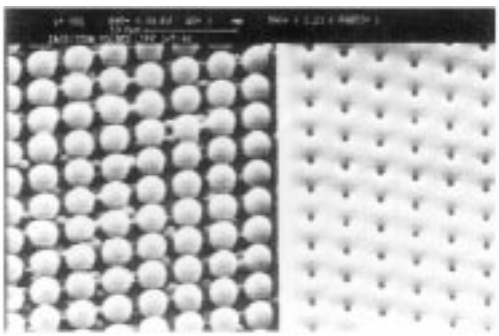

Fig. 4. *Examples of Defined Scaffold Microarchitecture. 3-dimensional textured low density poly(ethylene) scaffold. Spherical projections of 2.5 μm with ~ 1 μm diameter holes. Scaffold was fabricated by injection molding using textured silicon as a template (Kapur et al., 1999). Reprinted by kind permission of John Wiley & Sons.*

SFF) has been used to generate orthopedic implants to direct bony tissue growth (Chu et al., 1999). In direct ceramic freeform fabrication, a photocurable ceramic suspension in acrylates is used in place of the uv-curable resin used in conventional stereolithography. After polymerization, objects are sintered (1550°C) to burn off the polymer binder. While this technique is versatile and rapid, due to the side-scatter of light from ceramic particles the resolution of this technique is approximately 600 microns. Alternatively, stereolithographic molds can be utilized to cast thermally-cured versions of the ceramic/acrylate blend, a process known as indirect ceramic SFF. In Figure 5, a 40% v/v suspension of hydroxyapatite was prepared in acrylate monomer to build a bone tissue engineering scaffold with 420 micron channels. The construct was fabricated in order to study the influence of channel architecture on bone growth. This suspension was cast into stereolithographically-fabricated epoxy ''lost'' mold. The object is then heated to 60°C to polymerize the hydroxyapatite object around the epoxy mold. Finally, the object is heated further to pyrolyze the polyacrylate ceramic binder and then burn away the epoxy mold. This technique allows creation of 3-dimensional biomaterial scaffolds, though the efficiency of polyacrylate pyrolysis limits the ability to remove sections much smaller than 450 microns.

Finally, this approach has also been utilized in a process known as 3-dimensional printing, where a bed of polymer particles mixed with salt and are bound with droplets of a binding agent, layer-by-layer (Griffith et al., 1997; Kim et al., 1998). Ultimately, the unbound polymer will fall away and the salt can be dissolved to leave behind a porous polymer. This printing process has been proposed for construction of complex, vascularized solid organs such as the liver. The polymer particles utilized, were biodegradable polyesters that generate physiologic acids (glycolic and lactic) and can be altered in their relative proportion to adjust degradation rates, as has been previously characterized by Bob Langer's group at MIT (Langer et al., 1993). Current resolution of this technique is limited by polymer particle size and remains on the order of 200–300 microns. This technique may ultimately prove more useful for small tissue replacements and/or lumenal structures, since solid vascular organs such as the liver will require cell population of an entire polymer scaffold. This process is often limited by migration rates of seeded cells and diffusion of nutrients and oxygen. In this case, a better understanding of cell migration and fate in response to scaffold cues may be important to the successful implementation of this strategy.

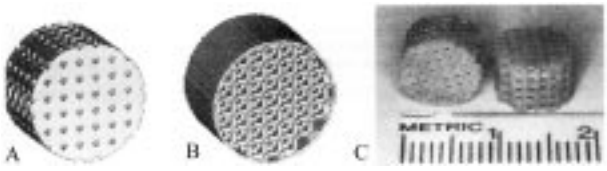

Fig. 5. *Hydroxyapapetite scaffold for bone tissue. Prepared by indirect ceramic stereolithography, a method of solid free form fabrication. A. Design B. Negative image of mold design displaying 420 micron channels. C. Hydroxyapapetite structure for implantation (Chu et al., 1999). Reproduced by kind permission of Materials Research Society.*

In the future, micro and macroscale structure will be key features of engineered tissues. In particular, systems that allow increased resolution (down to $\sim 1\,\mu m$) in the 3-dimensional fabrication have been made through generation of new photochemistries for light-activated fabrication. One study showed that a new class of initators allowed use of two-photon microscopy to create 3-dimensional elements such as photonic bandgap structures, optical data storage, cantilevers, and tapered waveguides (Cumpston et al., 1999). A similar approach to biological fabrication may introduce important advantages over SFF. Similarly, compatibility of fabrication techniques with synthetic or biological hydrogels (Suggs et al., 1998; Elisseeff et al., 1999) would allow current tissue engineering protocols to readily adapted to incorporate relevant architectural features. Synthesis of hydrogel structures under physiologic conditions could even allow incorporation of sensitive bioactive moieties and living cells.

Patterning of scaffold chemistry

Scaffolds for tissue engineering may incorporate specific bioactive chemical moieties to direct cell adhesion, migration, and tissue ingrowth and repair. To direct the adhesive moieties in specific patterns on the scaffolds, several techniques originally developed for *in vitro* 2-D model systems (see *Cells*) have been translated for this application. Protein and peptide localization can be achieved through a class of techniques known as protein stamping or ''microcontact printing''. Popularized by Whitesides at Harvard, this method for precise transfer of chemical groups from a texturized silicone surface is discussed in detail elsewhere (Kumar et al., 1994; Kumar and Whitesides, 1993; Singhvi et al., 1994). Recently, similar approaches have been utilized to print proteins directly (i.e., not a group that will later be linked to a protein). Texturized silicone stamps are dipped in protein, dried, and then transferred onto chemically-modified substrates. This has been accomplished for poly-L-lysine, laminin, and bovine serum albumin (James et al., 1998; Bernard et al., 1998; Branch et al., 1998; Wheeler et al., 1999). Uniformity of protein deposition depends upon various processing parameters (hydrophilicity of stamp, drying procedure, contact

pressure, etc). Stamping has been typically conducted onto flat surfaces, though microcontact printing with flexile stamps has previously been utilized on non-planar surfaces as. Thus, this technique may allow modification of accessible regions of pre-fabricated scaffolds.

Alternatively, a surface with topological features can be inverted and partially dipped in protein solutions- another mode of physical localization (Kapur et al., 1999; Kapur et al., 1996). This technique, though useful as an experimental tool, is limited by surface tension, feature size, and reproducibility. Similarly, localized protein deposition has been achieved through the use of microcapillaries filled with a highly hydrophilic hydrogel (Martin et al., 1998). The hydrogel, composed of repeating neutral sugar groups, resists protein adsorption, thereby promoting protein transfer to another surface. Currently, this technique is limited in resolution by the size of the capillary (10–80 μm) and the labor-intensive capillary loading and stamping process (2 seconds per spot).

Another mode of physical localization of bioactive moieties is achieved by microfluidic patterning. Delamarche et al. (1997) demonstrated use of a patterned PDMS stamp. When placed against a rigid surface, PDMS conforms to form an aqueous seal. In this study, capillary action was used to wick a protein solution into microchannels, resulting in deposition of a protein (Immunoglobulins) in linear strips on the underlying substrates. Alternatively, channels have been forcibly perfused with protein solutions or multiple different proteins, allowed to adsorb, and were shown to retain immunoreactivity for fluorescent staining and sufficient epitopes for cell binding (Folch and Toner, 1998). Finally, chemical reactions can be localized within elastomeric netowrks. Patel et al. (1998) demonstrated that biotinylated PLA (a biodegradable plymer) could be selectively bound to avidin by exposure through microfluidic localization. The resulting avidin pattern could be utilized to pattern an arbritary biotinylated ligand (RGD, IKVAV) and mediate cell attachment. Thus, relatively planar surfaces of biomaterial scaffolds may be selectively modified in contiguous patterns with resolution on the order of 10 microns. Patterning of discrete regions may require incorporation of other techniques, such as protein stamping or photochemical modification (Bearinger et al., 1997). Indeed, many photochemistries have been developed for modification of biomaterial scaffolds (Clemence et al., 1995; Herbert et al., 1997; Chen et al., 1997; Aldenhoff et al., 1997; Ito et al., 1996; Aldenhoff and Koole, 1995). Advances in optoelectronics and multi-photon microscopy will increase the versatility and utility of these light-based approaches- potential allowing selective chemical modification in a pre-specified scaffold voxel.

Finally, chemical modification of components to be used in solid free from fabrication, allows regional incorporation of specific moieties within a 3-dimensional construct (Park et al., 1998). This technique is limited by various processing parameters: retention of bioactivity of immobilized moiety in solvent, minimal achievable polymer particle size, and binder mechanics. However, the combination of architectural and technical techniques, conceptually points towards a future of precisely defined scaffolds that are tailored with respect to physicochemical material properties and material structure.

Living Hybrid (Cell/Scaffold) Constructs

The progress in understanding cellular behavior and manufacturing scaffolds, though still in their infancy, has led to a number of attempts to develop building blocks for multicellular tissue implants. Combining living cells and fabricated scaffolds into hybrid constructs, investigators have begun to address fundamental issues such as tissue integration, protection from immune responses, and vascularization. Tissue integration and/or wound healing can be promoted by exploiting the phenomena of contact guidance (see cells) where cells align and migrate along the major axis of a microtextured surface. For example, in percutaneous skull implants, groove orientation of titanium-coated substrates modified the down growth of adjacent epithelium (Brunette and Chehroudi, 1999; Chehroudi et al., 1990), a phenomena that may be useful for dental prosthesis. Animal studies are underway to examine the effects of implanted substrates on local tissue organization and bone deposition. A similar approach may be utilized in other areas (skin, muscle) where integration with host tissue is of paramount importance.

Another key factor influencing the success of tissue engineered constructs is the interaction with the immune response. Normal immune responses (cell-mediated and humoral) account for both short and long-term responses to foreign bodies from hypersensitivity, to foreign body reaction and fibrous encapsulation, to rejection. With regard to acute rejection that occurs due to antibody recognition and complement fixation, investigators have attempted to hinder passage of these effectors by encapsulating cells with size-selective polymer membranes. These membranes typically exhibit a range of pore sizes due to fabrication by phase separation or other techniques. Desai et al., 1999; (1998) have microfabricated silicon biocapsules with a uniform pore size distribution and demonstrated that immunoisolation effectiveness is greater with 18 nm pores than 66 nm (see Figure 6). Since the effective size of antibody and

complement components are estimated at 20–50 nm, the superiority of the smaller pore size may be due to effective suppression of a portion of the immune response. Long-term studies with this approach will need to specifically address the biocompatibility of silicon with regard to fibrous capsule formation as well as the potential induction of the immune response due to implanted cell products permeating through host tissue (''antigen shedding''). In the long term, scaffolds can become progressively encapsulated by scar tissue in the so-called 'foreign body response'. In the case of hybrid constructs, this would lead to device failure as the transport of nutrients to the implanted cells would be compromised leading to impaired cellular function. Ultimately, hybrid constructs may include immunomodulatory cytokines (interleukin-10) to further suppress the localized immune response.

Another key obstacle in the creation of tissue engineering of whole organs is the need for vascularization. With the exception of cartilage (chondrocytes normally live at very low oxygen tensions), most tissue engineered constructs will require an extensive capillary network as well as sites for surgical anastomosis to the patient's circulation. In the biological community, this is an active area or research and many angiogenic and angiostatic factors (both soluble and insoluble) are under investigation. In addition, the role of extracellular matrix is known to interact with the angiogenic pathway. Thus, extracellular matrix localization has been used *in vitro* to spatially direct capillary formation (Spargo et al., 1994). This may allow definition of capillary networks in engineered organs. In addition, capillaries formed by spatial localization were noted to form functional lumens when assessed by microinjection of fluorescent dyes, in 1–3 d as compared to 7–10 d under randomly-oriented configurations (see Figure 7). Thus, capillary ingrowth may ultimately be spatially specified and accelerated for earlier perfusion of tissue engineered constructs.

Finally, microfabrication techniques have been useful for production of building-blocks for tissue engineered constructs. For example, hepatocyte spheroids- an aggregate of hepatocytes known to stabilize hepatocyte function- are typically manufactured for use in extracorporeal bioreactors by agitation and aggregation of suspended cells on a non-adhesive substrate. This process creates a wide distribution of spheroid sizes, some of which will have oxygen limitations and necrosis at their core. To systemetize the process of spheroid formation, Yamazaki et al. used patterned thermoresponsive polymers to create islands of attached fibroblasts (Yamazaki et al., 1994). Upon lowering of substrate temperature below the polymer transition temperature, the polymer dissolved allowing formation of fixed diameter ''floating'' hepatocyte islands that later formed spheroids of the same size ($\sim$ 350 μm diameter).

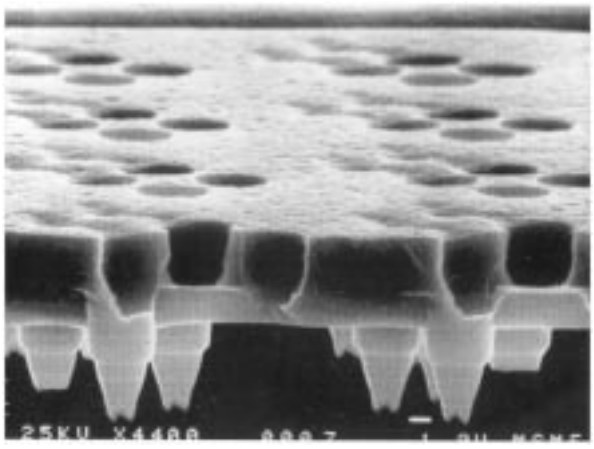

Fig. 6. *Microfabricated silicon biocapsule for islet transplantation. P-doped silicon, polysilicon, and a sacrificial oxide layer were used to define 18 nm pores for immunoisolation of encapsulated cells. Unencapsulated cells were rapidly destroyed whereas encapsulated cells survived for 8 days and retained ability to respond to glucose stimulation (Desai et al., 1999). Reproduced here by kind permission of Kluwer Academic Press.*

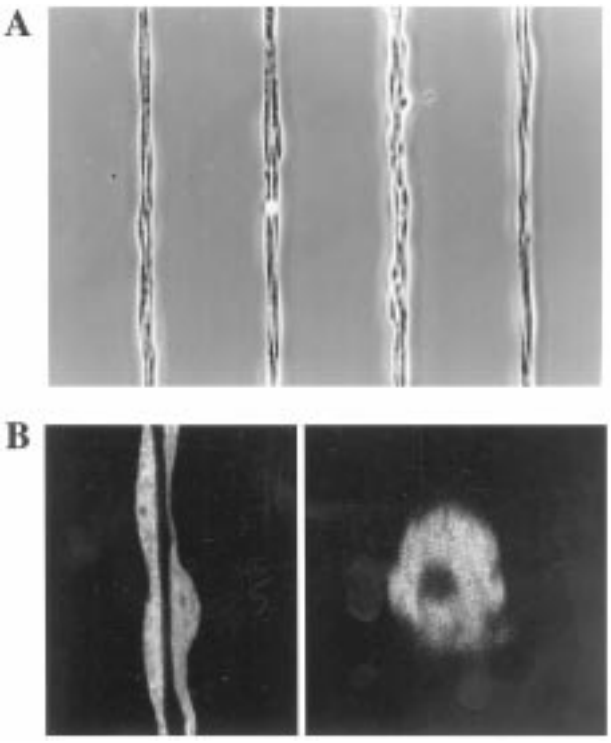

Fig. 7. *Microfabricated substrates used to induce capillary blood vessel formation. A. Capillary endothelial cells cultured on adhesive lines (10 um wide) are induced to differentiate and B. self-organize into patent tubes (Dike et al., 1999) as seen here in cross-section in two different planes. Reproduced here by kind permission of the Society for In Vitro Biology.*

This technique has also been used to formation of multicellular spheroids by layering hepatocytes on top of the fibroblasts prior to polymer dissolution (Takezawa et al., 1992). Resulting ''organoids'' could therefore be controlled by initial surface area and cellular composition. Immunostaining indicated presence of albumin (a marker of liver-specific function) up to 3 weeks. Recently, the resolution of this technique was demonstrated to be as good as 100 microns, offering the potential to create a wide range of dimensions (Ito et al., 1997). This microfabrication offers a method to reproducibly fabricate a building block for tissue engineered constructs.

Similarly, cubic tissue aggregates have been created by culture in microcontainers that have been precisely fabricated (Weibezahn et al., 1995; Knedlitschek et al., 1999). A brass mold was machined by diamond milling to create 300 μm square features. This mold is used to cast PMMA, which is subsequently milled to create micropores (< 10 μm) at the bottom of each well. Thus, cells are exposed to oxygenated media from top and bottom surface, and convective flow can be introduced to the system while minimizing shear stress to the cell surface. The porous structure allowed for oxygen transport while the macroscopic form allowed essentially tissue molding. These structures were modified by adsorption of dilute ECM and used for cell culture of immortalized cell lines as well as primary rat hepatocytes. Preliminary data suggest that cells remain viable and produced copious amounts of extracellular matrix. Indeed, when washed out with a ''jet'' of media, cellular aggregates preserved the three-dimensional shape of the well. Both these techniques while promising are very preliminary. Functional and biochemical assays should be conducted as well as examination of factors which influence stability of these structures, rearrangement of cellular aggregates, the role of other cell populations, and methods to incorporate these constructs in their intended application.

Future Directions

While the possibility of engineering tissues for clinical use is becoming a reality, many challenges remain for the future. Many advances from within cell biology, biomaterials, and fabrication continue to build the foundations demanded by tissue replacement therapies:

The rapid acquisition of experience with identifying, isolating, and controlling the differentiation pathways of embryonic stem cells suggests the eventual possibility of not only producing and harvesting the desired cell types of a given organ, but also directing the natural process of development to produce whole organs. Mesenchymal stem cells, which retain the ability to renew themselves as well as to differentiate into many different connective tissue cell types, are already being isolated and studied for replacement of cartilage, bone, and skin (Pittenger et al., 1999; McKay, 1997; Prockop, 1997). Similar efforts to isolate hematopoietic stem cells for bone marrow replacement have been in progress for several years (Berardi et al., 1995). The primary challenge for stem cell biology is to develop a detailed understanding of the cues, including the architecture of the extracellular matrix scaffold and cell-cell interactions as well as the influence of soluble factors, that could be used to guide the development of these cells into the desired organs. Understanding these environmental stimuli and their subsequent intracellular effects is a major focus across all of cell biology. To this end, the microfabrication industry has stepped in to provide tools to engineer well-controlled cellular environments as well as analytical approaches that will increase the pace of data acquisition. Specifically, the development of microarrays has enabled gene chip development for studying the gene expression patterns of cells under different conditions (genomics), and the possibility of for studying protein expression in the near future (proteomics), as well as the functional impact of such expression (phenomics) (see for review Ramsay, 1998).

In scaffold manufacturing, several advances are on the horizon. Whole new classes of materials continue to be developed. For example, using peptides as polymer bases themselves, entirely biocompatible polymers are being developed by genetic design (Tirrell, 1997; van Hest and Tirrell, 1998). The use of combinatorial approaches, made available by microarray techniques, allow organic chemists also to produce novel materials at ever increasing rates. Fabrication tools continue to be developed to increase the resolution of 3-D scaffold manufacturing. The need for high resolution fabrication techniques is clear in the field of tissue engineering, and may drive fabrication to be able to routinely manufacture micrometer and nanometer scale structures. In addition to spatial resolution, investigators are now realizing that incorporating active/dynamic elements within cellular constructs may be critical to match the dynamic needs of living tissues. A clear example of this is the development of micro-electronic drug delivery devices, such that concentrations of a therapy can be dynamically and rapidly titrated to the varying needs of a patient (Santini et al., 1999). Less obviously, the ability to control adhesivity, for example, over time within a implantable scaffold could add functionality to the device that would otherwise be unattainable. Finally, as optical tools advance, cell placement due to photoactivated chemistries as well as by optical forces (i.e., laser-

directed writing) may offer increased flexibility (Odde and Renn, 1998).

In each key area, cells, scaffolds, and hybrid constructs, the microfabrication community has made significant contributions. As tissue engineering capabilities transition from dream to reality, one of the critical roles of the microfabrication community will be to open communication channels with the tissue engineering community, so that as technologies are being developed, they can be appropriately geared for the applications. We have conceptually depicted some opportunities in tissue engineering for mirofabrication tools in Figure 8, although we anticipate that the potential synergy of these fields will only expand with time. As these products transition to the clinic, another landscape of opportunity will be uncovered- issues of safety and reproducibility of the manufactured tissue, preservation of the material, tracking of biological materials and cells, prevention of transfer of cancerous or infected materials, and standardization across the various stages of development of the product- and will be paramount to acceptance of these products by the medical establishment. The focus on replacement of diseased tissues has already created a make-shift community from diverse disciplines- from genetics to manufacturing. In the future, tissue engineering at the micro-scale will provide a unique opportunity and challenge to focus existing and developing technologies towards impacting clinical medicine.

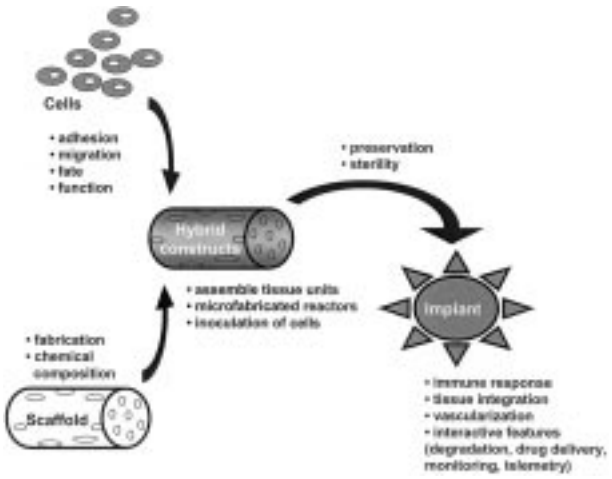

Fig. 8. *Opportunities in tissue engineering for microfabrication. Contributions in tissue engineering can be made at many levels: individual cells and cell structures, scaffolds, hybrid constructs, and constructs after implantation. Understanding of fundamental microenvironmental cues on cell processes will be important to achieve functional organs. Microfabricated cell cultures offer unique opportunities to study key features of the microenvironment on relevant length scales. Biomaterial scaffolds with precise architectural and chemical features will offer the ability to tailor both physiochemical scaffold properties (degradation, mechanical stability, porosity) as well as interactions with cells and host. Hybrid constructs can be manufactured from smaller tissue units using microfabrication, bioreactors for maturation of in vitro tissues may incorporate microfluidics, microactuators, or microsensors, and assembly of the constructs may require micro-delivery devices to innoculate scaffolds with cells. Hybrid constructs will require precise preservation and sterility requirements, both of which can be improved by microfabrication tools. Finally, implantation into the host requires immunoisolation, tissue integration, and may ultimately incorporate active elements (sensing, triggered drug delivery, telemetry)- areas where microfabrication may offer significant advantages over conventional approaches.*

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