

Introduction

One aspect of tissue engineering involves the development of artificial organs or replacement tissues for use in individuals with failing organs or tissues due to trauma, disease, or even old age. Researchers have been successful in developing potential tissue engineered solutions for several tissues such as skin, pancreas, blood, and cartilage. All of these “successful” solutions have a common thread- the tissues are generally amorphous or two-dimensional, isotropic, and have no orientation dependence. Attempts to develop tissue engineered solutions for more complex tissues, such as muscle, nervous, and lung tissues, have progressed slowly and generally amount to little more than three-dimensional cell culture.

Current attempts at organized cell and tissue growth in complex tissues have been thwarted by the fact that cells *in vitro* do not respond in the same way that they do *in vivo*. Most cells grow in a random fashion that does not approximate the natural growth of tissues in the body. For example, muscle cells must be highly oriented and organized into a coherent tissue in order to perform their actuation function. The random nature of the growth seems to be a function of the lack of external signals or cues to the developing cells. This random growth *in vitro* negates any attempt to grow functional complex tissues for later transplantation or testing.

Complex tissues appear to develop by the use of cues of signals between cells that direct the growth and development of individual cells. The signals include soluble molecules that are transported by the medium in which the cells are growing, signal molecules that reside on the surfaces of cells and the extracellular matrix, physical forces, and surface topography. The effect of surface topography on the development, motility, differentiation, orientation, and alignment of cells is often referred to as “contact guidance.”

Contact Guidance

“Contact guidance” was the term devised by Weiss in 1945 for the ability of an underlying substrate to direct or modify the response of a cell. A substrate can vary in a number of ways such as with absorbed protein gradients, or in levels of adhesiveness, but the strict definition of contact guidance includes only morphological variation in the surface. A number of morphologies have been studied including curved surfaces, steps, parallel grooves, and angled planes. These papers have similar conclusions and a comprehensive theory behind contact guidance is beginning to form, though some of the specifics are still being debated.

Considering that the effects of underlying surfaces on cells was first reported in 1912¹, relatively little is known about how to manipulate the surface morphology to direct cell growth and movement. The ability to manipulate cell shape, orientation, differentiation, and motility is of critical importance in tissue engineering. For tissue engineering to even begin to approach its potential, knowledge of how to cause cells to grow in specific patterns is essential. It is known that cell shape, orientation, differentiation, and motility is affected by a number of factors associated with the surface such as the chemical makeup of the surface (i.e. the presence of proteins such as laminin or fibronectin), bound chemo-attractants, the adhesiveness of the surface, and surface morphology². Recent strides have been made in this last area with the help of another booming area of research, micromachining.

Micromachining is basically a set of tools and techniques for fabrication of structures and devices on the nanometer to millimeter scale. Since most cells and cellular features are of the same scale, microfabrication technologies and microfabricated devices are ideal for study of cellular phenomena. Micromachining technologies have already been used to develop sensors for measuring electrical phenomena on the cellular level and to create highly sensitive biosensors. The application of micromachining, and other techniques, to the study of surface effects on cells is a relatively recent phenomenon and is a focus of this proposal.

A number of investigators have used microfabrication and other techniques to study the response of cells to microscale substrate surface properties with a variety of results. A short topical overview of their results is appropriate here.

Effects of Surface Roughness

Both random and regular surfaces have been found to affect the spreading, proliferation, and differentiation of cells *in vitro*. Schmidt and von Recum found in 1991³ that random textures with typical repetitions of about 5 μm produced the smallest macrophages and that macrophages with both smaller and larger dimensions were more spread out. A similar study done in 1995 involving the growth, proliferation, differentiation, and protein synthesis of human-osteoblast cells on titanium found that there were no spreading effects like those reported by Schmidt and von Recum, but there were several other significant effects such as decreased cell proliferation, increased cell differentiation, and increased protein synthesis and matrix production.⁴ Thus, the surface seemed to affect not only protein synthesis, but also gene expression.

Effects of Surface Texture

Surface texture research attempts to determine whether it is the bumps or the depressions that have a greater impact on cells. A paper by Green, et al.⁵ concluded that the upper surfaces of a substrate have the greatest effect on proliferation rates for fibroblasts on silicone (i.e. pillars have greater effect than do depressions). No alignment effects were found.

Effects of Convex Surfaces

Dunn and Heath⁶ reported in 1976 that convex surfaces play a significant role in the migration direction and aspect ratio of chick heart fibroblasts. They tested two types of convex surfaces: curved and angled. They found that on glass fibers with a radius of less than 100 μm the cells aligned with the axis of the fiber and movement of the fibroblasts was bidirectional along the axis of the fiber. For prisms with angles of less than 4°, the fibroblasts showed no effect from the barrier and crossed at will with no change in direction or speed. At angles greater than 4°, perpendicular travel over the barrier was almost eliminated, while travel at angles across the barrier were reduced and generally redirected along the peak rather than across it. For angles much greater than 4°, travel over the barrier was almost completely eliminated. Dunn in 1982⁷ found that cells on spheres with radius of less than 50 μm became almost completely round and immobile, seemingly trapped wherever they first landed.

Effects of Concave Surfaces

Dunn in 1982⁷ reported on the effects of a concave surface on the alignment and movement of fibroblasts. He found that fibroblasts tended to align perpendicularly to the concave axis, exactly opposite of that for convex surfaces.

Effects of Step Changes

The influence of step changes in surface morphology on cells was investigated by Clark and others in 1987⁸. They found that for epithelial (BHK) cells and chick embryonic neural cell processes a gradual inhibition of step crossing occurred as the steps became larger. They also found a corresponding increase in alignment along the step as the steps grew larger. Cell type had a large impact on whether the step had any affect. While fibroblasts, epithelial cells, and neurons all reacted strongly to the steps, neutrophils were relatively unaffected.

Effects of Grooves or Ridges

A number of researchers have found that cells will align with multiple grooves on a substrate.^{9,10,11,12,13,14,15,16,17,18} The degree of orientation depended on the cell type, the surface, groove density, groove width, and groove depth. The cell alignment occurred with both radial¹⁶ and parallel grooves. Some found increased proliferation rates with grooves versus smooth areas^{13,19} while other found no change in proliferation rates^{15,17}. For example, Chehoudi¹³ et al found that V grooves in titanium could highly align gingival epithelial cells that were round in smooth areas. They also found a 50% higher cell density in the grooved areas. In their *in vivo* studies they found that the V grooves significantly reduced cell downgrowth which leads to implant encapsulation. They suggested that the grooves could be used to reduce implant encapsulation in not only dental implants, but other biomedical implants as well. Clark in 1990¹⁴ in a follow up to his step research found that grooves provide a much higher alignment than would be predicted by his step research. Clark noted that cells with more highly organized cytoskeletal structures were more likely to be impacted by the surface morphology while cells such a neutrophils with a less defined structure were less affected. Muscle cells have a highly organized cytoskeletal structure and would be expected to follow this pattern. Another study by Clark²⁰ on the effects of extremely small grooves with 130 nm spacing. He found that cells would align with grooves even at these dimensions. Alignment and elongation appeared related to groove depth. Meyle¹⁵ et al chose to focus on smaller grooves (1, 1.5, and 2 μm wide and 1 μm deep) in silicon and found that the degree of alignment for fibroblasts at these dimensions was extremely high. They found that the extending lamella stayed exclusively on the ridges which may indicate why the cells aligned even though they made contact with the surface at all heights. A group in the Netherlands¹⁸ tried to quantitate the effect of grooves on cells and found that 2 μm grooves highly aligned and that 5 μm grooves significantly aligned rat dermal fibroblast while 10 μm grooves had little effect on cell alignment.

Other Topographical Effects

Several other applications have been developed that involve micromachining and topographical effects. Micro-topographical structures have been used to direct the growth of dendrites and axons in desired directions and locations to both build neural networks² and to study the dendritic tree in individual neurons²¹. Similar structures have been used to guide neurons past stationary microelectrodes for use in measuring action potentials and cell impedance²². Additionally, topographical effects have been used to reduce inflammatory effects in soft tissue²³.

Electrical and Magnetic Field Effects

A great deal of research has been done on the effects of electric and magnetic fields, due primarily to concerns regarding mutagenicity and toxicity of the fields. Little, if any, data has been reported regarding

orientation effects, and even less has been done with muscle cells. There is significant data, though, indicating that electric and magnetic fields do and can have significant effects on cells that are not always consistent or predictable. For example, in the last few years, several researchers have indicated that applied electric fields increase cell proliferation²⁴, growth rates²⁵, and permeability^{26,27}, as well as cause morphological changes^{27,28} in both embryonic and adult tissues. Osteoclast cells, though, were inhibited by electric fields²⁹. Additionally cell viability effects were found, as might be expected. The studies that determined morphological changes indicated the possibility that electric fields may be applied to orient and direct cells, though nothing regarding muscle cells has been reported in this area. Thus, there remains a significant need for study in this area.

Magnetic field studies have produced similar results, though some researchers are unsure whether the effects of the magnetic field are the result of induced electric fields (or possibly the other way around). Research into magnetic field effects finds results such as slowed cell growth and proliferation in plants^{30,31}, but higher growth rates and proliferation in bacteria^{32,33} and animals^{34,35,36,37}, increased collagen and proteoglycan production in cartilage³⁸, and cAMP production that varies with field strength³⁹. The most interesting paper, as related to this proposal, found that collagen growth could be oriented using magnetic fields⁴⁰, thus trapping the smooth muscle cells in the matrix and forcing them to grow in an aligned manner. This research suggests that there may be a possibility of orienting muscle cell growth using magnetic fields, especially considering that as myogenic cells develop and grow, they begin to deposit collagen. Magnetic fields, then, are a promising area for research into the *in vitro* alignment of skeletal muscle cells.

Effects Specific to Skeletal Muscle

The majority of research into contact guidance and other cell phenomena associated with surfaces has not involved muscle cells. Specific research with muscle cells is critical since muscle cells develop in unique ways and have a unique function when compared to most other cells. Mature muscle cells and fibers develop from the fusion of mononucleated myoblasts during embryogenesis. Thus, mature muscle fibers are multinucleate and relatively large compared to most cells. For this fusion to occur, the myoblasts must be linearly aligned, they must recognize each other and adhere to each other. There must be cues *in vivo* to direct this action, but *in vitro* these cues do not exist and myotubes grow randomly, without any orientation effects, and the mature muscle cells are highly branched rather than linear⁴¹. There is also a difference between embryonic myotubes and myotubes cultured from adult animals. Myotubes from adult animals react with and proliferate on laminin (a basic component of the basal lamina) while embryonic myotubes do not⁴². Thus, primary and secondary myotubes (as they are generally called) do not respond to the same cues equally. Specifically, secondary myotubes respond to the cues left by the primary myotubes. These cues appear to be a basic function of the basal lamina. Secondary myotubes are responsible for muscle growth after the primary myotubes have laid out the form of the muscle and for muscle repair in damaged muscle tissues. Without the cues of the basal lamina, secondary myotubes grow in random directions, even in primary myotube cultures in which no basal lamina has yet been deposited. In general, though, cultured muscle cells without any cues from their environment lose the majority of their inherent actuation function and some of their instructive value.

Because of the differences between muscle cells and most other cells previously studied, research into the specific reactions of muscle cells to surface morphology and patterning is of critical importance, especially since muscle cell engineering may benefit more from this type of research than other cell types due to their requirement of linear alignment for both fusion and function. A few groups have done some pioneering work in this area of providing cues for developing muscle cells. Isaeva⁴³ demonstrated that grooves can play a role in alignment and mobility of myogenic cells. John and Lawson⁴⁴ showed that collagen could play a role in muscle cell alignment during myogenesis. Several other groups have demonstrated that attachment and basal lamina proteins such as fibronectin⁴⁵, laminin⁴¹, and entactin⁴⁶ may also play a role in cell guidance and structure. Mechanical stresses and deformation have also been shown to be valuable in orienting muscle cells during development^{47,48,49,50}, even stresses that only apply to the underlying substrate (extracellular matrix) and not to the cells themselves (i.e. a prestressed substrate)⁴⁸.

Tissue engineering in three dimensions is a rapidly growing field and several researchers have performed work in the area of tissue engineering of muscle cells. Mulder and associates have demonstrated skeletal myogenesis in three-dimensional elastomeric substrates^{51,52}. Muscle cells have also been grown in three-dimensional culture media⁵³. The systems that have been most successful in aligning skeletal muscle fibers used applied forces to physically orient the cells or the mesh in which the cells were cultured. Okana, et.al. Demonstrated the ability to generally align mature cells by culturing the cells in rodlike meshes using centrifugal cell packing and cyclic tensile forces⁵⁴. Other groups^{55,56} have demonstrated similar work, though none of the groups reported angular alignment as high as expected due to the applied forces (i.e. none of the cells aligned directly with the applied forces, though the tendency was in that direction). Thus, while physical forces seem to be a factor in muscle

cell alignment, they are by no means the initial factor, nor do they suffice independently. All three-dimensional structures to date fail to provide most or any of the surface cues required by the developing cells for proper orientation, which are obviously required, for example, in the developing embryo where no tensile forces are present. Thus, even though the cells fully differentiate and proliferate, the cells are randomly oriented as they are in two-dimensional cultures unless physically forced to align. Thus three-dimensional models, while they do possess some advantages, provide no more information regarding the cues cells require for proper orientation and formation into tissues than do two-dimensional cultures without any added cues. Current manufacturing processes for three-dimensional gels and matrices for cell growth and culture, while they can control average pore size, density, and some other parameters, have not provided the ability to organize, control, or otherwise engineer specific pore sizes, patterns, or surface characteristics with any spatial resolution. Most gels and meshes also are physically weak and lack a constant structure when cells are introduced, though advances are being made in this area⁵⁷. Thus until manufacturing processes for highly controlled gels and matrices are developed, we are left using two-dimensional structures, which can be easily controlled and are physically stable, to investigate the response of cells to surface characteristics, patterns, and morphology.

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