Protein Printboard: Fibronectin Patterning to Control Cellular Organization

Asad Moten

Abstract

Cells are inherently sensitive to local mesoscale and microscale patterns of chemistry and topography. Recent research has investigated how surface mechanics might dictate cell behavior, affecting both cell function and differentiation. This study professes that alterations of the underlying matrix can dictate cell behavior and function. Through micro-orienting the substrate and micro-patterning protein, we were able to engineer a structural and biological backbone to regulate cellular behavior. The techniques involved in this study to control cell behavior include the micro-orienting of the ECM, electrospinning a 3D fibrous PMMA scaffold, and micro-patterning of fibronectin(Fn) for cellular attachment, The use of microscale structuring to restore tissue architecture and dictate cell behavior has several important implications for tissue engineering, cancer treatment, and stem cell differentiation.

1 Introduction

The human body consists of 10 trillion cells, which have been classified into more that 200 different cell types. Our bodies are organized in a hierarchical way: cells and extracellular matrix (ECM) form tissues, tissue form organs, and organs form complex organisms. Deciding which protein to express, when to divide, when to specialize, and when to commit suicide are all ongoing processes of the cells [1]. In addition to the intrinsic cell factors that regulate cell fate, extrinsic signals to the cell from surrounding ECM are essential in guiding it through distinct development paths. The difference between a clot of cells and matrix from a functional tissue is the well-defined organization of cells and ECM, which is closely associated with tissue function. The ECM is a 3D fibrous, highly oriented structure, which provides cells with numerous focal adhesion sites – points at which cells use their integrin receptors to attach to the surface. This attachment of the cell to its matrix is facilitated by fibronectin (Fn), which is an adhesive protein. The loss of this ECM architecture results in tissue malfunction and disease. Physiological processes such as development, tissue maintenance, angiogenesis, and wound healing require an orderly ECM architecture [2]. In order to intervene in any of these processes, it is necessary to have a comprehensive understanding of the molecular interactions that mediate cell motility and also of the highly complex pathways of signal transduction that regulate them.

Biology and medicine are currently undergoing a paradigm shift. Up until now, cellular transformations, whether in terms of stem cell differentiation or carcinogenesis, have been discussed in terms of genetic alterations that modify or deregulate cellular growth [2]. We have focused on rigorously characterizing the molecular components that comprise life, with the hope that such classification of all the parts will lead to a greater understanding of the whole. After the sequencing of the genomes of multiple organisms, it is now clear that the approach of reductionism provides only minimum understanding of tissue behavior. Consequently, biology is moving towards the development of methods and approaches to understand how complex cell and tissue behaviors emerge from collective interactions among multiple molecular components. It also seeks to describe molecular processes as integrated systems rather than numerous, isolated parts [3].

There is a very close relationship between the maintenance of the specific architecture of tissues and the original construction of those tissues. In morphogenesis, sets of cells are defined in the embryo by various organizer genes that in turn specify the anteroposterior axis, the dorsoventral axis, and, in Drosophila, segments and parasegments, ultimately forming blocks of cells with specific relations to each other [4]. Within these blocks, further separation and differentiation occurs, resulting in functional groups of cells in well-defined structural compartments. The fibrous extracellular matrix plays a very important role in molding the architecture of tissue as it provides support and anchorage for the cells, regulates intercellular communication, and provides necessary proteins for cellular adhesion and migration [5]. Cells maintain a characteristic morphology by adhering both to neighboring cells and the ECM. In vivo, the ECM is comprised of several proteins including collagen, fibronectin,

laminins and proteoglycans, which assemble in an intricate fibril network that is closely related to the plasma membrane of the cell. Cells bind to the proteins of the ECM through the transmembrane-surface receptors, which are in the group of macromolecules that includes members of the integrin, cadherin, immunoglobulin, selectin, and proteoglycan super families [6]. Of these, the cadherins, immunoglobulin-like receptors and selectins are involved in cell–cell adhesion, whereas the integrins and proteoglycans are involved mainly in cell–ECM binding. These receptors transmit mechanical stimuli from the matrix to the cytoskeleton of the cell [7]. The internal network of the cytoskeleton is a criss-cross network maintained by the opposing tension between microfilaments and microtubules that create a stable architecture to provide cell shape, in a phenomenon known as tensegrity. When these receptor molecules anchor the cells to the matrix, mechanical forces are carried through the cytoskeleton and these vibrations trigger cellular reaction, spreading, and protein expression [8].

Consequently, this study seeks to explain how cell and tissue behaviors emerge from collective interactions within complex molecular networks. The micro-manipulation of the 3D fibrous extracellular network will enable us to profoundly dictate cell behavior, affecting both cell function and differentiation. The techniques utilized in this study to control cell behavior involve the micro-orienting of the ECM, engineering of a 3D fibrous scaffold with the optimum diameter and orientation, and criss-cross micro-patterning of protein for cellular attachment. These micro-scale changes will trigger different intracellular signaling pathways through the cell surface receptors which in turn will lead to various cell responses. The use of microscale structuring to dictate cell behavior has several important implications for tissue engineering, cancer treatment, and stem cell differentiation.

2. Materials and Methods

Preparing the solutions- Various solutions were prepared for engineering the substrates. Thin fibers with optimum diameter and orientation were prepared using a solution with 0.8 g of 20% PMMA with 3.2 g of CH3Cl (Chloroform), THF (Tetrahydrofuran), and DMF (Dimethylformamide). A 60 mg of PMMA per 2 mL of toluene was prepared for spin casting the thin film. A 300 Angstrom thin film was prepared using 275 mg of Polybutadiene (PB) per 1 mL of toluene.

Preparing the thin film- Silicon was used as the substrate for our experiments. The silicon wafers were purified by immersing them in a boiling 1:1:4 solution of NH4OH: H2O2: H2O for 10 minutes at 80 degrees Celsius and then in a 1:1:3 solution of H2SO4:H2O2:H2O at the same temperature and time. Then, the wafers were submerged in a 1:3 solution of HF: H2O to create a hydrophobic surface. The thin film surfaces were spun cast at 2500 rpm by adding the varying solutions to the surfaces of the wafers. Using an ellipsometer, the thicknesses of these films were measured. The control thin film, which contained 20% PMMA and 2ml of toluene, was found to be approximately 1000 Angstroms, and the PB substrates were measured to be 300 Angstrom (hard film) and 3000 angstrom (soft film).

Electrospinning- Unless otherwise noted, all reagents were purchased from Sigma Chemical Company. The 20% PMMA solutions were placed into a 1.0 ml syringe mounted in a syringe pump that was subjected to a high electric voltage. The syringe was capped with an 18-gauge blunt end needle. The positive lead of the high voltage supply was attached to the external surface of the metal syringe needle. A rectangular grounded target fabricated from 202 stainless steel was mounted 4-6 inches from the syringe tip. The syringe pump was set to deliver the source solution at 25mL/h. In order to obtain oriented fibers, the electrospinning apparatus was modified to include a rotating mandrel. Simultaneously, the high voltage was applied across the source and the grounded target mandrel. The mandrel was set to rotate at 500 rpm.

Cell Attachment Assay (Cell Culturing/ Cell-Protein Seeding)- The various cells were stored in nitrogen and then thawed rapidly at 37 degrees Celsius and grown in DMEM supplemented with 10% bovine calf serum for approximately one week and were fed once every two days. Once cultures achieved 75% confluence, they were harvested by 0.1% trypsin on the surface to detach the cells. The trypsin was then neutralized by adding media to the solution. The cell density of these solutions was then verified using the hemacytometer. In the plating process, fibronectin was micro patterned onto the substrate in a criss-cross arrangement to help cellular adhesion. The cells were cultured for a day. Adipose, dermal fibroblasts, normal, and cancerous osteoblasts were cultured for approximately 48 hours before observation. All cells were cultured at 7% CO2 at 37 degrees Celsius.

Confocal Microscopy (Immunofluorenscence and Vinculin Staining) - Cells were fixed in 10% formaldehyde. For Immunofluorescence staining, Alexa Fluor 488 (actin stain) was used with

Propidium Iodide (nucleide stain) after cells were permeablized using a 0.4% solution of Triton with PBS.

Atomic Force Microscopy- The AFM was used to observe cell rigidity and detailed morphology of the PMMA fibers. Lateral Force Microscopy (LFM), which was used to measure the modulus of the cells on the substrates, is a secondary contact AFM mode that detects and maps relative differences in the frictional forces between the probe tip and the sample surface. In LFM, the scanning is always perpendicular to the long axis of the cantilever.

Scanning Electron Microscopy- PMMA fibers were rinsed four times in PBS for 30 minutes each and then overnight at room temperature. Samples were dehydrated through incubation for 20 minutes each at room temperature. PMMA fibers were incubated in 100% ethanol four times for 20 minutes each and then overnight at room temperature. Samples were then viewed using a JEOL JSM-840A scanning electron microscope with a DSG digital image acquisition system.

3. Results and Discussions

Characterization of Substrates- Three polymer biocompatible and biodegradable substrates, with varying characteristics, were engineered to test the cell behavior. The first substrate was made up of porous, elastic, electrospun PMMA fibers with optimum orientation and fiber diameter. Optimum diameter was achieved by assessing the quality of fibers made from the different solvents, which included Chloroform (CH3Cl), Tetrahydrofuran (THF), and Dimethyformamide (DMF). The fibers with different diameters were electrospun, while keeping the concentration of the PMMA constant at 20%, using several solvents with different dielectric constants which in turn allowed us to achieve fibers with varying diameter measures. The fibers formed using DMF were the thinnest with an average diameter of 200 nm, but consisted of numerous beads which hindered cell attachment by limiting focal adhesion sites [2]. Similarly, the THF solvent yielded fibers with an average diameter of 265 nm, but these fibers also consisted of several beads. The PMMA-Chloroform fibers, with an average diameter of 8 micrometers, were the best fibers because they yielded the greatest number of focal adhesion sites, indicated by the absence of beads. Consequently, chloroform was used as a solvent to engineer a 3D oriented substrate. The PMMA-Chloroform fibers were electrospun in a criss-cross orientation, in which the first layer was made up of horizontally placed fibers while the second layer consisted of vertically placed fibers. Previously, cells seeded on solvent casted films were shown to proliferate strictly in a 2D geometry. Thus, a more novel method of fabricating a 3D micro-porous scaffold was used to allow cells to grown both on and inside the fibrous substrate. The 3D oriented and random substrates' surface morphologies were evaluated using a scanning electron microscope, which also confirmed the average diameter of the chloroform fibers. Furthermore, spin casted films were fabricated using the polymer Poly-Butadiene (PB). Two concentrations of PB to toluene were spin casted in order

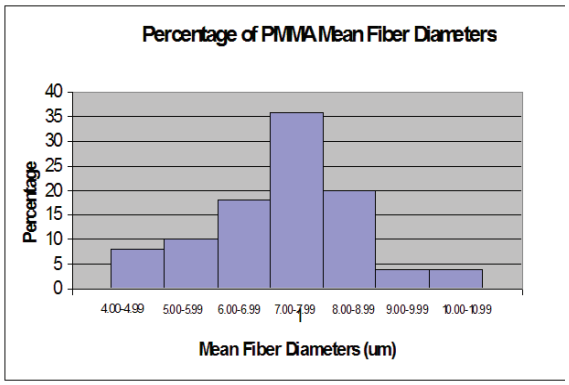

Figure 1. A. The table shows the average diameters of the three solvents used to determine the optimum diameter. B. shows the percentage of PMMA-Chloroform mean fiber diameter. It shows that the most frequently occurring diameter range is 7-7.99µm.

to make thin films of desired thickness: one of 300 Angstroms (hard film) and the other of 3000 Angstroms (soft film). The thin films were uniform and homogenous, as confirmed by the AFM images.

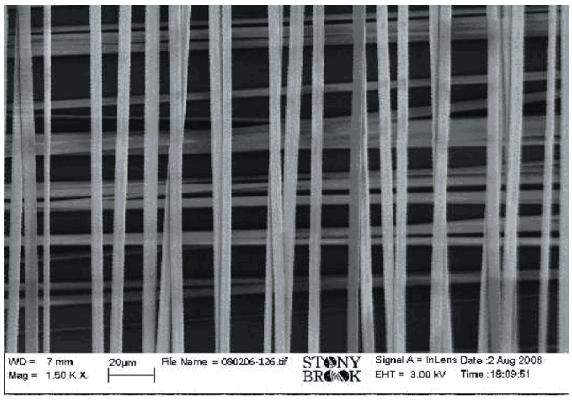

Figure 2. The thin PMMA-Chloroform fibers are micro-oriented in a criss-cross arrangement.

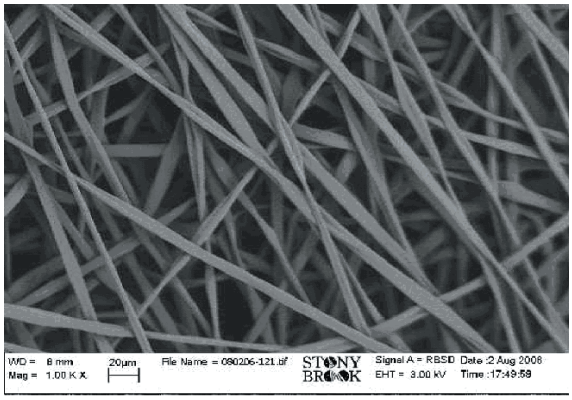

Figure 3. shows the random PMMA-Chloroform fibers under the SEM.

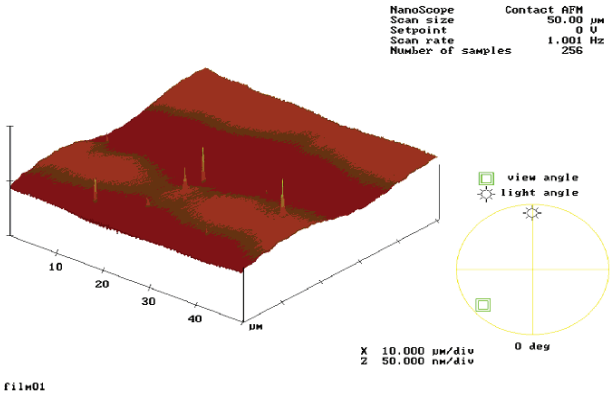

Figure 4. Shows the AFM image of the pb thin film on silicon substrate. The image shows that the thin film is uniform and homogenous and thus, suitable for further experimentation.

Substrate Induced Protein and Cell Organization - The oriented PMMA fibers were evaluated in vitro using normal 3T3 cells and fibronectin protein. Earlier studies have found that pore size, pore orientation, fiber structure, and fiber diameter can influence cell behavior. Among them, pore properties, such as porosity, pore dimensions, and pore volume, are parameters directly related to the cell behavior [5]. The PMMA fibrous scaffold, with more than 80% porosity, 8 micrometers median pore size, and 5m2 /g total pore area promoted cell growth and migration. To investigate the effect of this micro-oriented matrix on cell spatial organization and growth, the cultures of NIH 3T3 were carried out. The confocal images showed that the cells followed the fiber orienta-

tion by attaching their integrin receptors to focal adhesion sites on the fibers. Through vinculin staining, we discovered that these focal adhesion sites were present along the fibers and thus, dictated the cell linear spatial organization and in-migration on the fibrous substrate. As viewed by the confocal images, cells on fibers tended to spread and form extended, elaborate dendritic morphologies, while the cells on the thin film control adhered to their substrates in a flat 2D geometry. The protein micro-patterning was established through electrospining PMMA-chloroform fibers in micro-squares on the substrate and coating the electrospun membrane with fibronectin, which substantially enhanced cell attachment to the matrix. Fibronectin led to the greatest number of cells attaching to the matrix and formed the most elongated actin when compared to uncoated PMMA fibers and other proteins, such as collagen, laminin, and gelatin. The confocal images showed that cells were forced to follow the micro-printed squares on the surface and accordingly formulate their shapes. The cells formed elongated actin organization, which is suggestive of greater cell-cell and cell-matrix communication, within the micro-patterned squares on the substrate. Furthermore, fibers coated with fibronectin showed in-growth and migration of cells inside the 3D scaffold while the fibers not coated with fibronectin showed virtually no in-growth or cellular migration to the multiple layers of the scaffold. Consequently, cell behavior can be dictated through micro-patterning of the underlying substrates.

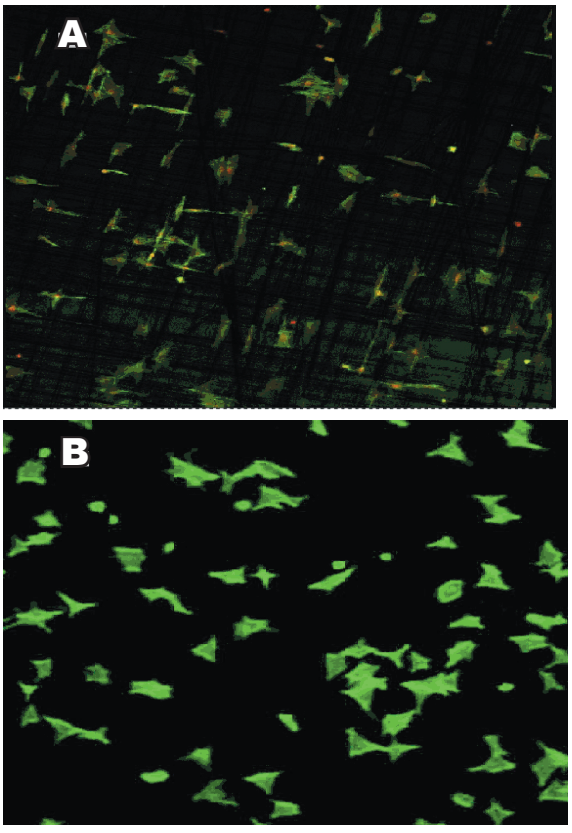

Figure 5. A. shows the cells, on the fibrous structure, following the orientation of the fibers both horizontally and vertically. B. shows disorganized cells, with folded, triangular morphologies, on the thin films.

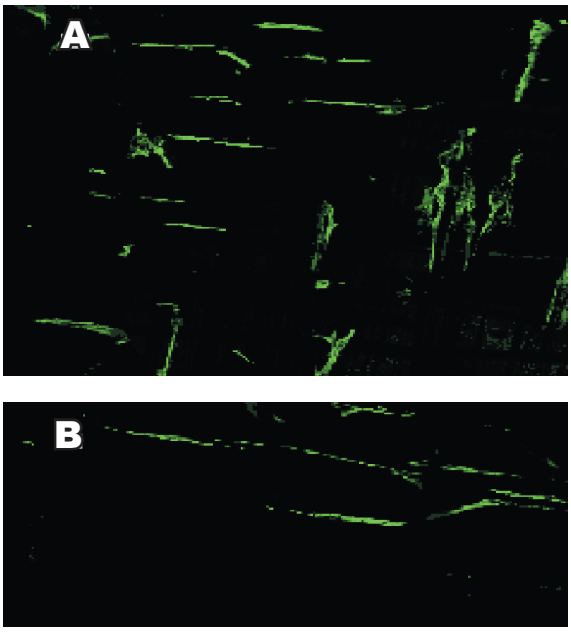

Figure 6. A. shows the migration, extensive spreading, and growth of cells inside the scaffold after a 48-hour incubation period on the fibrous scaffold with fibronectin coat. (20x Magnification) B. shows the fibers not coated with fibronectin and a lack of migration of cells into the fibrous microstructure.

4. Conclusions

Tissue engineering, cancer treatment, and stem cell differentiation necessitate the ability to biomimic and manipulate the extracellular matrix. This study professed that altering the mechanics of the underlying matrix can dictate cell behavior and function. This was determined through micro-orienting the substrate and micro-patterning the proteins to provide both a structural and biological backbone to influence cell mechanics.

References

[1] Altman G. et al., Cell Differentiation by Mechanical Stress FASEB Journal February 2002 Vol. 16: 270-272

[2] Beningo K.A., Dembo M., and Wang Y., Responses of Fibroblasts to Anchorage of Dorsal Extracellular Matrix Receptors Cell Biology Vol. 101(52): 18024-18029

[3] Brivanlou A.H. et al., Setting Standards for Human Embryonic Stem Cells, Science 9 May 2003 Vol. 300(5621): 913 – 916

[4] Chuenjitkuntaworn B., Damrongsri D., Pavasant P., and Supaphol P., 1895 Electrospun Polylactic Acid Fiber for Bone Regeneration IADR Brisbane Convention 30 June 2006

[5] Cohn V., "Role of Extracellular Matrix Factors in Stem Cell Differentiation" Stem Cells and Development Vol. 914: 740

[6] Cukierman E., Pankov R., and Yamada K. M., Cell Interactions with Three-Dimensional Matrices Current Opinion in Cell Biology 2002 Vol. 14: 633–6397] Fridrikh S.V., Yu J.H., Brenner M.P., and Rutledge G.C., Controlling the Fiber Diameter During Electrospinning The American Physical Society: Physical Review Letters 2003 Vol. 90

[8] Gronthos S. et al., Stem Cell Properties of Human Dental Pulp Stem Cells Journal of Dental Research 2002 Vol. 81(8): 531-535