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(54) Title: SYSTEM AND METHODS FOR PREFERENTIALLY ENHANCING ACTIVATION OF RAC GTPASE IN A CELL OR TISSUE

(57) Abstract: A system and methods for preferentially enhancing activation of Rac GTPase in a cell or tissue is disclosed. Rac activation in the cell or tissue is sustained and does not default to a Rho or Rho kinase regulated pathway. The system and methods of the invention can be used in a variety of *in vitro*, *in vivo*, and *ex vivo* applications including cell culture, tissue culture, tissue engineering, and organ culture.

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SYSTEM AND METHODS FOR PREFERENTIALLY ENHANCING ACTIVATION OF RAC GTPase IN A CELL OR TISSUE

This application is being filed on 01 March 2006, as a PCT International Patent application in the name of Donaldson Company, Inc., a U.S. national corporation, applicant for the designation of all countries except the US, and Melvin Steven Schindler, Sally Ann Meiners, Mohammed Shah Alam Nur-E-Kamal, Hoo Young Chung, Thomas M. Weik, and Timothy Grafe, all citizens of the U.S., applicants for the designation of the US only, and claims priority to U.S. Provisional Patent Application Serial Nos. 60/658,421, filed March 2, 2006, 60/719,850, filed September 22, 2005, and 60/735,312, filed November 11, 2005.

Background of the Invention

Cellular interactions between cell surface receptors and specific macromolecules within the extracellular matrix (ECM) promote the activation of signaling pathways that regulate all aspects of cell development, organization, integration, and function in tissues (Kalluri et al., 2003, Nat. Rev. Cancer, 3:422-433; Wozniak et al., 2004, Biochem. Biophys. Acta, 1692: 103-119; Geiger et al., Nature Rev. Mol. Cell Biol., 2:793-805). For most cells, this regulation occurs through interactions with a specialized form of ECM called the basement membrane (Kalluri et al., 2003, Nat. Rev. Cancer, 3:422-433; Boudreau, 2003, Sci. STKE, 196:pe34). The basement membrane is predominantly composed of laminin-1, type IV collagen and to a lesser extent fibronectin, glycosaminoglycans, proteoglycans and growth factors (Kalluri et al., 2003, Nat. Rev. Cancer, 3:422-433; Wozniak et al., 2004, Biochem. Biophys. Acta, 1692: 103-119; Geiger et al., Nature Rev. Mol. Cell Biol., 2:793-805). Structurally, these macromolecules assemble into a complex array of interacting nanofibrillar networks that form a mechanically flexible, nano-contoured and porous surface for cell attachment and migration (Abrams et al., 2000, Cell Tissue Res., 299:39-46).

Most investigations to define the mechanisms of ECM/basement membrane (BM) mediated activation of cell signaling pathways have focused on the role of specific macromolecules of the ECM/BM in promoting cellular signaling (Kalluri et al., 2003, Nat. Rev. Cancer, 3:422-433; Wozniak et al., 2004, Biochem. Biophys. Acta, 1692: 103-119). In most of these experiments, ECM/BM components are applied to the surface of tissue culture plates and are therefore presented to the cell surface in the form of a two dimensional (2D) film. However, in a number of approaches utilizing three dimensional (3D) matrices derived from detergent-extracted mouse embryo sections (Cukierman et al., 2001, Science, 294:1708-1712) or from 3D preparations of ECM/BM (Schmeichel and Bissell, 2003, J. Cell Sci., 116:2377-2388), cell measurements of morphology, proliferation, and cytoskeletal organization were observed to be markedly different when compared with similar culture on 2D surfaces (Wozniak et al., 2004, Biochem. Biophys. Acta, 1692: 103-119; Cukierman et al., 2001, Science, 294:1708-1712; Schmeichel and Bissell, 2003, J. Cell Sci., 116:2377-2388). These results provided strong support for the emerging view that cells require flexible 3D environments to achieve more physiologically relevant phenotypes, in particular, regulating the assembly state and dynamics of the cytoskeleton and its associated effector molecules (Wozniak et al., 2004, Biochem. Biophys. Acta, 1692: 103-119; Abott, 2003, Nature, 424:870-872).

The cytoskeleton has been shown to integrate and propagate physical and chemical signals from the extracellular matrix (ECM) to the cell, leading to changes in cell morphology, cell-cell interactions, and function (Wozniak et al., 2004, Biochem. Biophys. Acta, 1692: 103-119; Geiger et al., Nature Rev. Mol. Cell Biol., 2:793-805; Boudreau, 2003, Sci. STKE, 196:pe34). This regulation is achieved through the selective activation of members of the Rho family of small GTPases (Burridge and Wennerberg, 2004, Cell, 116:167-179; Nobes and Hall, 1995, Cell, 81:53-62). The Rho GTPases Rho, Rac, and Cdc42 in conjunction with their downstream effectors regulate cell adhesion, cell migration, cell polarity, endocytosis, vesicle trafficking, cell cycle progression, differentiation, oncogenesis, and gene transcription (Etienne-Manneville and Hall, 2002, Nature, 420:629-635). Two-dimensional solid planar culture surfaces predominantly activate Rho, which through its effector Rho kinase (ROCK) induces the assembly of focal adhesions and stress fibers (Wozniak et al., 2004, Biochem. Biophys. Acta, 1692: 103-119; Geiger et al., Nature Rev. Mol. Cell Biol., 2:793-805; Burridge and Wennerberg, 2004, Cell, 116:167-179). However, efforts to induce morphogenesis and differentiation in cell culture using hydrogels and specific substrate adhesion molecules have indicated that these global changes in cell physiology and cell-cell interactions are initiated by the activation of Rac, with a concomitant decrease in the activation state of either Rho and/or ROCK (Sander et al., 1999, J. Cell Biol., 147:1009-1021; Connolly et al., 2002, Mol. Biol. Cell, 13:2474-2485; Tsuji et al., 2002, J. Cell Biol., 157:819-830). Rac activation induces lamellipodia, membrane ruffles, and filipodia microspikes and is known to contribute to activation of angogenesis and cell proliferation (Bishop and Hall, 2000, Biochem. J., 348:241-255; Hassanain et al., 2005, Surgery, 137:92-101).

There is a significant need for a system and methods for growing cells or tissue that promotes *in vivo*-like cell structure and function and provides the ease of use associated with standard two dimensional tissue and cell culture plate techniques. Given the pivotal role of Rac signaling in initiating cellular transformations, a system comprising a simple or composite growth surface that provides the ease of use, uniformity, quality control, and flexibility associated with the standard tissue culture plate, does not default to a Rho/ROCK regulated pathway, and is permissive for sustained Rac activation would be highly desirable and useful.

Summary of the Invention

The invention provides a system and methods for preferentially enhancing activation of Rac GTPase in a cell or tissue. The system and methods of the invention provide Rac activated cells having *in vivo*-like morphology and fibrillar organization that are capable of proliferating and self-renewing. Rac activated cells or tissue of the invention are useful in a variety of *in vitro*, *in vivo*, and *ex vivo* applications including cell culture, tissue culture, tissue engineering, tissue regeneration, organ culture, and organ regeneration.

The system of the invention comprises one or more cells and a growth surface. The growth surface comprises nanotopography that mimics the nanotopography of extracellular matrix (ECM) or basement membrane (BM). The nanotopography of the growth surface can be defined by surface roughness parameters including, but not limited to, average surface roughness (Ra), maximum peak height (Rp), maximum valley depth (Rv), total roughness (Rt), average peak spacing (S), and peak count (Pc). Examples of growth surfaces include, but are not limited to, a network of one or more nanofibers, a nanofibrillar structure, glass, silicon, or plastic comprising an etched or micropatterned surface, glass, silicon, or plastic surface comprising macropores or nanopores, or a polymer scaffold. In an embodiment, the growth surface comprises Ra of about 13 nm to about 1200 nm, S of about 50 nm to about 800 nm, Pc of about 1 to about 30, and elongation of about 25 percent to about 500 percent.

The amount of activated Rac in the cells or tissue on the growth surface is at least about one fold greater, more preferably at least about two fold greater, more preferably at least about four fold greater, more preferably at least about eight fold greater, more preferably at least about ten fold greater than the amount of activated Rac in a cell or tissue on a two dimensional culture surface. Preferably, Rac activation in the cell or tissue is sustained and does not default to a Rho or Rho kinase regulated pathway. In an embodiment, Rac activation is sustained for at least about 6 hr. In an embodiment, Rac GTPase activation in a cell or tissue on the growth surface is at least about 2 fold greater, more preferably at least about 4 fold greater, more preferably at least about 8 fold greater, more preferably at least about 10 fold greater than Rho GTPase activation or Cdc42 GTPase activation in the cell or tissue when compared to culture on a two dimensional surface.

Additional physical and chemical properties of the growth surface can be engineered to create a nano- or microenvironment that promotes or induces Rac activation or *in vivo*-like differentiation and morphogenesis. Physical properties of the growth substrate such as adhesivity, porosity, solidity, elasticity, geometry, interconnectivity, surface to volume ratio, solubility/insolubility, hydrophilicity/hydrophobicity, and density can be engineered to desired parameters.

Specific chemical properties and recognition motifs that promote one or more selected cellular activities, such as polypeptides, lipids, carbohydrates, amino acids, nucleotides, nucleic acids, polynucleotides, or polysaccharides including, but not limited, bioactive molecules such as growth factors and differentiation factors, fibrous proteins, adhesive proteins, glycoproteins, functional groups, adhesive compounds, deadhesive compounds, and targeting molecules can be engineered into the growth surface. Amino acids, peptides, polypeptides, and proteins can include any type of such molecules of any size and complexity as well as combinations of such molecules including, but not limited to, structural proteins, enzymes, and peptide hormones. In an embodiment, one or more of the bioactive molecules is a growth factor, differentiation factor, adhesive protein, or bioactive peptide derived from an adhesive protein. Examples of suitable growth factors include VEGF, bone morphogenic factor β, EGF, PDGF, NGF, FGF, IGF, or TGF. Examples of suitable differentiation factors include neurotrophin, CSF, or TGF. Additionally, nano- and micro-environments that promote cellular activity of a particular cell or tissue, such as sustained activation of Rac, can be constructed by layering growth surfaces that have selected physical and/or chemical properties.

The growth surface can be a network of one or more nanofibers. The nanofiber network can be deposited on a surface of a substrate. In an embodiment, the nanofiber network comprises a fiber diameter of about 30 nm to about 1200 nm, average interfiber spacing of about 100 nm to about 600 nm, and solidity of about 70 percent or less. The nanofibers can be fabricated from a variety of polymers or polymer systems. Preferably the polymer or polymer system is non-cytotoxic. In an embodiment, the nanofibers are fabricated from a polyamide or polyester. The polyamide can be nylon 6, nylon 66, nylon 610 or other biocompatible polyamides. The polyester can be poly(ε-caprolactone), poly(lactate) or poly(glycolate). In an embodiment, the polyamide or polyester is suitable for *in vivo* human application.

The growth surface can be a nanofibrillar structure comprising one or more nanofibers. The network of one or more nanofibers defines the nanofibrillar structure. In an embodiment, the nanofiber network is deposited on a surface of a substrate. The substrate can be glass or plastic. The substrate can be a surface of a culture container, coverslip, or film. The film can be water soluble or water insoluble, biodegradable or biodissolvable. Preferably the film is non-cytotoxic. In an embodiment, the film comprises polyvinyl alcohol, polychlorotrifluoroethylene, polystyrene, polymethylpentene, or polycylo-olefin. The nanofibrillar structure can be utilized singly or layered to form a multi-layered assembly of nanofibrillar structures for cell or tissue culture. In an embodiment, the nanofibrillar structure comprises a spacer. The spacer can function as a support structure. The spacer provides sufficient openings to permit cells to penetrate and attach to the nanofiber network. The spacer can be water soluble or water insoluble, porous or non-porous, biodegradable or biodissolvable. Preferably the spacer is biocompatible.

Another aspect of the invention includes methods for preferentially enhancing activation of Rac GTPase in a cell or tissue. The methods of the invention comprise growing the cell or tissue on a growth surface comprising biomimetic nanotopography. The nanotopography of the growth surface preferentially enhances activation of Rac in a cell or tissue. Examples of growth surfaces include, but are not limited to, nanofibrillar growth surface, nanofibrillar structure, glass, silicon, or plastic comprising an etched or micropatterned surface, glass, silicon, or plastic surface comprising macropores or nanopores, polymer scaffold, hydrogel, or peptide scaffold. Cells or tissue can be grown on the growth surface *in vivo*, *in vitro*, or *ex vivo* using known methods.

In an embodiment, the methods of the invention provide a cell or tissue comprising an amount of activated Rac at least one fold greater, more preferably at least two fold greater, more preferably at least four fold greater, more preferably at least eight fold greater than the amount of activated Rac in a cell or tissue on a control surface. The control surface can be a standard glass or plastic solid planar culture surface. The Rac GTPase can be Rac 1, Rac2, Rac3, or a combination thereof.

Cells useful in the system and methods of the invention include stem cells, somatic cells, committed stem cells, differentiated cells, and tumor cells. The cells can be from a mammal. The mammal can be human. The cells can be a tissue. Examples of tissue include skin, bone, liver, heart, kidney, bladder, muscle, ligament, tendon, cartilage, brain, retina, cornea, and pancreas. Examples of cells useful in the system and methods of the invention include, but are not limited to, osteoblasts, myoblasts, neuroblasts, fibroblasts, glioblasts, germ cells, stem cells, hepatocytes, chondrocytes, keratinocytes, smooth muscle cells, cardiac muscle cells, connective tissue cells, glial cells, epithelial cells, endothelial cells, hormone-secreting cells, neurons, and lymphoid cells such as B cells, T cells, macrophages, and neutrophils. Examples of stem cells include, but are not limited to, embryonic stem cells, mesenchymal stem cells, bone marrow stem cells, and umbilical cord stem cells. The stem cells can be mammalian stem cells.

The system and methods of the inventions have many useful applications including cell culture, tissue culture, tissue engineering, analysis of therapeutic compounds, drug sensitivity analysis, and wound treatment. The growth surface of the system and methods of the invention provides an environment for the cells or tissue to more closely mimic the *in vivo* nature of the cells or tissue in an *ex vivo* environment. The system and methods of the invention can be used to induce *in vivo*-like cell differentiation and morphogenesis and enhance proliferation. The system and methods of the invention can be used in analysis of therapeutic compounds and drug sensitivity analysis. The system and methods of the invention can be used to grow and maintain stem cells in an undifferentiated state on a synthetic growth substrate. In an embodiment, the growth surface comprises a nanofibrillar growth surface. In an embodiment, embryonic stem cells on the nanofibrillar growth substrate proliferate, self renew, and maintain their undifferentiated state. In an embodiment, the stem cells proliferate and maintain pluripotency. Preferably, the stem cells maintain the ability to differentiate after proliferation. In an embodiment, the stem cells proliferate but do not differentiate until a differentiation factor is administered to the cells.

The system and methods of the invention are useful for treating wounds including cuts, lacerations, incisions, abrasions, and penetrations of a tissue, burns, and dermal ulcers, wounds requiring neovascularization or re-endothelialization, and regenerating tissue at the site of a wound. The wound can be cutaneous, subcutaneous, or internal. In an embodiment, the tissue is vascular tissue. The vascular tissue can be skin. A dressing comprising a growth surface of the invention can be applied directly to the site of the wound. The growth surface of the dressing or bandage preferentially enhances Rac activation in cells or tissue at the site of the wound. In an embodiment, the growth surface of the dressing or bandage comprises one or more of the following: Ra of about 13 nm to about 1200 nm; Rp of about 13 nm to about 1200 nm; Rv of about 13 nm to about 1200 nm; Rt of about 26 nm to about 2400 nm; S of about 50 nm to about 800 nm; Pc of about 1 to about 30; and elongation of about 25 percent to about 500 percent. The dressing can be a bandage. In an embodiment, the bandage comprises a backing, a pad comprising a growth surface of the invention, and an adhesive.

Brief Description of the Drawings

Figure 1 shows an SEM micrograph of a glass coverslip coated with a nanofibrillar growth surface. Scale bar, 2 μm.

Figures 2A-2C show AFM analysis of the nanofibrillar growth surface. Figure 2A shows fiber diameter. Figure 2B shows smoothness of a single fiber in the nanofibrillar growth surface. Figure 2C shows the diameter of a pore within the network of nanofibers forming the nanofibrillar growth surface.

Figure 3 shows a histogram of nanofiber diameters within the network of nanofibers forming the nanofibrillar growth surface.

Figure 4 shows the stress-strain curve for polyamide nanofiber material.

Figures 5A-5H show indirect immunofluorescence micrographs of NIH 3T3 fibroblasts on glass (Figs. 5A, 5C, 5E, 5G) and the nanofibrillar growth surface (Figs. 5B, 5D, 5F, 5H) stained with phalloidin-Alexa Fluor 488 (Figs. 5A and 5B), vinculin antibodies (Figs. 5C and 5D), FAK Y397 antibodies (Figs. 5E and 5F), and fibronectin antibodies (Figs. 5G and 5H). The extent of nuclear labeling was variable. Scale bar, 10 μm.

Figures 6A-6F show indirect immunofluorescent micrographs of NIH 3T3 fibroblasts stained with fibronectin antibody following 2 and 3 days of culture on glass (Figs. 6A and 6C) and the nanofibrillar growth surface (Figs. 6B and 6D), respectively. Arrows in Figures 6A and 6C show a parallel array of fibrillar adhesions composed of fibronectin on the basal surface of the cell. Double arrow in Figure 6B shows thicker fibrils composed of fibronectin on the apical surface. Figure 6E shows an indirect immunofluorescent micrograph of NIH 3T3 fibroblasts stained with anti-vinculin after two days of culture. In contrast, fibroblasts grown on the nanofibrillar growth surface demonstrate labeling that occurs predominantly in filopodia and lamellipodia (Fig. 6F).

Figures 7A-7J show indirect immunofluorescence micrographs of NRK cells on glass (Figs. 7A, 7C, 7E, 7G, 7I) and the nanofibrillar growth surface (Figs. 7B, 7D, 7F, 7H, 7J) stained with phalloidin-Alexa Fluor 488 (Figs. 7A and 7B), vinculin antibodies (Figs. 7C and 7D), FAK Y397 antibodies (Figs. 7E and 7F), fibronectin antibodies (Figs. 7G and 7H), and β1 integrin antibodies (Figs. 7I and 7J). The extent of nuclear labeling was variable. Scale bar, 10 μm.

Figures 8A and 8B show the results of a proliferation assay for NIH 3T3 fibroblasts (Fig. 8A) and NRK cells (Fig. 8B) cultured on glass (gray bars) and the nanofibrillar growth surface (black bars). Each bar is an average of 6 experiments.

Figures 9A-9F show a series of confocal micrographs of a multicellular spheroid composed of T47D breast epithelial cells grown on the nanofibrillar growth surface and stained with phalloidin-Alexa Fluor 488. Note the lumen extending through the spheroid. Sections were taken at 0 (Fig. 9A), 20 (Fig. 9B), 34 (Fig. 9C), and 48 (Fig. 9D) micron steps from the top. A fluorescent image a tubule is shown in Figure 9E. Figure 9F shows T47D cells after 10 days of culture on glass. Scale bar, 10 μm.

Figures 9G and 9H show confocal micrographs of MCF-7 cells grown on glass (Fig. 9G) and the nanofibrillar growth surface (Fig. 9H) stained with phalloidin-Alexa Fluor 488. Scale bar, 10 μm.

Figure 10 shows a western blot analysis of activated Rac, Rho, and Cdc42 in NIH 3T3 fibroblasts or NRK cells cultured on glass or the nanofibrillar growth surface for 1 hr or 6 hr. Results for cells grown on glass are shown in the column labeled "G". Results for cells cultured on the nanofibrillar growth surface are shown in the column labeled "N".

Figures 11A and 11B shows a densitometric analysis of the western blots shown in Figure 10 for activated Rac, Rho, and Cdc42 in NIH 3T3 fibroblasts (Fig. 11A) or NRK cells (Fig. 11B). The results are shown as a ratio of expression of activated Rac, Rho, or Cdc42 for cells grown on the nanofibrillar surface versus cells grown on glass.

Figure 12 shows a western blot analysis of activated Rac, Rho, and Cdc42 in MES cells cultured on glass or the nanofibrillar growth surface. Results for MES cells cultured on glass are shown in the column labeled "2D". Results for MES cells cultured on the nanofibrillar growth surface are shown in the column labeled "3D".

Figure 13 shows a densitometric analysis of the western blot shown in Figure 12 for activated Rac, Rho, and Cdc42 in MES cells on glass (black bars) or the nanofibrillar growth surface (gray bars).

Figure 14 shows MES cell colonies cultured on glass coverslips stained with alkaline phosphatase.

Figure 15 shows MES cell colonies cultured on glass coverslips coated with a nanofibrillar growth surface stained with alkaline phosphatase.

Figure 16 shows proliferation of MES cells cultured on glass (gray bars) or the nanofibrillar growth surface (black bars).

Figures 17A and 17B show SEM micrographs of the organization of F-actin within fibroblasts cultured on a nanofibrillar growth surface. Figure 17A shows NIH 3T3 fibroblasts cultured on glass coverslips and stained for F-actin. Arrowhead points to stress fibers. Figure 17B shows NIH 3T3 fibroblasts cultured on nanofiber coated coverslips and stained for F-actin. Double arrowhead points to F-actin containing ruffles of the dorsal membrane and a triple arrowhead points to ruffles containing F-actin at the edge of lamellipodia. Scale bar, 10 μm.

Figure 18 shows the number of colonies for MES cells cultured on glass or the nanofibrillar growth surface. Control cells were transfected with a control plasmid vector, dnRac cells were transfected with a vector encoding a dominant negative Rac mutant, and caRac cells were transfected with a vector encoding a constitutively active Rac mutant.

Figure 19A shows phosphorylation of AKT in MES cells cultured on glass or the nanofibrillar growth surface.

Figure 19B shows Nanog expression in MES cells cultured on glass or the nanofibrillar growth surface.

Figures 19C-19E show inhibition of Nanog expression in MES cells cultured on the nanofibrillar growth surface by Wortmannin (Fig. 19C), caffeine (Fig. 19D), or retinoic acid (Fig. 19E).

Figure 19F shows inhibition of Nanog transcription in MES cells cultured on the nanofibrillar growth surface by DMSO (control), retinoic acid, caffeine, or Wartmannin.

Figure 19G shows a Western blot analysis of the expression of c-Fos in feeder cells only (MEFs) or MES cells cultured on glass or the nanofibrillar growth surface.

Figure 20 shows induction of GFAP and nestin expression by retinoic acid in MES cells cultured on the nanofibrillar growth surface.

Figure 21 shows expression of nestin in MES cells cultured on a nanofibrillar growth surface coated with laminin-1.

Detailed Description of the Invention

I. Definitions

A "growth surface" is a synthetic surface that supports growth of cells or tissue. The growth surface comprises three-dimensional structure and geometry that preferentially enhances activation of Rac GTPase in a cell or tissue on the growth surface.

"Preferentially enhances Rac GTPase activation" or "preferentially enhances activation of Rac GTPase" as used herein means the ratio of activated Rac GTPase to activated Rho GTPase in a cell or tissue on the growth surface is greater than the ratio of activated Rac GTPase to activated Rho GTPase in a cell or tissue on a control surface. The control surface can be a standard glass or plastic solid planar culture surface. The control surface has average surface roughness of less than about 10 nm. In an embodiment, the control surface has average surface roughness of about 0.01 nm to about 10 nm. In an embodiment, the control surface has average surface roughness of about 5 nm to about 10 nm.

The amount of activated Rac in the cells or tissue on the growth surface is at least about one fold greater, more preferably at least about two fold greater, more preferably at least about four fold greater, more preferably at least about eight fold greater, more preferably at least about ten fold greater than the amount of activated Rac in a cell or tissue on a control surface. Preferably, Rac activation in the cell or tissue is sustained. In an embodiment, Rac activation is sustained for at least about 6 hr. In an embodiment, Rac activation is sustained such that one or more of the pathways initiated by Rac GTPase is activated. In an embodiment, a pathway that results in cell differentiation or cell morphogenesis is initiated. In an embodiment, Rac activation induces lamellipodia and membrane ruffles. In an embodiment, Rac activation induces filopodia microspikes. In an embodiment, Rac activation induces angiogenesis.

The amount of activated Rho or Cdc42 in a cell or tissue on the growth surface can be similar to or less than the amount of activated Rho or Cdc42 in the cell or tissue on the control surface. In an embodiment, activation of Rac in a cell or tissue on a growth surface is associated with a concomitant decrease in activation of Rho or Rho kinase (ROCK) in the cell or tissue. In an embodiment, Rac GTPase activation in a cell or tissue on the growth surface is at least about 2 fold greater, more preferably at least about 4 fold greater, more preferably at least about 8 fold greater, more preferably at least about 10 fold greater than Rho GTPase activation or Cdc42 GTPase activation in a cell or tissue on the control surface.

The terms "Rac GTPase" and "Rac" are used interchangeably to refer to Rac, a member of the Rho family of small GTPases (Burridge and Wennerberg 2004, Cell, 116:167-179; Nobes and Hall, 1995, Cell, 81:53-62). The terms encompass Rac1, Rac2, Rac3, and combinations thereof. "GTP-Rac" or "GTP*Rac" refers to Rac that is activated.

The terms "Rho GTPase" and "Rho" are used interchangeably to refer to Rho, a member of the Rho family of small GTPases (Burridge and Wennerberg 2004, Cell, 116:167-179; Nobes and Hall, 1995, Cell, 81:53-62). "GTP-Rho" or "GTP*Rho" refers to Rho that is activated.

The terms "Cdc42 GTPase" and "Cdc42" are used interchangeably to refer to Cdc42, a member of the Rho family of small GTPases (Burridge and Wennerberg 2004, Cell, 116:167-179; Nobes and Hall, 1995, Cell, 81:53-62). "GTP-Cdc42" or "GTP*Cdc42" refers to CDC42 that is activated.

The term "nanofibrillar growth surface" as used herein means a growth surface defined by a network of one or more nanofibers. The nanofibrillar growth surface can be deposited on a surface of a substrate such as, for example, silicon, glass, or plastic. The substrate can be a culture container. The nanotopography of the nanofibrillar growth surface (*e.g.* topography of the nanofiber network and the arrangement of the nanofibers in space) can be engineered to provide an *in vitro* biomimetic substratum that promotes homotypic or heterotypic cell growth and/or cell differentiation. The nanofibrillar growth surface can be a surface of a nanofibrillar structure.

The term "nanofiber" as used herein means a polymer fine fiber comprising a diameter of about 1200 nanometers or less. The polymer is preferably a non-cytotoxic polymer. The polymer can be water soluble or water insoluble. The polymer can be biodegradable and/or biodissolvable. In an embodiment, a nanofiber comprises an average surface roughness of about 0.01 nm to about 5 nm over an evaluation length of about 1.5 μm.

The term "network" as used herein means a random or oriented distribution of nanofibers in space that is controlled to form an interconnecting net with spacing between fibers selected to preferentially enhance activation of Rac GTPase in a cell or tissue. The network has small spaces between the fibers that form pores or channels in the network. The pores or channels can have a diameter of about 20 nm to about 2000 nm, through a thickness. A network can comprise a single layer of nanofibers, a single layer formed by a continuous nanofiber, multiple layers of nanofibers, multiple layers formed by a continuous nanofiber, or mat. The network can be unwoven or net. A network can have a thickness of about the diameter of a single nanofiber to about 250 μm. Physical properties of the network including, but not limited to, texture such as surface roughness, peak height, peak depth, total roughness, spacing between peaks, or peak count, elongation, rugosity, adhesivity, porosity, solidity, elasticity, geometry, interconnectivity, surface to volume ratio, fiber diameter, fiber solubility/insolubility, hydrophilicity/hydrophobicity, fibril density, and fiber orientation can be engineered to desired parameters using known methods.

In some embodiments, the nanofiber comprises one or more bioactive molecules. The bioactive molecule can be a peptide, polypeptide, lipid, carbohydrate, polysaccharide, amino acid, nucleotide, nucleic acid, polynucleotide, or hybrid molecule thereof. The bioactive molecule can be a growth factor or differentiation factor. The nanofiber can release one or more growth factors or differentiation factors. The rate of release is determined by the rate of degradation and/or dissolution of the nanofiber. In some embodiments, the nanofiber comprises one or more alcohol, aldehyde, amino, carboxy, sulphydryl or photoactivatable functional groups. Preferably the photoactivatable group is a carbene or nitrene.

The term "nanofibrillar structure" as used herein means a structure comprising an environment for growth of living cells comprising one or more nanofibers, wherein the structure is defined by a network of one or more nanofibers. In some embodiments, the nanofibrillar structure comprises a substrate wherein the nanofibrillar structure is defined by a network of one or more nanofibers deposited on a surface of the substrate. The nanotopography (*e.g.* the topography of the nanofiber network and the arrangement of the nanofibers of the nanofiber network in space) of the nanofibrillar structure can be engineered to provide an *in vitro* biomimetic substratum that promotes homotypic or heterotypic cell growth and/or cell differentiation. The nanofibrillar structures can be layered to form a multi-layered nanofibrillar assembly, cellular array, or tissue structure.

The term "substrate" as used herein means any surface on which nanofiber or network of nanofibers is deposited. The substrate can be any surface that offers structural support for the deposited network of nanofibers. The substrate can comprise glass or plastic. Preferably the plastic is non-cytotoxic. The substrate can be a film or culture container.

The substrate can be water soluble or water insoluble. A substrate that is water soluble is preferably a polyvinyl alcohol film. The substrate can be porous or non-porous. Porosity of the substrate is determined by cellular penetration. A cell is able to penetrate a porous substrate but is not able to penetrate a non-porous substrate. Preferably the pores in a porous substrate have a diameter of about 2 μm to about 10 μm. The substrate can be biodegradable and/or biodissolvable. Preferably the substrate is biocompatible.

The substrate can comprise one or more bioactive molecules. Preferably one of the bioactive molecules is a peptide, polypeptide, lipid, carbohydrate, polysaccharide, amino acid, nucleotide, nucleic acid, polynucleotide, or hybrid molecule thereof. The substrate can comprise one or more alcohol, aldehyde, amino, carboxy, sulphydryl or photoactivatable functional groups. Preferably the photoactivatable group is a carbene or nitrene. The substrate can comprise one or more growth factors and/or differentiation factors. The substrate can release one or more growth factors and/or differentiation factors. The rate of release is determined by the rate of dissolution or degradation of the substrate.

The term "spacer" as used herein means a layer separating growth surfaces that are stacked or layered upon one another such that opposing growth surfaces are separated by the thickness of the layer. In an embodiment, the spacer separates a nanofiber or nanofiber network from a surface of a substrate or a surface of a first nanofibrillar structure from a surface of a second nanofibrillar structure such that the structures are separated by the diameter or thickness of the spacer. The spacer can comprise a polymer fine fiber or film. Preferably the film has a thickness of about 10 microns to about 50 microns. The spacer can comprise a polymer including cellulose, starch, polyamide, polyester, or polytetrafluoroethylene. The fine fiber can comprise a microfiber. A microfiber is a polymer fine fiber comprising a diameter of about 1.0 μm to about 10 μm. The microfiber can be unwoven or net.

The spacer can be water soluble or water insoluble. The spacer can be porous or non-porous. Porosity of the spacer is determined by cellular penetration. A cell is able to penetrate a porous spacer but is not able to penetrate a non-porous spacer. The spacer can be biodegradable and/or biodissolvable. Preferably the spacer is biocompatible.

The spacer can comprise one or more bioactive molecules. Preferably one of the bioactive molecules is a peptide, polypeptide, lipid, carbohydrate, nucleotide, nucleic acid, polynucleotide, polysaccharide, amino acid, or hybrid molecule thereof. The spacer can comprise one or more alcohol, aldehyde, amino, carboxy, sulphydryl or photoactivatable functional groups. Preferably the photoactivatable group is a carbene or nitrene. The spacer can comprise one or more growth factors and/or differentiation factors. The spacer can release one or more growth factors and/or differentiation factors. The rate of release is determined by the rate of dissolution or degradation of the spacer.

The term "bioactive molecule" as used herein means a molecule that has an effect on a cell or tissue. The term includes human or veterinary therapeutics, nutraceuticals, vitamins, salts, electrolytes, amino acids, peptides, polypeptides, proteins, carbohydrates, lipids, polysaccharides, nucleic acids, nucleotides, polynucleotides, glycoproteins, lipoproteins, glycolipids, glycosaminoglycans, proteoglycans, growth factors, differentiation factors, hormones, neurotransmitters, pheromones, chalones, prostaglandins, immunoglobulins, monokines and other cytokines, humectants, minerals, electrically and magnetically reactive materials, light sensitive materials, anti-oxidants, molecules that can be metabolized as a source of cellular energy, antigens, and any molecules that can cause a cellular or physiological response. Any combination of molecules can be used, as well as agonists or antagonists of these molecules. Glycoaminoglycans include glycoproteins, proteoglycans, and hyaluronan. Polysaccharides include cellulose, starch, alginic acid, chytosan, or hyaluronan. Cytokines include, but are not limited to, cardiotrophin, stromal cell derived factor, macrophage derived chemokine (MDC), melanoma growth stimulatory activity (MGSA), macrophage inflammatory proteins 1 alpha (MIP-1 alpha), 2, 3 alpha, 3 beta, 4 and 5, interleukin (IL) 1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, TNF-alpha, and TNF-beta. Immunoglobulins useful in the present invention include, but are not limited to, IgG, IgA, IgM, IgD, IgE, and mixtures thereof. Amino acids, peptides, polypeptides, and proteins can include any type of such molecules of any size and complexity as well as combinations of such molecules. Examples include, but are not limited to, structural proteins, enzymes, and peptide hormones.

The term bioactive molecule also includes fibrous proteins, adhesion proteins, adhesive compounds, deadhesive compounds, targeting compounds, growth inhibitors, and differentiation inhibitors. Fibrous proteins include collagen and elastin. Adhesion/deadhesion compounds include fibronectin, laminin, thrombospondin and tenascin C. Adhesive proteins include actin, fibrin, fibrinogen, fibronectin, vitronectin, laminin, cadherins, selectins, intracellular adhesion molecules 1, 2, and 3, and cell-matrix adhesion receptors including but not limited to integrins such as $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_1\beta_2$, $\alpha_2\beta_3$, and $\alpha_6\beta_4$. Growth inhibitors include bone marrow stromal cell derived growth inhibitor, p21(WAF1/Cip1) cell cycle inhibitor, and taxol. Differentiation inhibitors include thrombospondin and Nogo-A.

The term bioactive molecule also includes leptin, leukemia inhibitory factor (LIF), RGD peptide, tumor necrosis factor alpha and beta, endostatin, angiostatin, thrombospondin, osteogenic protein-1, bone morphogenic proteins 2 and 7, osteonectin, somatomedin-like peptide, osteocalcin, interferon alpha, interferon alpha A, interferon beta, interferon gamma, interferon 1 alpha, and interleukins 2, 3, 4, 5 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17 and 18.

The term "growth factor" as used herein means a bioactive molecule that promotes the proliferation of a cell or tissue. Growth factors useful in the present invention include, but are not limited to, transforming growth factor-alpha. (TGF-alpha), transforming growth factor-beta. (TGF-beta), platelet-derived growth factors including the AA, AB and BB isoforms (PDGF), fibroblast growth factors (FGF), including FGF acidic isoforms 1 and 2, FGF basic form 2, and FGF 4, 8, 9 and 10, nerve growth factors (NGF) including NGF 2.5s, NGF 7.0s and beta NGF and neurotrophins, brain derived neurotrophic factor, cartilage derived factor, bone growth factors (BGF), basic fibroblast growth factor, insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), EG-VEGF, VEGF-related protein, Bv8, VEGF-E, granulocyte colony stimulating factor (G-CSF), insulin like growth factor (IGF) I and II, hepatocyte growth factor, glial neurotrophic growth factor (GDNF), stem cell factor (SCF), keratinocyte growth factor (KGF), transforming growth factors (TGF), including TGFs alpha, beta, beta1, beta2, and beta3, skeletal growth factor, bone matrix derived growth factors, and bone derived growth factors and mixtures thereof. Some growth factors can also promote differentiation of a cell or tissue. TGF, for example, can promote growth and/or differentiation of a cell or tissue. Some preferred growth factors include VEGF, NGFs, PDGF-AA, PDGF-BB, PDGF-AB, FGFb, FGFa, and BGF.

The term "differentiation factor" as used herein means a bioactive molecule that promotes the differentiation of cells. The term includes, but is not limited to, neurotrophin, colony stimulating factor (CSF), or transforming growth factor. CSF includes granulocyte-CSF, macrophage-CSF, granulocyte-macrophage-CSF , erythropoietin, and IL-3. Some differentiation factors can also promote the growth of a cell or tissue. TGF and IL-3, for example, can promote differentiation and/or growth of cells.

The term "adhesive compound" as used herein means a bioactive molecule that promotes attachment of a cell through the formation of focal adhesion complexes to a fiber surface comprising the adhesive compound. Examples of adhesive compounds include, but are not limited to, fibronectin, vitronectin, laminin, or fragments thereof.

The term "deadhesive compound" as used herein means a bioactive molecule that promotes the detachment of a cell through the disassembly or prevention of formation of focal adhesion complexes from a fiber comprising the deadhesive compound. Examples of deadhesive compounds include, but are not limited to, thrombospondin and tenascin C.

The term "targeting compound" as used herein means a bioactive molecule that functions as a signaling molecule inducing recruitment and/or attachment of cells to a fiber comprising the targeting compound. Examples of targeting compounds and their cognate receptors include attachment peptides including RGD peptide derived from fibronectin and integrins, growth factors including EGF and EGF receptor, and hormones including insulin and insulin receptor.

The term "lipid" as used herein means an organic molecule that is insoluble in water but tends to dissolve in nonpolar organic solvents. The term includes lipophilic molecules, including, but not limited to plant and animal triglycerides, sterols, phosphatidylcholine materials, including lysophosphatidylcholine, phosphatidylcholine, sphingomyelin, and cholesterol.

The term "culture container" as used herein means a receptacle for holding media for culturing a cell or tissue. The culture container can be glass or plastic. Preferably the plastic is non-cytotoxic. The term culture container includes, but is not limited to, single and multiwell culture plates, chambered and multi-chambered culture slides, coverslips, cups, flasks, tubes, bottles, roller bottles, spinner bottles, perfusion chambers, bioreactors and fermenters.

The term "mat" as used herein means a densely interwoven, tangled or adhered mass of nanofibers. The distribution of nanofibers in the mat can be random or oriented. A mat can be unwoven or net. A mat may or may not be deposited on a substrate. A mat can have a thickness of about the diameter of a single nanofiber to about 5000 nm. In an embodiment, the thickness comprises about 30 nm to about 5000 nm, about 3000 nm to about 5000 nm, about 1000 nm to about 2000 nm, about 1000 nm to about 1500 nm, about 30 nm to about 2000 nm, about 100 nm to about 1000 nm, or about 500 nm to about 1500 nm.

"Average surface roughness" or "Ra" is a measurement of the nanotopography of the growth surface that is calculated from a surface profile of the growth surface. Average surface roughness is the arithmetic average of the absolute values of measured profile height deviations from a center line divided by the evaluation length. The evaluation length is the entire length of a profile over which data has been collected. Average surface roughness of the growth surface can be calculated in one or two dimensions from analog or digital data. In some embodiments, average surface roughness is the integral of the absolute value of the roughness profile height of the growth surface over the evaluation length:

$$R_a = \frac{1}{L}\int_0^L |r(x)|\,\mathrm{d}x .$$

In some embodiments, the integral is approximated by a trapezoidal rule:

$$R_a = \frac{1}{N}\sum_{n=1}^{N} |r_n| .$$

"Surface profile" as used herein means is the contour of the growth surface in a plane measured normal, or perpendicular, to the growth surface unless another angle is specified.

"Nanotopography" as used herein means the three-dimensional representation of the structure and geometry of the growth surface obtained by measurement at the nanoscale.

"Maximum peak height" or "Rp" or "peak roughness" are used interchangeably and are a measurement of the nanotopography of the growth surface calculated from a surface profile of the growth surface. Maximum peak height is the height of the highest peak in the roughness profile of the growth surface over the evaluation length of the growth surface.

"Maximum valley depth" or "Rv" are used interchangeably and are a measurement of the nanotopography of the growth surface calculated from a surface profile of the growth surface. Maximum valley depth is the depth of the deepest valley in the roughness profile of the growth surface over the evaluation length.

"Total roughness" or "Rt" is the sum of Rp and Rv and represents the vertical distance of the deepest valley to highest peak in the roughness profile of the growth surface over the evaluation length.

"Peak count" or "Pc" is a measurement of the nanotopography of the growth surface calculated from a surface profile of the growth surface. Pc is the number of peaks in the roughness profile of the growth surface over the evaluation length. In an embodiment, the evaluation length is 1 μm.

"Average spacing between peaks" or "S" is a measurement of the nanotopography of the growth surface calculated from a surface profile of the growth surface. S is the average spacing between adjacent peaks of the measured surface profile within the roughness evaluation length. In an embodiment, S is calculated by:

$$S_m = ({}^{1}/_{N}) \sum_{n=1}^{N} S_n \quad .$$

II. Modes for Carrying Out the Invention

Rac signaling plays an important role in initiating cellular transformations such as morphogenesis and differentiation. Global changes in cell physiology and cell-cell interactions are believed to be initiated by activation of Rac, with a concomitant decrease in the activation state of either Rho and/or ROCK (Sander et al., 1999, *J. Cell Biol.*, 147:1009-1021; Connolly *et al.*, 2002, 13:2474-2485; Tsuji *et al.*, 2002, *J. Cell. Biol.*, 157:819-830). The invention provides a system and methods for preferentially enhancing activation Rac GTPase in a cell or tissue. The system and methods of the invention provide Rac activated cells having *in vivo*-like morphology and fibrillar organization, including membrane ruffling and lamellipodia (Figs. 5B, 17A, and 17B), that are capable of proliferating and self-renewing. The Rac activated cells of the invention are useful in a variety of *in vitro*, *in vivo*, and *ex vivo* applications including cell culture, tissue culture, tissue engineering, tissue regeneration, organ culture, and organ regeneration.

A. System for inducing Rac activation

One aspect of the invention provides a system for preferentially enhancing activation of Rac GTPase in a cell or tissue. The system comprises one or more cells and a growth surface that preferentially enhances activation of Rac GTPase in the cells. The Rac GTPase can be Rac 1, Rac2, Rac3, or a combination thereof. The growth surface comprises a nanofibrillar growth surface or a surface that comprises a nanotopography having physical and geometric properties similar to the nanofibrillar growth surface.

While not wishing to be bound by theory, it is believed the physical and geometric properties of the nanotopography of the nanofibrillar growth surface mimic the nanotopography of the ECM or BM. The cytoskeleton has been shown to integrate and propagate physical and chemical signals from the extracellular matrix (ECM) to the cell, leading to changes in cell morphology, cell-cell interactions, and function (Wozniak et al., 2004, Biochem. Biophys. Acta, 1692: 103-119; Geiger et al., Nature Rev. Mol. Cell Biol., 2:793-805; Boudreau, 2003, Sci. STKE, 196:pe34). As shown in the Examples, synthetic growth surfaces comprising these structural features provided sufficient *in vivo*-like spatial cues to preferentially enhance activation of the Rac pathway in a cell on the growth surface.

The physical and geometric properties of the biomimetic nanotopography of the nanofibrillar growth surface can be characterized by surface profile parameters. Useful surface profile parameters include, but are not limited to, average surface roughness (Ra), maximum peak height (Rp), maximum valley depth (Rv), total roughness (Rt), average peak spacing (S), and peak count (Pc). Methods for calculating and visualizing surface profile parameters of the growth surface in one, two, or three dimensions are known and routine. See, for example, Oberg et al., Machinery's Handbook 27th Ed., McCauley et al ed., New York 2004; http://www-predev-com/smg/parameters/htm; http://www-pacificnanotech-com/nano-roughness_single-html.; and http://www-mitutoyo-com/pdf/1685.pdf. Optical methods including microscopy, optical profilers, and scatterometry, electron and ion beam methods, and mechanical profilers including surface profilers such as the SURFTEST™ (Mitutoyo America Co., Aurora, IL) or SURFOMETER® (Precision Devices, Inc., Milan, MI) and atomic force microscopy (AFM) such as NANO-R™ or NANO-I™ AFM (Pacific Nanotechnology, Santa Clara, CA) are useful for analyzing surface profiles of the growth surface at the micro and nanoscale. Computer software useful for calculating and visualizing surface profile parameters of the growth surface is known and commercially available. Examples of such software include, but are not limited to, SURFPAK PRO™ (Mitutoyo America Co., Aurora, IL) and NANORULE+™ (Pacific Nanotechnology, Santa Clara, CA).

In an embodiment, the growth surface comprises one or more of the following: Ra of about 13 nm to about 1200 nm; Rp of about 13 nm to about 1200 nm; Rv of about 13 nm to about 1200 nm; Rt of about 26 nm to about 2400 nm; S of about 50 nm to about 800 nm; Pc of about 1 to about 30; and elongation of about 25 percent to about 500 percent. Ra of the growth surface can be from about 13 nm to about 1200 nm, from about 50 nm to about 600 nm; from about 50 nm to about 400 nm, or from about 100 nm to about 300 nm. Rp of the growth surface can be from of about 13 nm to about 1200 nm, from about 50 nm to about 600 nm, or from about 100 nm to about 300 nm. Rv of the growth surface can be from about 13 nm to about 1200 nm, from about 50 nm to about 600 nm, or from about 100 nm to about 300 nm. Rt of the growth surface can be from about 26 nm to about 2400 nm, from about 100 nm to about 1200 nm, or from about 200 nm to about 600 nm. S of the growth surface can be from about 20 nm to about 2000 nm, from about 20 nm to about 1000 nm, from about 20 nm to about 700 nm, from about 100 nm to about 600 nm, from about 20 nm to about 400 nm, or from about 50 nm to about 200 nm. Pc of the growth surface can be from about 1 to about 30 per μm, from about 1 to about 20 per μm, from about 1 to about 10 per μm, from about 1 to about 5 per μm, or from about 5 to about 10 per μm. Elongation of the growth surface can be from about 25 percent to about 500 percent, from about 50 percent to about 200 percent, from about 100 percent to about 500 percent, or from about 100 percent to about 200 percent.

In an embodiment, the growth surface comprises Ra of about 13 nm to about 1200 nm, S of about 50 nm to about 800 nm, Pc of about 1 to about 30 nm; and elongation of about 25 percent to about 500 percent. In another embodiment, the growth surface comprises Ra of about 75 nm to about 300 nm; S of about 100 nm to about 600 nm, Pc of about 1 to about 10 nm; and elongation of about 100 percent to about 500 percent.

Additional physical and chemical properties of the growth surface can be engineered to create a nano- or microenvironment that promotes or induces Rac activation or *in vivo*-like differentiation and morphogenesis. Physical properties of the growth substrate such as adhesivity, porosity, solidity, elasticity, geometry, interconnectivity, surface to volume ratio, solubility/insolubility, hydrophilicity/hydrophobicity, and density can be engineered to desired parameters. Functional groups can be incorporated into or on the growth surface. Functional groups can promote or inhibits cellular activity, such as for example growth, differentiation, targeting, and adhesion. Functionalized surfaces can be reacted to bind recognition motifs such as peptides, polypeptides, lipids, carbohydrates, amino acids, nucleotides, nucleic acids, polynucleotides, or polysaccharides including, but not limited to, growth factors, differentiation factors, fibrous proteins, adhesive proteins, glycoproteins, adhesive compounds, deadhesive compounds, and targeting molecules to promote appropriate cellular activity, including cell growth and/or differentiation. In an embodiment, the functionalized surfaces are reacted to bind one or more bioactive molecules. Preferably one or more of the bioactive molecules is a growth factor, differentiation factor, adhesive protein, or bioactive peptide derived from an adhesive protein. The growth factor can be VEGF, bone morphogenic factor β, EGF, PDGF, NGF, FGF, IGF, or TGF. The differentiation factor can be neurotrophin, CSF, or TGF. The bioactive peptide can be an RGD peptide.

In an embodiment, functional groups are deposited on the growth surface by plasma deposition. Plasma deposition creates local plasmas on the growth surface. The treated surface is then reacted with gaseous molecules, such as allylamine and/or allyl alcohol, in a reaction chamber. In another embodiment, functional groups are introduced during manufacturing of the growth surface. For example, dodecyl amine, dodecyl aldehyde, dodecyl thiol, or dodecyl alcohol can be added to a polymer solution during the manufacturing process. A portion of the added amines, aldehydes, sulphydryl, or alcohol moieties, respectively, are exposed to the cells or tissue.

Nano- and micro-environments that promote cellular activity of a particular cell or tissue can, such as sustained activation of Rac, be constructed by layering growth surfaces that have selected physical and/or chemical properties. The physical and/or chemical properties can be engineered into the individual growth surface of the assembly as described herein. In an embodiment, multiple cell types are cultured on individual growth surfaces under different culture conditions and then assembled, manually or mechanically, layer by layer into a specific multi-layered assembly. Nano- and/or micro-environments that promote cellular activity of particular cell types can be engineered within an individual growth surface by varying and/or modifying selected physical and/or chemical properties of the growth surface or within the assembly by selectively layering the individual growth surfaces to obtain the desired nano- or micro-environment.

A spacer can separate the growth surfaces. In an embodiment, the spacer comprises a fine fiber or film. The film can have a thickness of not more than about 10 to about 50 microns. The fine fiber can comprise a microfiber. Preferably the microfiber has a diameter of about 1 micron to about 10 microns. The microfiber can be unwoven or net. The microfiber can be fabricated from many polymers including cellulose, polyamide, polyester, and polytetrafluoroethylene. The spacer can be water soluble or water insoluble. The spacer can be porous or non-porous. In an embodiment, the pores spacer have a diameter of about 2 μm to about 10 μm. The spacer can be biodegradable and/or biodissolvable. Preferably the spacer is biocompatible.

In an embodiment, the spacer comprises one or more bioactive molecules. The bioactive molecules can be incorporated into the spacer during fabrication of the spacer or can be attached to a surface of the spacer via a functional group. Functional groups can be incorporated onto a surface of the spacer as described herein. The functionalized surfaces of the spacer can be reacted to bind a peptide, carbohydrate, polysaccharide, lipid, nucleotide, nucleic acid, polynucleotide, or other bioactive molecule to the surface of the spacer

The growth surface can be synthetic. Preferably, the growth surface is biocompatible. In an embodiment, the growth surface is non-toxic. In an embodiment, the growth surface is implantable in a mammal, such as a human. Examples of growth surfaces include, but are not limited to, a network of one or more nanofibers; a nanofibrillar structure; glass, silicon, or plastic comprising an etched or micropatterned surface; glass, silicon, or plastic surface comprising macropores or nanopores; or a polymer scaffold.

In some embodiments, the growth surface comprises an etched surface. The etched surface can be glass, silicon, or plastic. Examples of glass or plastic include, but are not limited to, a coverslip, disc, or slide. Examples of silicon include, but are not limited to, a chip, disc, or wafer. Nanofabrication and microfabrication techniques for etching glass, silicon, or plastic surfaces are known and include, for example, wet etching (U.S. 6,734,000), photoelectrochemical etching (U.S. 6,734,000), electrochemical etching (U.S. 6,734,000), plasma etching, micromachining, and reactive ion etching (U.S. 6,734,000; Gray et al., 2002, Biomed. Microdevices, 4:9-16).

In some embodiments, the growth surface comprises macropores or nanopores. The growth surface can be glass, plastic, or silicon. Examples of glass or plastic include, but are not limited to, a coverslip, disc, or slide. Examples of silicon include, but are not limited to, a chip, disc, or wafer. Nanofabrication and microfabrication techniques for creating macropores or nanopores in a solid surface are known and include, for example, wet etching (U.S. 6,734,000), photoelectrochemical etching (U.S. 6,734,000), electrochemical etching (U.S. 6,734,000), and reactive ion etching (U.S. 6,734,000) techniques.

In some embodiments, the growth surface comprises a micropatterned surface. The growth surface can be glass, plastic, or silicon. Examples of glass or plastic include, but are not limited to, a coverslip, disc, or slide. Examples of silicon include, but are not limited to, a chip, disc, or wafer. Methods for micropatterning a glass, plastic, or silicon surface are known in the art. These methods include, but are not limited to, micromachining, photochemical resist photolithography (Mrksich and Whitesides, 1996, Ann. Rev. Biophys. Biomol. Struct. 25:55;-78; U.S. 6,734,000), photochemical resist stamping (Singhvi et al., 1994, Science, 264:696-698), microfluid stamping (U.S. 6, 686,184), elastomeric stenciling (WO03/0629920), and electron beam irradiated polymer grafting and localized laser ablation (Yamato et al., 2003, J. Biomed. Mater Res. A., 67:1065-1071) methods. In an embodiment, one or more extracellular matrix ligands are immobilized on the micropatterned surface.

In some embodiments, the growth surface comprises a polymer scaffold. Methods for constructing and assembling a polymer scaffold are known. See, for example, U.S. 6,228,117; U.S. 6,103, 255; U.S. 5,041,138, and Levenberg et al., 2003, Proc. Natl. Acad. Sci. U.S.A., 100:12741-12746. In an embodiment, the polymer comprises polyester and/or polyamide. The polyester can be polyglcolate or polylactate. The polyamide can be nylon. In an embodiment, the nylon is poly epsilon caprolactone. In an embodiment, the polymer scaffold comprises a sponge. In an embodiment, the polymer scaffold comprises a solidity of about 3 percent to about 70 percent. In an embodiment, the polymer scaffold comprises a solidity of about 3 percent to about 50 percent. In an embodiment, the polymer scaffold comprises a solidity of about 3 percent to about 30 percent.

The growth surface can comprise a hydrogel or a peptide scaffold. Methods for making hydrogels and peptide scaffolds are known in the art. See, for example, WO04/0097683; WO03/080653; and WO02/062969. Peptides comprising the scaffold or hydrogel can be synthetic. The peptide scaffold or hydrogel can be self-assembling. In an embodiment, the growth surface comprises a self-assembling peptide hydrogel. In an embodiment, the hydrogel or peptide scaffold comprises a solidity of about 3 percent to about 70 percent. In an embodiment, the hydrogel or peptide scaffold comprises a solidity of about 3 percent to about 50 percent. In an embodiment, the hydrogel or peptide scaffold comprises a solidity of about 3 percent to about 30 percent.

1. Nanofibrillar growth surface

In some embodiments, the growth surface comprises a network of one or more nanofibers. The nanofiber(s) preferably comprises a non-cytotoxic polymer. The polymer can be water soluble or water insoluble. The polymer can be biodegradable and/or biodissolvable. The polymer can comprise a first polymer and a second, but different polymer (differing in polymer type, molecular weight or physical property) conditioned or treated at elevated temperature.

A nanofiber network can have a thickness of about the diameter of a single nanofiber or greater. In an embodiment, the nanofiber network comprises a thickness less than about 250 μm. In an embodiment, the thickness comprises about 150 μm to about 250 μm. In an embodiment, the thickness comprises about 50 μm to about 100 μm. In an embodiment, the thickness comprises about 10 μm to about 50 μm. In an embodiment, the thickness comprises about 5 μm to about 10 μm. In an embodiment, the thickness comprises about 30 nm to about 5000 nm. In an embodiment, the thickness comprises about 3000 nm to about 5000 nm. In an embodiment, the thickness comprises about 1000 nm to about 2000 nm. In an embodiment, the thickness comprises about 1000 nm to about 1500 nm. In an embodiment, the thickness comprises about 30 nm to about 2000 nm. In an embodiment, the thickness comprises about 100 nm to about 1000 nm. In an embodiment, the thickness comprises about 500 nm to about 1500 nm.

i. Polymer and polymer systems

The polymer can be reacted and formed into a single chemical specie or can be physically combined into a blended composition by an annealing process. Annealing implies a physical change, like crystallinity, stress relaxation or orientation. Preferred materials are chemically reacted into a single polymeric specie such that a Differential Scanning Calorimeter analysis reveals a single polymeric material. Such a material, when combined with a preferred additive material, can form a surface coating of the additive on the nanofiber that provides oleophobicity, hydrophobicity or other associated improved stability when contacted with high temperature, high humidity and difficult operating conditions. The nanofiber can have a diameter of about 1200 nm to less than about 5 nm. Such fibers can have a smooth surface comprising a discrete layer of the additive material or an outer coating of the additive material that is partly solubilized or alloyed in the polymer surface, or both. Preferred materials for use in the blended polymeric systems include nylon 6; nylon 66; nylon 6-10; nylon (6-66-610) copolymers and other linear generally aliphatic nylon compositions. A preferred nylon copolymer resin (SVP-651) was analyzed for molecular weight by the end group titration. (J.E. Walz and G.B. Taylor, determination of the molecular weight of nylon, Anal. Chem. Vol. 19, Number 7, pp 448-450 (1947). A number average molecular weight (W_n) was between 21,500 and 24,800. The composition was estimated by the phase diagram of melt temperature of three component nylon, nylon 6 about 45%, nylon 66 about 20% and nylon 610 about 25%. (Page 286, Nylon Plastics Handbook, Melvin Kohan ed. Hanser Publisher, New York (1995)).

Reported physical properties of SVP 651 resin are:

-Property	ASTM Method	Units	Typical Value
Specific Gravity	D-792	--	1.08
Water Absorption (24 hr immersion)	D-570	%	2.5
Hardness	D-240	Shore D	65
Melting Point	DSC	°C(°F)	154 (309)
Tensile Strength @ Yield	D-638	MPa (kpsi)	50 (7.3)
Elongation at Break	D-638	%	350
Flexural Modulus	D-790	MPa (kpsi)	180 (26)
Volume Resistivity	D-257	ohm-cm	10^{12}

A polyvinyl alcohol having a hydrolysis degree of from 87 to 99.9+% can be used in such polymer systems. These are preferably crosslinked, and they are most preferably crosslinked and combined with substantial quantities of the oleophobic and hydrophobic additive materials.

The polymer can be a single polymeric material optionally combined with an additive composition to improve fiber lifetime or operational properties. The preferred polymers useful in this aspect of the invention include nylon polymers, polyvinylidene chloride polymers, polyvinylidene fluoride polymers, polyvinyl alcohol polymers and, in particular, those listed materials when combined with strongly oleophobic and hydrophobic additives that can result in a microfiber or nanofiber with the additive materials formed in a coating on the fine fiber surface. Again, blends of similar polymers such as a blend of similar nylons, similar polyvinylchloride polymers, blends of polyvinylidene chloride polymers are useful in this invention. Further, polymeric blends or alloys of differing polymers are also contemplated by the invention. In this regard, compatible mixtures of polymers are useful in forming the nanofiber or microfiber materials of the invention.

Additive compositions can be organic or inorganic, metals or non-metals. In an embodiment, the polymer solution comprises from about 0.25 percent to about 70 percent w/w additive composition. In a further embodiment, the additive composition is a bioactive molecule. In another further embodiment, the additive composition is a ceramic. The additive composition can be an optical additive that increases or decreases inherent fiber fluorescence for microscopy. In an embodiment, the optical additive is a quantum dot. In another embodiment, the optical additive minimizes fluorescent background of the fiber by enhancing the signal to noise ratio. Examples of optical additives include, but are not limited to quantum dots or Fluoroblok™ (Bectin Dickinson, Franklin Lakes, NJ). Additive composition compositions can be bioactive molecules. In an embodiment, an additive composition that influences packing of the polymer is a bioactive molecule. The bioactive molecule can be a lipid. Preferably the lipid is lysophosphatidylcholine, phosphatidylcholine, sphingomyelin, cholesterol, and mixtures thereof.

Polymer materials that can be used in the polymeric compositions of the invention include both addition polymer and condensation polymer materials such as polyolefin, polyacetal, polyamide, polyester, cellulose ether and ester, polyalkylene sulfide, polyarylene oxide, polysulfone, modified polysulfone polymers and mixtures thereof. Preferred materials that fall within these generic classes include polyethylene, poly(ε-caprolactone), poly(lactate), poly(glycolate), polypropylene, poly(vinylchloride), polymethylmethacrylate (and other acrylic resins), polystyrene, and copolymers thereof (including ABA type block copolymers), poly(vinylidene fluoride), poly(vinylidene chloride), polyvinyl alcohol in various degrees of hydrolysis (87% to 99.5%) in crosslinked and non-crosslinked forms. Preferred addition polymers tend to be glassy (a Tg greater than room temperature). This is the case for polyvinylchloride and polymethylmethacrylate, polystyrene polymer compositions or alloys or low in crystallinity for polyvinylidene fluoride and polyvinyl alcohol materials.

Aliphatic polyesters such as poly(ε-caprolactone), poly(lactate), poly(glycolate), and their copolymers are biodegradable, and biocompatible and among the few synthetic polymers approved by the Food and Drug Administration (FDA) for certain human clinical applications such as surgical sutures and some implantable devices. In an embodiment, the nanofibers are fabricated from an aliphatic polyester suitable for *in vivo* human application. Preferably the polyester is poly(ε-caprolactone), poly(lactate) or poly(glycolate). In an embodiment, the nanofibers are fabricated from a polymer solution comprising at least about 10% poly(ε-caprolactone) w/w in chloroform. In another embodiment, the nanofibers are fabricated from a polymer solution comprising at least about 15% poly(ε-caprolactone) w/w in chloroform.

One class of polyamide condensation polymers is nylon materials. The term "nylon" is a generic name for all long chain synthetic polyamides. Typically, nylon nomenclature includes a series of numbers such as in nylon-6,6 which indicates that the starting materials are a C_6 diamine and a C_6 diacid (the first digit indicating a C_6 diamine and the second digit indicating a C_6 dicarboxylic acid compound). Another nylon can be made by the polycondensation of epsilon caprolactam in the presence of a small amount of water. This reaction forms a nylon-6 (made from a cyclic lactam - also known as epsilon-aminocaproic acid) that is a linear polyamide. Further, nylon copolymers are also contemplated. Copolymers can be made by combining various diamine compounds, various diacid compounds and various cyclic lactam structures in a reaction mixture and then forming the nylon with randomly positioned monomeric materials in a polyamide structure. For example, a nylon 6,6-6,10 material is a nylon manufactured from hexamethylene diamine and a C_6 and a C_{10} blend of diacids. A nylon 6-6,6-6,10 is a nylon manufactured by copolymerization of epsilon aminocaproic acid, hexamethylene diamine and a blend of a C_6 and a C_{10} diacid material.

Block copolymers are also useful in the system and methods of the invention. With such copolymers the choice of solvent swelling agent is important. The selected solvent is such that both blocks were soluble in the solvent. One example is a ABA (styrene-EP-styrene) or AB (styrene-EP) polymer in methylene chloride solvent. If one component is not soluble in the solvent, it will form a gel. Examples of such block copolymers are Kraton® type of AB and ABA block polymers including styrene/butadiene and styrene/hydrogenated butadiene(ethylene propylene), Pebax® type of epsilon-caprolactam/ethylene oxide, Sympatex® polyester/ethylene oxide and polyurethanes of ethylene oxide and isocyanates.

Addition polymers like polyvinylidene fluoride, syndiotactic polystyrene, copolymer of vinylidene fluoride and hexafluoropropylene, polyvinyl alcohol, polyvinyl acetate, amorphous addition polymers, such as poly(acrylonitrile) and its copolymers with acrylic acid and methacrylates, polystyrene, poly(vinyl chloride) and its various copolymers, poly(methyl methacrylate) and its various copolymers, can be solution spun with relative ease because they are soluble at low pressures and temperatures. However, highly crystalline polymer like polyethylene and polypropylene require high temperature, high pressure solvent if they are to be solution spun. Therefore, solution spinning of the polyethylene and polypropylene is very difficult. Electrostatic solution spinning is one method of making nanofibers and microfiber.

We have also found a substantial advantage to forming polymeric compositions comprising two or more polymeric materials in polymer admixture, alloy format or in a crosslinked chemically bonded structure. We believe such polymer compositions improve physical properties by changing polymer attributes such as improving polymer chain flexibility or chain mobility, increasing overall molecular weight and providing reinforcement through the formation of networks of polymeric materials.

In one embodiment of this concept, two related polymer materials can be blended for beneficial properties. For example, a high molecular weight polyvinylchloride can be blended with a low molecular weight polyvinylchloride. Similarly, a high molecular weight nylon material can be blended with a low molecular weight nylon material. Further, differing species of a general polymeric genus can be blended. For example, a high molecular weight styrene material can be blended with a low molecular weight, high impact polystyrene. A Nylon-6 material can be blended with a nylon copolymer such as a Nylon-6; 6,6; 6,10 copolymer. Further, a polyvinyl alcohol having a low degree of hydrolysis such as a 87% hydrolyzed polyvinyl alcohol can be blended with a fully or super hydrolyzed polyvinyl alcohol having a degree of hydrolysis between 98 and 99.9% and higher. All of these materials in admixture can be crosslinked using appropriate crosslinking mechanisms. Nylons can be crosslinked using crosslinking agents that are reactive with the nitrogen atom in the amide linkage. Polyvinyl alcohol materials can be crosslinked using hydroxyl reactive materials such as monoaldehydes, such as formaldehyde, ureas, melamine-formaldehyde resin and its analogues, boric acids and other inorganic compounds. dialdehydes, diacids, urethanes, epoxies and other known crosslinking agents. Crosslinking technology is a well known and understood phenomenon in which a crosslinking reagent reacts and forms covalent bonds between polymer chains to substantially improve molecular weight, chemical resistance, overall strength and resistance to mechanical degradation.

Nanofibers having a smaller diameter provide a surface that promotes multipoint attachments between nanofibers and cells, a characteristic of cell attachment to the extracellular matrix *in vivo*. Electrospinning produces a population of nanofibers that can differ in diameter. In an embodiment, the nanofibers comprise a diameter of about 30 nm to about 1200 nm. In an embodiment, the nanofibers comprise a diameter of about 30 nm to about 600 nm. In an embodiment, the nanofibers comprise a diameter of about 30 nm to about 500 nm. In another embodiment, the nanofibers comprise a diameter of about 30 nm to about 400 nm. In another embodiment, the nanofibers comprise a diameter of about 100 nm to about 300 nm. In another embodiment, the nanofibers comprise a diameter of about 30 nm to about 200 nm. In another embodiment, the nanofibers comprise a diameter of about 180 nm.

In an embodiment, the polymeric material is combined with an additive composition that influences packing of the polymer such that electrospinning of the polymer results in the production of a population of nanofibers having a greater number or percentage thin fibers as compared to a population of nanofibers electrospun form a polymer solution not containing the additive composition. In an embodiment, the polymer solution comprises from about 0.25% to about 15% w/w additive composition. In another embodiment, the polymer solution comprises from about 1% to about 10% w/w additive composition.

In an embodiment, thin fibers comprise a diameter of about 5 to about 600 nm. In an embodiment, the thin fibers comprise a diameter of about 50 nm to about 400 nm. In another embodiment, the thin fibers comprise a diameter of about 5 nm to about 200 nm. In another embodiment, the thin fibers comprise a diameter of about 5 nm to about 100 nm. In another embodiment, the thin fibers comprise a diameter of about 5 nm to about 50 nm.

In an embodiment, at least about 25% of the population of nanofibers are thin fibers. In an embodiment, at least about 30% percent of the population of nanofibers are thin fibers. In another embodiment, at least about 40% of a polydisperse plurality of nanofibers is thin fibers. In another embodiment, at least about 50% of polydisperse plurality of nanofibers is thin fibers. In another embodiment, at least about 60% of polydisperse plurality of nanofibers is thin fibers. In another embodiment, at least about 70% of polydisperse plurality of nanofibers is thin fibers.

Preferably the additive composition is non-cytotoxic. The additive composition may or may not affect the activity of cells, including migration or cell attachment to the nanofibers. In an embodiment, a nanofiber comprising the additive composition does not affect the activity of cells. Preferably, the additive composition comprises one or more bioactive molecules. One or more of the bioactive molecules can be a lipid. Preferably the lipid is cholesterol. In another embodiment, a nanofiber comprising the additive composition can affect the activity of cells. Such a nanofiber can induce cell migration or enhance attachment of cells to the nanofiber.

The polymer or polymer system can comprise one or more bioactive molecules including, but not limited to, lipids or lipophilic molecules, fibrous proteins, adhesion proteins, growth factors, and differentiation factors. In an embodiment, at least one of the bioactive molecules comprises a lipid. The lipid molecules can function as signaling molecules inducing recruitment and attachment of cells to the fiber. The lipid molecules can also cause the cells to proliferate or differentiate. Preferably the lipid is lysophosphatidylcholine, phosphatidylcholine, sphingomyelin, or mixtures thereof. In an embodiment, one or more of the bioactive molecules is a growth factor, differentiation factor, fibrous protein, and/or adhesive protein. Examples of useful growth factors include, but are not limited to, VEGF, bone morphogenic factor β, EGF, PDGF, NGF, FGF, IGF, or TGF. Examples of useful differentiation factor include, but are not limited to, neurotrophin, CSF, or TGF. Preferably the differentiation factor is neurotrophin, CSF, or TGF.

The polymer systems of the invention have adhering characteristic such that when contacted with a cellulosic, polyvinyl, polyester, polystyrene, or polyamide substrate adheres to the substrate with sufficient strength such that it is securely bonded to the substrate and can resist delaminating effects associated with mechanical stresses. The nanofibers of the invention can be used to construct three-dimensional growth surface for functional tissues, including muscle and tendon. In such a mode, the polymer material must stay attached to the substrate while undergoing mechanical stresses associated with, for example, contraction of a muscle or tendon. Adhesion of the nanofiber to the substrate can arise from solvent effects of fiber formation as the fiber is contacted with the substrate or the post treatment of the fiber on the substrate with heat or pressure. However, polymer characteristics appear to play an important role in determining adhesion, such as specific chemical interactions like hydrogen bonding, contact between polymer and substrate occurring above or below Tg, and the polymer formulation including additives. Polymers plasticized with solvent or steam at the time of adhesion can have increased adhesion.

ii. Nanofiber network

Polymer selection and/or the process by which the nanofibers are fabricated and/or directed and oriented onto a substrate allow for specific selection and manipulation of physical properties of the nanofiber network. Physical properties of a growth surface, including fiber size, fiber diameter, fiber spacing, matrix density, fiber texture and elasticity, have been demonstrated to be important considerations for organizing the cytoskeletal networks in cells and the exposure of cell signaling motifs in extracellular matrix proteins (Meiners, S. and Mercado, M.L., 2003, *Mol.*

Neurobiol., 27(2), 177-196). Physical properties of the nanofiber network that can be engineered to desired parameters include, but are not limited to, surface roughness including Ra, Rp, Rv, Rt, S, and Pc, adhesivity, porosity, solidity, elasticity, geometry, interconnectivity, surface to volume ratio, fiber size, fiber diameter, fiber solubility/insolubility, hydrophilicity/hydrophobicity, and fibril density.

One or more of the physical properties of the fiber network of the nanofibrillar growth surface can be varied and/or modified to create a specifically defined environment for cell growth and/or differentiation. For example, the physical and geometric properties of the nanotopography of the nanofibrillar growth surface can be engineered to mimic the nanotopography of the ECM or BM. In an embodiment, the nanofiber network of the nanofibrillar growth surface comprises the growth surface comprises Ra of about 75 nm to about 300 nm; S of about 50 nm to about 600 nm, Pc of about 1 to about 10 nm; and elongation of about 100 percent to about 500 percent.

The porosity of the nanofibrillar growth surface, for example, can be engineered to enhance diffusion of ions, metabolites, and/or bioactive molecules and/or allow cells to penetrate and permeate the nanofibrillar growth surface to grow in an environment that promotes multipoint attachments between the cells and the nanofiber network. Interconnectivity of the nanofiber network can be engineered to facilitate cell-cell contacts. Elasticity of the nanofiber network can be increased or decreased by adding a bioactive molecule to the polymer solution from which the nanofibers are fabricated. In an embodiment, the bioactive molecule is a lipid. In a further embodiment the lipid is cholesterol. Homogeneous or heterogeneous nanofiber compositions can be selected to optimize growth or differentiation activity of the cells. For example, the nanofibrillar structure can be comprised of multiple nanofibers having different diameters and/or multiple nanofibers fabricated from different polymers.

Solubility or insolubility of the nanofibers of the nanofiber network can be engineered to control the release of bioactive molecules from nanofibrillar structure. In an embodiment, the rate of release of bioactive molecules is determined by the rate of biodegradation or biodissolution of the nanofibers of the nanofiber network. Hydrophobicity and hydrophilicity of the nanofiber network can be engineered to promote specific cell spacing. Solidity of the nanofibrillar structure can be engineered to promote cell growth and/or differentiation. In an embodiment, the nanofibrillar growth surface has a solidity of about 3 percent to about 70 percent. In another embodiment, the nanofibrillar growth surface has a solidity of about 3 percent to about 50 percent. In another embodiment, the nanofibrillar growth surface has a solidity of about 3 percent to about 30 percent. In another embodiment, the nanofibrillar growth surface has a solidity of about 3 percent to about 10 percent. In another embodiment, the nanofibrillar growth surface has a solidity of about 3 percent to about 5 percent. In another embodiment, the nanofibrillar growth surface has a solidity of about 10 percent to about 30 percent.

Electrospun nanofiber networks can be produced having random or directed orientations. The electrospinning process uses an electric field to control the formation and deposition of polymers. A polymer solution is injected with an electrical potential. The electrical potential creates a charge imbalance that leads to the ejection of a polymer solution stream from the tip of an emitter such as a needle. The polymer jet within the electric field is directed toward a grounded substrate, during which time the solvent evaporates and fibers are formed. The resulting single continuous filament collects as a nonwoven fabric on the substrate.

Random fibers can be assembled into layered surfaces and bonded to form an interlocking network. The nanofiber interlocking networks have relatively small spaces between the fibers. In an embodiment, the spacing between fibers comprises about 20 nm to about 2000 nm. In an embodiment, the spacing between fibers comprises about 20 nm to about 1000 nm. In an embodiment, the spacing between fibers comprises about 20 nm to about 700 nm. In an embodiment, the spacing between fibers comprises about 20 nm to about 400 nm. In an embodiment, the spacing between fibers comprises about 100 nm to about 600 nm. In an embodiment, the spacing between fibers comprises about 50 nm to about 200 nm.

Interfiber spaces form pores or channels in the nanofiber network allowing for diffusion of ions, metabolites, proteins, and/or bioactive molecules and/or allowing cells to penetrate and permeate the network and grow in an environment that promotes multipoint attachments between cells and the nanofibers. In an embodiment, the pores or channels comprise a diameter of about 20 nm to about 2000nm. In an embodiment, the pores or channels comprise a diameter of about 20 nm to about 1000 nm. In an embodiment, the pores or channels comprise a diameter of about 20 nm to about 700 nm. In an embodiment, the pores or channels comprise a diameter of about 100 nm to about 600 nm. In an embodiment, the pores or channels comprise a diameter of about 20 nm to about 400 nm. In an embodiment, the pores or channels comprise a diameter of about 50 nm to about 200 nm.

Nanofiber networks can be electrospun in an oriented manner. Such oriented electrospinning allows for the fabrication of a nanofiber network comprising a single layer of nanofibers or a single layer formed by a continuous nanofiber wherein the network has a height of the diameter of a single nanofiber. Physical properties such as surface roughness including Ra, Rp, Rv, Rt, S, and Pc, porosity, solidity, fibril density, and fiber orientation of the single layer network can be selected by controlling the direction and/or orientation of the nanofiber onto the substrate during the electrospinning process. In an embodiment, the pore size allows cells to penetrate and/or migrate through the nanofiber network.

iii. Functionalized Surfaces

Functional groups can be incorporated at the outside surface of the nanofibers. These functionalized surfaces can be reacted to bind a peptide, polypeptide, lipid, carbohydrate, polysaccharide, amino acid, nucleotide, nucleic acid, polynucleotide, or other bioactive molecule to the surface of the nanofiber. In an embodiment, the functionalized surfaces of the nanofiber are reacted to bind one or more bioactive molecules. Preferably one or more of the bioactive molecules is a growth factor, differentiation factor, adhesive protein, or bioactive peptide derived from an adhesive protein. The growth factor can be VEGF, bone morphogenic factor β, EGF, PDGF, NGF, FGF, IGF, or TGF. The differentiation factor can be neurotrophin, CSF, or TGF.

As a result of the high surface to volume ratio of the nanofibrillar growth surface, the amount of bioactive molecules bound to the nanofibrillar growth surface is significantly higher than the amount of bioactive molecules absorbed to a planar cell culture surface. In an embodiment, the density of bioactive molecules attached to the nanofibrillar growth matrix is 1 fold greater, 2 fold greater, 3 fold greater, 4 fold greater, or 5 fold greater than the density of bioactive molecules attached to a planar cell culture surface. The higher density of bioactive molecules on the nanofibrillar growth surface enhances the avidity of the interactions between cells and the nanofiber network to promote biological responses.

In an embodiment, the bioactive molecule is an extracellular matrix (ECM) molecule or fragment thereof. The ECM molecule can be naturally occurring or a synthetic peptide derived from a naturally occurring ECM molecule. Examples of ECM molecules include, but are not limited to, fibronectin, fibrinogen, laminin, and tenascin-C. Examples of synthetic ECM derived peptides include, but are not limited to, synthetic peptides comprising RGD (SEQ ID NO:1) derived from fibronectin (Meiners et al., 2003, Mol. Neurobiol., 27:177-96; Shin et al., 2003, Biomaterials, 24:4353-4364), VFDNFVLKIRDTKKQ (SEQ ID NO:2) derived from tenascin-C (Meiners et al., 2003, Mol. Neurobiol., 27:177-96), YIGSR (SEQ ID NO:3) derived from laminin-1 (Meiners et al., 2003, Mol. Neurobiol., 27:177-96; Shin et al., 2003, Biomaterials, 24:4353-4364), and IKAVAV (SEQ ID NO:4) derived from laminin-1(Meiners et al., 2003, Mol. Neurobiol., 27:177-96; Shin et al., 2003, Biomaterials, 24:4353-4364).

The bioactive molecules can be adsorbed or covalently attached to the nanofibrillar growth surface. Cysteines can be introduced at the N-terminal end of the peptides to provide a functional group for attachment to amines on the nanofiber surface and glycines can be added as spacers. Examples of synthetic ECM peptides that can be covalently attached to the nanofibrillar growth surface include, but are not limited to, CGGRGDSPG (SEQ ID NO:5), CGGIKAVAV (SEQ ID NO:6), CGGDPGYIGSR (SEQ ID NO:7), and CADEGVFDNFVLKIRDTKKQ (SEQ ID NO:8) (Meiners et al., 2003, Mol. Neurobiol., 27:177-96; Shin et al., 2003, Biomaterials, 24:4353-4364).

In an embodiment, functional groups are deposited on the outside surface of a nanofiber by plasma deposition. Plasma deposition creates local plasmas at the surface of the nanofiber. The treated surface is then reacted with gaseous molecules, such as allylamine and/or allyl alcohol, in a reaction chamber. In another embodiment, functional groups are introduced onto the surface of the nanofibers during the electrospinning process. Dodecyl amine, dodecyl aldehyde, dodecyl thiol, or dodecyl alcohol can be added to the polymer solution. The polymer solution is than electrospun into nanofibers in which a portion of the added amines, aldehydes, sulphydryl, or alcohol moieties, respectively, are exposed on the outside surface of the nanofibers.

iv. Fluorescent Marker

The nanofibers can comprise a fluorescent marker. The fluorescent marker allows for visualization of a nanofiber, identification of specific nanofibers within a nanofiber blend, identification of a chemical or physical property of a nanofiber, and evaluation of the degradation of and/or redistribution of implantable growth surfaces comprising a network or one or more nanofibers. The fluorescent marker can be photobleachable or non-photobleachable. The fluorescent marker can be pH sensitive or pH insensitive. Preferably the fluorescent marker is non-cytotoxic.

The fluorescent marker can comprise an organic dye fluorophore including, but not limited to, Texas Red®, BIODIPY®, Oregon Green®, Alexa Fluor®, fluorescein, Cascade Blue®, Dapoxyl®, coumarin, Rhodamine, *N*-methyl-4-hydrazine-7- nitrobenzofurazan, dansyl ethylenediamine, dansyl cadaverine, dansyl hydrazine, or mixtures thereof. More information on these and other suitable organic dye fluorophores can be found at www-probes-com (Molecular Probes, Eugene, OR). In an embodiment, the fluorophore is added to the polymer prior to electrospinning of a nanofiber. In another embodiment, the fluorophore is conjugated to a nanofiber via a functional group incorporated at the surface of the nanofiber. In another embodiment, the fluorophore is conjugated to a bioactive molecule that is attached to a nanofiber.

The fluorescent marker can comprise colloidal inorganic semiconductor nanocrystals. In an embodiment the nanocrystals comprise a CdSe core and ZnS cap. In another embodiment the nanocrystals comprise quantum dots. More information on nanocrystals and quantum dots can be found at www-evidenttech-com and www-quantumdots-com. The absorption spectra of the nanocrystal can be broad, extending from ultraviolet to a cutoff in the visible spectrum. The emission spectra of the nanocrystal can be narrow, preferably 20-40 nm full width at half maximum centered at a wavelength that is characteristic of the particle size of the selected nanocrystal. Preferably the nanocrystals are photochemically stable and/or non-cytotoxic.

Fluorescent markers are useful for identifying a chemical and/or physical property of the nanofiber. In an embodiment, a fluorescent marker is assigned to a chemical or physical property of the nanofiber and the nanofiber is labeled with the assigned fluorescent marker. Such chemical and physical properties include, but are not limited to, fiber diameter, bioactive molecules, functional groups, dissolution or degradation rate of fiber, composition of polymer comprising the nanofiber, hydrophobicity or hydrophilicity of the fiber; solubility of the polymer comprising the nanofiber, toxicity of the polymer, toxicity of bioactive molecules, or combinations thereof. In an embodiment, the bioactive molecule is a growth factor, differentiation factor, an adhesion molecule, or mixtures thereof.

The fluorescent marker can comprise bioactive fluorescent probes to determine changes in a biochemical environment. In an embodiment, the nanofiber comprises a fluorescent marker wherein the fluorescence or fluorescent intensity of the marker is dependent upon ion concentration. Such an ion sensing element is useful to detect changes in ion concentration including pH and calcium, sodium, or phosphate flux. In an embodiment, the fluorescent marker comprises SNARF, SNAFL, calcium green, or mixtures thereof. In another embodiment, the nanofiber contains dyes capable of changing their fluorescent properties as a result of complexion with other molecules.

2. Nanofibrillar structure

In some embodiments, the growth surface comprises a nanofibrillar structure. The nanofibrillar structure comprises an environment for growth of living cells comprising one or more nanofibers. The nanofibrillar structure is defined by a network of one or more nanofibers as described herein for the nanofibrillar growth surface. In some embodiments, the nanofibrillar structure comprises a substrate wherein the nanofibrillar structure is defined by a network of one or more nanofibers deposited on a surface of the substrate.

The nanofiber(s) comprising the nanofibrillar structure comprise a polymer or polymer system as described herein for the nanofibrillar growth surface. In an embodiment, the nanofibers are fabricated from a polymer suitable for *in vivo* human application. The nanofiber can be fabricated by many techniques, including preferred electrospinning techniques. Phase separation techniques and expansion techniques can also be used to fabricate the nanofibrillar structure.

The phase separation process typically includes polymer dissolution, phase separation and gelatin, solvent extraction from the gel with water, freezing, and then freeze drying under a vacuum. A typical procedure can be used as follows: polymer is added to solvent such as THF was added to make a solution about 1% (wt/v) to 15% (wt/v). The solution is stirred until uniform. Polymer solution (prewarmed to 50°C) is added into a Teflon vial. The vial containing polymer solution is then rapidly chilled to gel. The gel-time depends on temperature, solvent, and the polymer concentration. The gel is kept at temperature for at least 120 minutes. The gel is than immersed into distilled water for solvent exchange for 2 days. Following solvent exchange, the gel is removed from water, dried with filter paper, and frozen at -18°C. The frozen gel is than transferred into a freeze-drying vessel at about -10°C under vacuum lower than 0.5 mm Hg for 1 week. The dried scaffolds are then maintained in a desiccator.

Expansion techniques can be used to expand or stretch a polymer into a microporous or nanoporous structure. Examples of suitable polymers include, but are not limited to, polytetrafluoroethylene (PTFE), polyethylene, polypropylene, high density polyethylene (HDPE), poly(4-methyl-1-pentene) (PMP), polyoxymethylene (POM), and poly(vinyl fluoride) (PVDF). Methods for expanding or stretching a polymer into a microporous or nanoporous structure are known. See, for example, U.S. 5,869,156. In an embodiment, a polymer film is extruded, annealed, and stretched. In an embodiment, the polymer is polytetrafluoroethylene. The polymer can be extruded by ram extrusion or melt extrusion. In an embodiment, the extruded polymer is transversely stretched. In an embodiment, the extruded polymer is uniaxially stretched. In an embodiment, the polymer is stretched at a stretch rate of less than 10% per second at a temperature below the melt point of the polymer. The porous structure can be treated with a fluorinated organic polymer fluid including, but not limited to, a perfluoroether fluid or fluorinated polyalkene fluid. The polymer can be treated with the fluorinated organic polymer fluid before or after stretching. The pore structure and permeability of the expanded polymer can be analyzed by AFM, SEM, or Gurley number measurements.

The expanded porous structure comprises pores and fibrils. In an embodiment, the fibrils comprise a diameter of about 30 nm to about 1200 nm. In an embodiment, the fibrils comprise a diameter of about 30 nm to about 600 nm. In an embodiment, the fibrils comprise a diameter of about 30 nm to about 500 nm. In another embodiment, the fibrils comprise a diameter of about 30 nm to about 400 nm. In another embodiment, the fibrils comprise a diameter of about 100 nm to about 300 nm. In another embodiment, the fibrils comprise a diameter of about 30 nm to about 200 nm. In another embodiment, the fibrils comprise a diameter about 50 nm to about 100 nm.

In an embodiment, the pores comprise a diameter of about 20 nm to about 2000 nm. In an embodiment, the pores comprise a diameter of about 20 nm to about 1000 nm. In an embodiment, the pores comprise a diameter of about 20 nm to about

700 nm. In an embodiment, the pores comprise a diameter of about 100 nm to about 600 nm. In an embodiment, the pores comprise a diameter of about 20 nm to about 400 nm. In an embodiment, the pores comprise a diameter of about 50 nm to about 200 nm.

The nanotopography of the nanofibrillar structure can be engineered according to the surface roughness parameters described herein. The nanofiber networks of the nanofibrillar structure can be oriented or random as described herein. Layering of individual single layer networks form channels in the nanofibrillar structure allowing diffusion of ions, metabolites, proteins, and/or bioactive molecules and allowing cells to penetrate the nanofibrillar structure and grow in an environment that promotes multipoint attachments between the cells and the nanofiber network.

The nanofibers comprising the nanofibrillar structure can comprise one or more bioactive molecules as described herein for the nanofibrillar growth surface. The bioactive molecules can be incorporated into the nanofiber network during fabrication of the network or can be attached to a surface of the network via a functional group. In an embodiment, the polymer or polymer system from which the nanofiber is fabricated comprises one or more bioactive molecules. The one or more bioactive molecules can be a lipid, growth factor, differentiation factor, fibrous protein, adhesive protein, or combination thereof. The lipid can be lysophosphatidylcholine, phosphatidylcholine, sphingomyelin, or mixtures thereof. Examples of growth factors include, but are not limited to, VEGF, bone morphogenic factor β, EGF, PDGF, NGF, FGF, IGF, or TGF. Examples of differentiation factors include, but are not limited to, neurotrophin, CSF, or TGF.

Functional groups can be incorporated onto a surface of the network as described for the nanofibrillar growth surface. The functionalized surfaces of the network can be reacted to bind a peptide, polypeptide, lipid, carbohydrate, polysaccharide, nucleotide, nucleic acid, polynucleotide, or other bioactive molecule to the surface of the network. In an embodiment, the functionalized surfaces of the network are reacted to bind one or more bioactive molecules. Preferably one or more of the bioactive molecules is a growth factor, differentiation factor, fibrous protein, and/or adhesive protein. Preferably the growth factor is VEGF, bone morphogenic factor β, EGF, PDGF, NGF, FGF, IGF, or TGF. Preferably the differentiation factor is neurotrophin, CSF, or TGF.

i. Substrate

Structural properties of the nanofibrillar structure such as strength and flexibility can be provided in part by the substrate on which the nanofiber network is deposited. In an embodiment, the substrate comprises cellulose, silicon, glass, or plastic. Preferably the substrate is non-cytotoxic. The substrate can be a film or culture container. In an embodiment, the film comprises a thickness of not more than about 10 to about 1000 microns. The film can comprise polyvinyl alcohol, polychlorotrifluoroethylene, polystyrene, polymethylpentene, or polycylo-olefin. The substrate can be water soluble or water insoluble. A substrate that is water soluble is preferably a polyvinyl alcohol film and can be used with a polyvinyl alcohol fiber matrix. The substrate can be porous or non-porous. Porosity of the substrate can be determined by cellular penetration. A cell is able to penetrate a porous substrate but is not able to penetrate a non-porous substrate. Preferably the pores in a porous substrate have a diameter of about 2 μm to about 10 μm. The substrate can be biodegradable and/or biodissolvable. Preferably the substrate is biocompatible.

In an embodiment, the substrate comprises one or more bioactive molecules. The bioactive molecules can be incorporated into the substrate during fabrication of the substrate or can be attached to a surface of the substrate via a functional group. Functional groups can be incorporated onto a surface of the substrate as described for the nanofibrillar growth surface. The functionalized surfaces of the substrate can be reacted to bind a peptide, carbohydrate, polysaccharide, lipid, nucleotide, nucleic acid, polynucleotide, or other bioactive molecule to the surface of the substrate.

In an embodiment, the functionalized surfaces of the substrate are reacted to bind one or more bioactive molecules. Preferably one or more of the bioactive molecules is a growth factor, differentiation factor, fibrous protein, and/or adhesive protein. Examples of growth factors include, but are not limited to, VEGF, bone morphogenic factor β, EGF, PDGF, NGF, FGF, IGF, or TGF. Examples of differentiation factors include, but are not limited to, neurotrophin, CSF, or TGF. The substrate can release one or more bioactive molecules. The rate of release can be determined by the rate of dissolution and/or degradation of the substrate.

ii. Spacer

Structural properties of a nanofibrillar structure, such as strength and flexibility, can be provided by a spacer. Spacers can also provide sufficient separation between a nanofiber network and a substrate or sufficient separation between two or more nanofibrillar structures to permit cells to penetrate and attach to the nanofibers. In an embodiment, the spacer comprises a first and second surface wherein the first surface of the spacer contacts a surface of the nanofiber network deposited on a substrate and the second surface of the spacer contacts a surface of the substrate such that the nanofiber network and substrate are separated by the diameter or thickness of the spacer. In another embodiment, the spacer comprises a first and second surface wherein the first surface of the spacer contacts a surface of a first nanofibrillar structure and the second surface of the spacer contacts a surface of a second nanofibrillar structure such that the two nanofibrillar structures are separated by the diameter or thickness of the spacer.

In an embodiment, the spacer comprises a fine fiber or film. The film can have a thickness of not more than about 10 to about 50 microns. The fine fiber can comprise a microfiber. Preferably the microfiber has a diameter of about 1 micron to about 10 microns. The microfiber can be unwoven or net. The microfiber can be fabricated from many polymers including cellulose, polyamide, polyester, and polytetrafluoroethylene. The spacer can be water soluble or water insoluble. The spacer can be porous or non-porous. Porosity of the substrate can be determined by cellular penetration. A cell is able to penetrate a porous spacer but is not able to penetrate a non-porous spacer. Preferably the pores in a porous spacer have a diameter of about 2 μm to about 10 μm. The spacer can be biodegradable and/or biodissolvable. Preferably the spacer is biocompatible.

In an embodiment, the spacer comprises one or more bioactive molecules. The bioactive molecules can be incorporated into the spacer during fabrication of the spacer or can be attached to a surface of the spacer via a functional group. Functional groups can be incorporated onto a surface of the spacer as described herein. The functionalized surfaces of the spacer can be reacted to bind a peptide, carbohydrate, polysaccharide, lipid, nucleotide, nucleic acid, polynucleotide, or other bioactive molecule to the surface of the spacer.

In an embodiment, the functionalized surfaces of the spacer are reacted to bind one or more bioactive molecules. Preferably one or more of the bioactive molecules is a growth factor, differentiation factor, fibrous protein, and/or adhesive protein. Examples of growth factors include VEGF, bone morphogenic factor β, EGF, PDGF, NGF, FGF, IGF, or TGF. Examples of differentiation factors include neurotrophin, CSF, or TGF. The spacer can release one or more bioactive molecules. The rate of release can be determined by the rate of dissolution and/or degradation of the spacer.

iv. Multi-Layered Assembly

In an embodiment, the nanofibrillar structure can be layered to form a multi-layered nanofibrillar assembly. A diverse array of growth environments for a cell or tissue can be constructed by engineering specific chemical and physical properties into the nanofiber network, substrate, and/or spacers comprising the individual nanofibrillar structure and/or sequentially layering individual nanofibrillar structures.

Specific nano- and/or micro-environments can be engineered within individual nanofibrillar structures or within an assembly comprising two or more layered nanofibrillar structures. Physical properties and/or characteristics of individual nanofibrillar structures including, but not limited to, surface roughness, adhesivity, porosity, solidity, elasticity, geometry, interconnectivity, surface to volume ratio, fiber diameter, fiber solubility/insolubility, hydrophilicity/hydrophobicity, fibril density, and fiber orientation can be engineered to mimic the nanotopography of ECM or BM. For example, the physical and geometric properties of the nanotopography of the individual nanofibrillar structures of the assembly can be engineered to mimic the nanotopography of the ECM or BM. In an embodiment, the nanofibrillar structure comprises Ra of about 75 nm to about 300 nm; S of about 50 nm to about 600 nm, Pc of about 1 to about 10 nm; and elongation of about 100 percent to about 500 percent.

Specific recognition motifs such as peptides, polypeptides, lipids, carbohydrates, amino acids, nucleotides, nucleic acids, polynucleotides, or polysaccharides including, but not limited to, growth factors, differentiation factors, fibrous proteins, adhesive proteins, glycoproteins, functional groups, adhesive compounds, deadhesive compounds, and targeting molecules can be engineered into the nanofibrillar network, substrate, and/or spacers of the individual nanofibrillar structures or multi-layered assembly either isotropically or as gradients to promote appropriate cellular activity, including cell growth and/or differentiation. Embodiments involving amino acids, peptides, polypeptides, and proteins can include any type of such molecules of any size and complexity as well as combinations of such molecules. Examples include, but are not limited to, structural proteins, enzymes, growth factors, differentiation factors, and peptide hormones.

In an embodiment, viable cells are deposited on a nanofibrillar structure. Nano- and/or micro-environments that promote cellular activity of a particular cell or tissue can be engineered into the nanofibrillar structure by varying and/or modifying selected physical and/or chemical properties of the nanofiber network. The physical and/or chemical properties can be engineered into the individual nanofibrillar structures as described herein. The nanofibrillar structure comprising the cells is cultured under conditions that promote cellular activity, including growth and/or differentiation.

In another embodiment, two or more nanofibrillar structures are layered to form a multi-layered nanofibrillar assembly. Nano- and/or micro-environments that promote cellular activity of a particular cell or tissue can be constructed by layering nanofibrillar structures that have selected physical and/or chemical properties. The physical and/or chemical properties can be engineered into the individual nanofibrillar structures as described herein. Viable cells are deposited onto the multi-layered nanofibrillar assembly and the assembly is cultured under conditions that promote growth and/or differentiation of the deposited cells.

In another embodiment, multiple cell types are cultured on individual nanofibrillar structures under different culture conditions and then assembled, manually or mechanically, layer by layer into a specific multi-layered nanofibrillar assembly. Nano- and/or micro-environments that promote cellular activity of particular cell types can be engineered within an individual nanofibrillar structure by varying and/or modifying selected physical and/or chemical properties of the nanofibrillar structure or within the assembly by selectively layering the individual nanofibrillar structures to obtain the desired nano- or micro-environment. The physical and/or chemical characteristics can be engineered as described herein. The multi-layered nanofibrillar assembly is than cultured under conditions that promote cellular activity, including cell growth and/or differentiation.

In another embodiment, multiple cell types are cultured on individual nanofibrillar structures under different culture conditions. The physical and chemical properties of the individual nanofibrillar structures can be customized for a particular cell type. The substrate and/or spacers of the nanofibrillar structures are biodegradable and/or biodesolvable allowing for controlled release of bioactive molecules during culture. The bioactive molecules are selected to promote a desired cellular activity, including growth and/or differentiation. The individual nanofibrillar structures are then assembled, manually or mechanically, layer by layer under sterile conditions into a multi-layered nanofibrillar assembly. The multi-layered assembly can be layered to create nano- and/or micro-environments that promote a desired cellular activity, including growth and/or differentiation. Biodegradable and/or biodesolvable spacers comprising selected bioactive molecules can be inserted between the layered nanofibrillar structures comprising the assembly to fine tune nano- and/or micro-environments within the assembly. The rate of release of the bioactive molecules from the spacer can be determined by the rate of biodegradation and/or biodissolution of the polymer comprising the spacer. The assembled cellular array is than cultured under conditions that promote cellular activity, including cell proliferation and/or differentiation.

B. Methods

Another aspect of the invention includes methods for preferentially enhancing activation of Rac GTPase in a cell or tissue. The methods of the invention comprise growing the cell or tissue on a growth surface comprising biomimetic nanotopography. Growth surfaces useful in the methods of the invention are described herein. Examples of useful growth surfaces include, but are not limited to, nanofibrillar growth surface; nanofibrillar structure; glass, silicon, or plastic comprising an etched or micropatterned surface; glass, silicon, or plastic surface comprising macropores or nanopores; polymer scaffold; hydrogel; or peptide scaffold. Cells or tissue can be grown on the growth surface *in vivo*, *in vitro*, or *ex vivo* using known methods. Culture conditions for fibroblasts, kidney cells, and stem cells are described in the Examples.

In an embodiment, the methods of the invention provide a cell or tissue comprising an amount of activated Rac at least one fold greater, more preferably at least two fold greater, more preferably at least four fold greater, more preferably at least eight fold greater than the amount of activated Rac in a cell or tissue on a control surface. The control surface can be a standard glass or plastic solid planar culture surface. The Rac GTPase can be Rac 1, Rac2, Rac3, or a combination thereof. The amount of activated Rho or Cdc42 in a cell or tissue on the growth surface can be similar to or less than the amount of activated Rho or Cdc42 in the cell or tissue on the control surface. In some embodiments, activation of Rac in the cell or tissue is associated with a concomitant decrease in activation of Rho or Rho kinase.

The nanotopography of the growth surface preferentially enhances activation of Rac in cells or tissue. In an embodiment, Rac activation in the cells or tissue is sustained and does not default to a Rho or Rho kinase (ROCK) activated pathway. In an embodiment, the Rac activation is sustained for at least 6 hr. While not wishing to be bound by a particular theory, it is believed the nanotopography of the growth surface comprises physical and geometric properties that mimic the nanotopography of the ECM or BM.

In an embodiment, the growth surface comprises one or more of the following: Ra of about 13 nm to about 1200 nm; Rp of about 13 nm to about 1200 nm; Rv of about 13 nm to about 1200 nm; Rt of about 26 nm to about 2400 nm; S of about 50 nm to about 800 nm; Pc of about 1 to about 30; and elongation of about 25 percent to about 500 percent. Ra of the growth surface can be from about 13 nm to about 1200 nm, from about 50 nm to about 600 nm; from about 50 nm to about 400 nm, or from about 100 nm to about 300 nm. Rp of the growth surface can be from of about 13 nm to about 1200 nm, from about 50 nm to about 600 nm, or from about 100 nm to about 300 nm. Rv of the growth surface can be from about 13 nm to about 1200 nm, from about 50 nm to about 600 nm, or from about 100 nm to about 300 nm. Rt of the growth surface can be from about 26 nm to about 2400 nm, from about 100 nm to about 1200 nm, or from about 200 nm to about 600 nm. S of the growth surface can be from about 20 nm to about 2000 nm, from about 20 nm to about 1000 nm, from about 20 nm to about 700 nm, from about 100 nm to about 600 nm, from about 20 nm to about 400 nm, or from about 50 nm to about 200 nm. Pc of the growth surface can be from about 1 to about 30 per μm, from about 1 to about 20 per μm, from about 1 to about 10 per μm, from about 1 to about 5 per μm, or from about 5 to about 10 per μm. Elongation of the growth surface can be from about 25 percent to about 500 percent, from about 50 percent to about 200 percent, from about 100 percent to about 500 percent, or from about 100 percent to about or from about 200 percent.

In an embodiment, the growth surface comprises Ra of about 13 nm to about 1200 nm, S of about 50 nm to about 800 nm, Pc of about 1 to about 30 nm; and elongation of about 25 percent to about 500 percent. In another embodiment, the growth surface comprises Ra of about 75 nm to about 300 nm; S of about 100 nm to about 600 nm, Pc of about 1 to about 10 nm; and elongation of about 100 percent to about 500 percent.

In an embodiment, the method of the invention comprises depositing viable cells on a growth surface. Nano- and/or micro-environments that promote cellular activity of a particular cell or tissue can be engineered into the growth surface by varying and/or modifying selected physical and/or chemical properties of the growth surface, including structural and geometric properties of the nanotopography of the growth surface to preferentially enhance activation of Rac GTPase. In an embodiment, the growth surface comprises a network of one or more nanofibers. The physical and/or chemical properties of the growth surface can be engineered as described herein. The growth surface comprising the cells is cultured under conditions that promote cellular activity, including growth and/or differentiation.

In an embodiment, the method of the invention comprises layering or stacking two or more growth surfaces to form a multi-layered assembly. Nano- and micro-environments that promote cellular activity of a particular cell or tissue can be constructed by layering growth surfaces that have selected physical and/or chemical properties. In an embodiment, the growth surface is a nanofibrillar structure. The physical and/or chemical properties can be engineered into the individual growth surface of the assembly as described herein Viable cells are deposited onto the multi-layered assembly and the assembly is cultured under conditions that promote growth and/or differentiation of the deposited cells.

In another embodiment, the method of the invention comprises culturing multiple cell types on individual growth surfaces under different culture conditions and then assembling, manually or mechanically, the growth surface layer by layer under into a specific multi-layered assembly. In an embodiment, the growth surfaces are nanofibrillar structures. Nano- and/or micro-environments that promote cellular activity of particular cell types can be engineered within an individual growth surface by varying and/or modifying selected physical and/or chemical properties of the growth surface, including geometric properties of the nanotopography of the growth surface to preferentially enhance Rac activation, or within the assembly by selectively layering the individual surfaces to obtain the desired nano- or micro-environment. The physical and/or chemical characteristics can be engineered as described herein. The multi-layered assembly is than cultured under conditions that promote cellular activity, including cell growth and/or differentiation.

C. Cells

Cells useful in the system and methods of the invention include stem cells, somatic cells, committed stem cells, differentiated cells, and tumor cells. The cells can be from a mammal. The mammal can be human. The cells can be a tissue. Examples of tissue include skin, bone, liver, heart, kidney, bladder, muscle, ligament, tendon, cartilage, brain, retina, cornea, and pancreas. Examples of cells useful in the system and methods of the invention include, but are not limited to, osteoblasts, myoblasts, neurons, fibroblasts, glioblasts, germ cells, stem cells, hepatocytes, chondrocytes, keratinocytes, smooth muscle cells, cardiac muscle cells, connective tissue cells, glial cells, epithelial cells, endothelial cells, hormone-secreting cells, neurons, and lymphoid cells such as B cells, T cells, macrophages, and neutrophils. Examples of stem cells include, but are not limited to, embryonic stem cells, mesenchymal stem cells, bone marrow stem cells, and umbilical cord stem cells. The stem cells can be mammalian stem cells. In an embodiment, the stem cells are human or murine stem cells. In an embodiment, the stem cells are embryonic stem cells.

The cells can be cultured or grown *in vitro*, *in vivo*, or *ex vivo* using known methods. The cells can be derived from a natural source, genetically engineered, or produced by any other means. Any natural source of eukaryotic cells can be used. In an embodiment, the natural source is a mammal. In an embodiment, the mammal is human. In some embodiments, the cells are implanted into a mammal such as a human. The cells can be from the recipient, a nonspecific donor from the same species, or a donor from a different species. Cells harvested from a natural source and cultured prior to use are included.

The cells can be engineered to express one or more genes, repress the expression of one or more genes, or both. An example of genetically engineered cells useful in the system and methods of the present invention are cells engineered to make and secrete one or more desired bioactive molecules. When these cells are implanted in an organism, the bioactive molecules produced by the cells can produce a local effect or a systemic effect. Examples of bioactive molecules include growth factors, differentiation factors, and hormones. Examples of hormones include insulin, human growth factor, erythropoietin, thyroid stimulating hormone, estrogen, or progesterone. Cells can be engineered to produce an antigen. These cells can be implanted into an organism to produce an immune response. Cells can be engineered to produce bioactive molecules that inhibit or stimulate inflammation, facilitate healing, resist immuno-rejection, provide hormone replacement, replace neurotransmitters, inhibit or destroy cancer cells, promote cell growth, inhibit or stimulate formation of blood vessels, augment tissue, and promote or induce supplementation or replacement of skin, synovial fluid, tendons, cartilage, ligaments, bone, muscle, organs, dura, blood vessels, bone marrow, and extracellular matrix.

Genetic engineering can involve, for example, adding or removing genetic material to or from a cell, altering existing genetic material, or both using standard recombinant methods. Embodiments in which cells are transfected or otherwise engineered to express a gene can use transiently or permanently transfected genes, or both. Gene sequences can be full or partial length, cloned or naturally occurring.

D. Uses

The system and methods of the invention have many applications including cell culture, tissue culture, tissue engineering, analysis of therapeutic compounds, drug sensitivity analysis, and wound treatment. Rac signaling has been shown to play an important role in initiating cellular transformation such as morphogenesis and differentiation (Sander et al., 1999, J. Cell Biol., 147:1009-1021; Connolly et al., 2002, 13:2474-2485; Tsuji et al., 2002, J. Cell. Biol., 157:819-830). The system and methods of the invention can be used to induce *in vivo*-like cell differentiation and morphogenesis and enhance proliferation. The system and methods of the invention can be used in analysis of therapeutic compounds and drug sensitivity analysis. The growth surface of the system and methods of the invention provides an environment for the cells or tissue to more closely mimic the *in vivo* nature of the cells or tissue in an *ex vivo* environment.

Rac activated cells produced by the system and methods of the invention have many uses. Growth of cells on a growth substrate that preferentially enhances Rac activation facilitates characterizations of biochemical pathways and activities of the cells, including gene expression, receptor expression, and polypeptide production. The Rac activated cells and system of the invention can be used *in vitro*, *in vivo*, or *ex vivo* for applications including wound repair, growth of artificial skin, veins, arteries, tendons, ligaments, cartilage, heart valves, or organ cultures, tissue engineering, tissue regeneration, organ regeneration, spinal cord injury repair, treatment of burns, and bone grafts.

Some embodiments use atypical or abnormal cells such as tumor cells. Tumor cells cultured on a growth substrate that preferentially enhances Rac activation can provide more accurate representations of the native tumor environment in the body for the assessment of drug treatments. The physical and/or chemical properties of the growth surface, including nanotopography, growth factors, and differentiation factors, on which such cells are grown can be engineered to mimic the native *in vivo* nano- or micro-environment of the tumor. Growth of tumor cells on a growth substrate that preferentially enhances Rac activation facilitate characterizations of biochemical pathways and activities of the tumor, including gene expression, receptor expression, and polypeptide production, in an *in vivo*-like environment allowing for the development of drugs or therapeutic compounds that specifically target the tumor.

The system and methods of the invention can be used to grow and maintain stem cells in an undifferentiated state in the absence of a feeder cell layer on a synthetic growth substrate. Stem cells on the nanofibrillar growth substrate proliferated, self renewed, and maintained their undifferentiated state in the absence of a feeder cell layer (Examples 6-8). In an embodiment, the stem cells proliferate and maintain pluripotency. In an embodiment, the stem cells comprise human or murine stem cells. In an embodiment, the stem cells comprise embryonic stem cells. Preferably the stem cells maintain the ability to differentiate. In an embodiment, the stem cells proliferate but do not differentiate until a differentiation factor is administered to the cells. Examples of suitable differentiation factors include neurotrophin, VEGF, CSF, retinoic acid, or TGF. In an embodiment, the differentiation factor comprises retinoic acid.

Rac activated stem cells produced by the system and method of the invention have many uses including wound repair, growth of artificial skin, veins, arteries, tendons, ligaments, cartilage, or heart valves, tissue regeneration, organ cultures, treatment of burns, and bone grafts. In some embodiments, it is not necessary to pre-select the type of stem cell to be used because many types of stem cells can be induced to differentiate in an organ specific pattern by engineering the physical and/or chemical properties of the growth surface including, growth factors, differentiation factors, and surface roughness properties such as Ra, Rp, Rv, Rt, S, and Pc. In some embodiments, stem cells can be induced to proliferate in an organ specific pattern by delivering the growth surface or a multi-layered assembly to a given organ. For example, stem cells can be induced to become liver cells by growing the stem cells on a growth surface comprising nanotopography that preferentially enhances Rac activation. The growth surface can than be implanted into the liver. The stem cells on the growth surface can provide cell seeding for regenerating liver tissue or produce bioactive molecules that support regeneration of liver tissue.

Activation of Rac in cells at the site of a wound has been shown to accelerate the wound healing process in mammals (Hassanain et al., 2005, Surgery, 137:92-101). Rac activation triggers overexpression of growth factors and cytokines required for the wound repair process and is believed to induce angiogenesis at the wound and accelerate healing of the wound via cell proliferation (Hassanain et al., 2005, Surgery, 137:92-101). The system and methods of the invention are therefore useful for treating wounds including cuts, lacerations, incisions, abrasions, and penetrations of a tissue, burns, and dermal ulcers including pressure sores, venous ulcers, hemophiliac ulcers, and diabetic ulcers, wounds requiring neovascularization or re-endothelialization, and regenerating tissue at the site of a wound. The wound can be cutaneous, subcutaneous, or internal. In an embodiment, the incision is a surgical incision. In an embodiment, the tissue is vascular tissue. The vascular tissue can be skin. Assays for detecting wound-healing activity are known and described, for example, in Winter, Epidermal Wound Healing, Maibach, H. I. and Rovee, D. T., eds. (Year Book Medical Publishers, Inc., Chicago, IL) pp. 71-112; Eaglstein and Mertz, 1978, J. Invest. Dermatol., 71: 382-384; and Hassanain et al., 2005, Surgery, 137:92-101.

A dressing comprising a growth surface of the invention can be applied directly to the site of the wound. Many types of wound dressings are known. Examples of a dressing useful in the invention include, but are not limited to an adhesive absorbent dressing, antiseptic dressing, dry dressing, fixed dressing, occlusive dressing, pressure dressing, tie-over dressing, or water dressing. Methods for making a dressing for a wound are known. See, for example, Remington's Pharmaceutical Science, Gennaro et al eds., Lippincott (Philadelphia, PA). In an embodiment, the dressing can be a film, membrane, or mat comprising a network of one or more nanofibers as described herein. The nanofibers can be unwoven or net.

In an embodiment, the nanofiber network is deposited on a substrate. The substrate can be unwoven or net. In an embodiment, the nanofiber network is deposited on an unwoven cellulose material. In an embodiment, the nanofiber network is deposited on gauze. In an embodiment, the nanofiber network is deposited on a polymer film or membrane.

A dressing comprising a growth surface of the invention can be a bandage. Many types of bandages are known. Examples of a bandage useful in the invention include, but are not limited to, an adhesive bandage, gauze bandage, or roller bandage. Methods for making bandages are known. See, for example, Remington's Pharmaceutical Science, Gennaro et al eds., Lippincott (Philadelphia, PA). In an embodiment, the bandage comprises a backing, a pad comprising a growth surface of the invention, and an adhesive. The adhesive can be pressure sensitive. Adhesives, including pressure sensitive adhesives, useful for bandage applications are known. In an embodiment, the bandage comprises a backing, an adhesive on a first surface of the backing, and a pad proximate the first surface of the backing. The bandage can include a release area or liner. The adhesive on the backing can be releasably adhered to the release area or liner.

In an embodiment, the pad comprises a nanofibrillar growth surface. In an embodiment, the nanofibrillar growth surface comprises a network of one or more nanofibers as described herein, a nanofibrillar structure of the invention, or a nanofiber mat. The nanofiber network or mat can be unwoven or net. The nanofiber network can be deposited on a woven or non-woven. In an embodiment, the nanofiber network is deposited on a polymer or plastic backing. In an embodiment, the nanofiber network is deposited on a non-woven cellulose backing.

The growth surface of the dressing or bandage preferentially enhances Rac activation in cells or tissue of the wound as described herein. In an embodiment, the growth surface preferentially enhances Rac activation in a cell or tissue at the site of the wound. In an embodiment, the growth surface of the dressing or bandage comprises one or more of the following: Ra of about 13 nm to about 1200 nm; Rp of about 13 nm to about 1200 nm; Rv of about 13 nm to about 1200 nm; Rt of about 26 nm to about 2400 nm; S of about 50 nm to about 800 nm; Pc of about 1 to about 30; and elongation of about 25 percent to about 500 percent. In another embodiment, the growth surface comprises Ra of about 13 nm to about 1200 nm, S of about 50 nm to about 800 nm, Pc of about 1 to about 30 nm; and elongation of about 25 percent to about 500 percent. In another embodiment, the growth surface comprises Ra of about 75 nm to about 300 nm; S of about 100 nm to about 600 nm, Pc of about 1 to about 10 nm; and elongation of about 100 percent to about 500 percent.

Cells closing the wound can adhere to the nanofibrillar growth surface of the dressing or bandage. In order to facilitate removal of the dressing or bandage, the nanofibers can be biodegradable or biodesolvable. The nanofibers can be engineered to dissolve or degrade within a desired amount of time. In an embodiment, the nanofibers dissolve or degrade in about 7 to 14 days. In another embodiment, the nanofibers dissolve or degrade in about 2 to 5 days. In another embodiment, the nanofibers dissolve or degrade in about 2 to 3 days.

A bandage or dressing comprising a growth surface of the invention is useful for keeping a wound clean and non-infected, including wounds with a high risk of infection such as an abdominal wound. An antibiotic can be attached to growth surfaces comprising a functionalized surface. A dressing or bandage can include a system of the invention. In an embodiment, the dressing or bandage comprises one or more stem cells and a growth surface that preferentially enhances activation of Rac GTPase in the stem cells.

A dressing or bandage comprising a growth surface of the invention can be used to promote endothelialization in vascular graft surgery. In the case of vascular grafts using either transplanted vessels or synthetic material, for example, the growth surface can be applied to the surfaces of the graft and/or at the junctions of the graft and the existing vasculature to promote the growth of vascular endothelial cells. A dressing or bandage comprising a growth surface of the invention can be used to promote or induce angiogenesis at the site of the wound. For example, a dressing or bandage comprising a growth surface of the invention can be applied to full-thickness burns and injuries where angiogenesis is required to prepare the burn or injured site for a skin graft or flap. In this case the dressing or bandage is either applied directly to the site or applied to the skin or flap that is being transplanted prior to grafting. In a similar fashion, a dressing or bandage comprising a growth surface of the invention can be used in plastic surgery when reconstruction is required following a burn or other trauma, or for cosmetic purposes.

Angiogenesis is also important in keeping wounds clean and non-infected. A dressing or bandage comprising a growth surface of the invention can therefore be used in association with general surgery and following the repair of cuts and lacerations. It is particularly useful in the treatment of abdominal wounds with a high risk of infection. Neovascularization is also key to fracture repair, since blood vessels develop at the site of bone injury. Applying a bandage or dressing comprising a growth surface of the invention to the site of a fracture is therefore another utility.

For the wounds and traumatic indications referred to above, a dressing or bandage comprising a growth surface of the invention can applied to the wound consistent with good medical practice taking into account the specific type of wound to be treated, the condition of the individual patient, the site of the wound, the method of applying the dressing or bandage to the wound, and other factors known to practitioners. Antibiotics and other bioactive molecules including growth factors, differentiation factors, and adhesion factors can be engineered into the growth surface as described herein to promote wound healing including angiogenesis and cellular proliferation.

EXAMPLES

The invention is illustrated by the following Examples, which serve to exemplify the embodiments. Many variations and embodiments can be made to the disclosed invention. The Examples are not intended to limit the invention in any way.

Example 1

Physical and Structural Characteristics of Nanofibrillar Growth Surface on Coverslips

The physical and structural characteristics of the nanofibrillar growth surface on glass coverslips coated with nanofibers (ULTRAWEB™ Synthetic ECM, Part No. P607532, Donaldson, Bloomington, MN) were evaluated. The polyamide nanofibers formed a three-dimensional growth surface that had similar structural features, physical characteristics, and dimensional organization of fibers as ECM and basement membrane. The three-dimensional organization of nanofibers in the nanofibrillar growth surface was similar to the three-dimensional organization of fibers in corneal basement membranes (Abrams et al., 2000, Cell Tissue Res., 299:39-46), human cornea and sclera (Meller et al., 1997, Cell Tissue Res., 288:111-118), mammalian cornea (Hayashi et al., 2002, 254:247-258), and naturally deposited three-dimensional ECMs of NIH 3T3 fibroblasts denuded of cells (Cukierman et al., 2001, Science, 294:1708-1712).

Samples of the nanofibrillar growth surface were sputter coated with gold and examined under high-vacuum using a JEOL model JSM-5900 scanning electron (SEM) microscope. SEM analysis of the polyamide growth surface demonstrated an integrated network of overlapping nanofibers and pores (Fig. 1). The nanofibers were structurally continuous with each other at crossing points. Scanning force microscopy (SFM) imaging of the growth surface was performed in ambient air using a Digital Instruments Nanoscope IIIa operated in tapping mode with etched silicon probes, each with a nominal tip radius of curvature of 5-10 nm. Analysis of individual nanofibers from the growth surface using SFM showed a fiber with a diameter of approximately 180 nm (Fig. 2A), surface smoothness to within 5 nm over a length of 1.5 microns (Fig. 2B), and a pore diameter of approximately 700 nm (Fig. 2C). Characterization of nanofiber diameters demonstrated a distribution centered at approximately 180 nm (Fig. 3).

The Young's modulus of the polyamide used to make the nanofibers was determined. A polymer film of the same polyamide used to make nanofibers was cast out of a polymer solution under low drying rate conditions to avoid the development of cavities. A Deben 300N Tensile Tester was used to measure the physical and mechanical properties of the polyamide film. The sample dimensions were as follows: the distance between grips holding the film was 10 mm, the width of the film was 10 mm, and the thickness of the film was 25 μm. Stress-strain measurements of the polyamide film yielded a Young's modulus of 5 GPa (Fig. 4). Collagen-based tissues have been found to have similar stress-strain values (Saski et al., 1996, J. Biomech., 29:1131-1136).

Example 2

Distribution Pattern of Focal Adhesion Components in Cells Cultured on Nanofibrillar Growth Surface Indicate *In Vivo*-like Cellular Differentiation and Morphogenesis

To determine if the nanotopography of the synthetic nanofibrillar growth surface described in Example 1 is biomimetic of the ECM or basement membrane, we examined fibril matrix formation and the distribution of actin and focal adhesion components vinculin and focal adhesion kinase (FAK) in cells cultured on the nanofibrillar growth surface for distribution patterns known to correlate with cellular differentiation and morphogenesis *in vivo*.

Vinculin is a prominent component of focal complexes and focal adhesions that line the cytoskeleton, plasma membrane, and ECM (Zamir et al., 2001, J. Cell. Sci., 114:3583-3590; Geiger et al., 2001, Nat. Rev. Mol. Cell Biol., 2:793-805; Katz et al., 2000, Mol. Biol. Cell, 11:1047-1060). FAK has been proposed to function as a central mechanosensing transducer in cells (Wang et al., 2002, Proc. Natl. Acad. Sci. U.S.A., 98:11295-11300). Phosphorylation of FAK at tyrosine 397 in fibroblasts has been shown to be a key signaling event (Wang et al., 2002, Proc. Natl. Acad. Sci. U.S.A., 98:11295-11300; Schwartz et al., 2002, J. Cell Sci., 114:2553-2560). Distribution patterns for vinculin and FAK in cells *in vivo* are known. Changes in the pattern of vinculin and FAK labeling have been shown to correlate with cellular differentiation and morphogenesis *in vivo* (Wozniak et al., 2003, J. Cell Biol., 163:583-595; Cukierman et al., 2001, Science, 294:1708-1712).

Methods

NIH 3T3 fibroblasts were seeded at 5 x 10^4 cells/ml on glass coverslips (18mm, No. 1; Fisher Scientific) or glass coverslips coated with nanofibers (ULTRAWEB™ Synthetic ECM, Part No. P607532, Donaldson, Bloomington, MN) in 12 well cell culture plates (Corning, Corning, NY) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA) (4.5 g/l glucose) in the presence of 10% calf serum at 5% CO_2 and 37° C.

For analysis of the actin network, cells were rinsed once with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde in PBS (15 min), washed with PBS, treated with 0.5% Triton X-100 (5 min), washed with PBS, incubated with phalloidin-Alexa Fluor 488 (Sigma, Co. St. Louis, MO) diluted 1:100 in PBS containing 0.3% Tween-20 for 1 hr, washed with PBS (3X, 5 min per wash) and then mounted on a slide with GelMount.

For analysis of vinculin distribution, the cells were rinsed once with PBS, fixed with 4% paraformaldehyde in PBS (15 min), washed with PBS. The cells were then treated with 0.5% Triton X-100 (5 min), washed with PBS, blocked with normal goat serum (diluted with PBS/0.3 % Tween-20) for 30 min at room temperature, washed with PBS (3X, 5 min per wash), and incubated with mouse anti-human vinculin monoclonal antibody (Sigma Co., St. Louis, MO) diluted 1:400

with PBS/0.3 % Tween-20. The cells were then washed with PBS (3X, 5 min per wash), incubated for 1 hr with the secondary antibody goat anti-mouse IgG*CY3 (diluted with PBS/0.3 % Tween-20), washed with PBS (3X, 5 min per wash), and then mounted on a slide with GelMount.

For analysis of FAK distribution, the cells were rinsed once with PBS, fixed with 4% paraformaldehyde in PBS (15 min), and washed with PBS. The cells were then treated with 0.5% Triton X-100 (5 min), washed with PBS, blocked with normal donkey serum (diluted with PBS/0.3 % Tween-20) for 30 min at room temperature, washed with PBS (3X, 5 min per wash), and incubated with FAK (PY^{397}) rabbit polyclonal antibody (Biosource, Camarillo, CA) diluted 1:500 with PBS/0.3 % Tween-20. The cells were then washed with PBS (3X, 5 min per wash), incubated for 1 hr with the secondary antibody donkey anti-rabbit IgG*CY3 (diluted with PBS/0.3 % Tween-20), washed with PBS (3X, 5 min per wash), and then mounted on a slide with GelMount.

For analysis of fibril matrix formation, the cells were rinsed once with PBS, fixed with 4% paraformaldehyde in PBS (15 min), washed with PBS. The cells were then treated with 0.5% Triton X-100 (5 min), washed with PBS, blocked with normal goat serum (diluted with PBS/0.3 % Tween-20) for 30 min at room temperature, washed with PBS (3X, 5 min per wash), and incubated with monoclonal cellular fibronectin antibody (Sigma Co., St. Louis, MO) diluted 1:500 with PBS/0.3 % Tween-20. The cells were then washed with PBS (3X, 5 min per wash), incubated for 1 hr with the secondary antibody goat anti-mouse IgG*CY3 (diluted with PBS/0.3 % Tween-20), washed with PBS (3X, 5 min per wash), and then mounted on a slide with GelMount.

Imaging was performed with a Zeiss Axioplan Epi-Fluorescent Microscope. Confocal imaging was performed with a Zeiss LSM-410 microscope. Routine controls for immunohistofluorescence staining were performed.

Results

The actin, fibronectin, vinculin, and FAK data shown herein demonstrate that the three-dimensional structure and geometry of the nanofibrillar growth surface promotes differentiation and morphogenesis of cells similar to that of the ECM or basement membrane.

F-actin distribution within NIH 3T3 fibroblasts (Figs. 5A and 5B) and NRK cells (Figs. 7A and 7B) was first observed after 24 hr of growth utilizing phalloidin-

Alexa Fluor 488. As shown in Fig. 5A, fibroblasts plated on glass were well spread with an elaborate checkerboard pattern of stress fibers. In contrast, cells plated on the nanofibrillar growth surface were more elongated and bipolar with thinner actin fibers arranged parallel to the long axis of the cell. In cells grown on the nanofibrillar growth surface, a notable increase in formation of actin-rich lamellipodia, membrane ruffles, and cortical actin was observed (Fig. 5B, 17A, and 17B). Similar changes have been described for cells cultured on cell-free three-dimensional matrices derived from detergent-extracted mouse embryo sections or from naturally deposited three-dimensional ECMs of NIH 3T3 fibroblasts denuded of cells (Cukierman et al., 2001, Science, 294:1708-1712). Therefore, the nanofibrillar growth surface appears to support a more biologically relevant form of cell growth *in vitro* than do the more commonly used two-dimensional tissue culture surfaces or three-dimensional hydrogels which surround cells on all surfaces.

Vinculin staining in the fibroblasts was arranged in parallel streaks after 24 hr of cells growth on the glass coverslips (Fig. 5C). This distribution pattern is characteristic for adhering fibroblasts cultured on two-dimensional glass or tissue culture plastic (Cukierman et al., 2002, Curr. Opin. Cell Biol., 14:633-639; Zamir et al., 2001, J. Cell. Sci., 114:3583-3590; Geiger et al., 2001, Nat. Rev. Mol. Cell Biol., 2:793-805). In contrast, streaked staining for vinculin within fibroblasts cultured on the nanofibrillar growth surface was limited to the edge of lamellipodia with a more diffuse staining throughout the cell cytoplasm (Fig. 5D). Such changes in the pattern of vinculin labeling have been shown to correlate with cellular differentiation and morphogenesis *in vivo* (Wozniak et al., 2003, J. Cell Biol., 163:583-595; Deroanne et al., 2001, Cardiovasc. Res., 49:647-648).

Fibroblasts cultured on glass (Fig. 5E) demonstrated a streaky pattern of labeling which was similar to vinculin staining in fibroblasts cultured on glass (Fig. 5C). Like vinculin, the FAK PY^{397} labeling in fibroblasts cultured on glass is characteristic of localization at focal adhesions (Kisidau et al., 2002, Proc. Natl. Acad. Sci. U.S.A., 99:9996-10001; Geiger et al., 2001, Nat. Rev. Mol. Cell Biol., 2:793-805; Katz et al., 2000, Mol. Biol. Cell, 11:1047-1060).

Loss of FAK PY^{397} localization at focal adhesions has been correlated with morphogenesis and differentiation in breast epithelial cells (Wozniak et al., 2003, J. Cell Biol., 163:583-595). The localization of FAK PY^{397} for fibroblasts cultured on the nanofibrillar growth surface was more punctate and less well defined (Fig. 5F), similar to that observed for vinculin in fibroblasts cultured on the nanofibrillar growth surface (Fig. 5D). Notably, Cukierman et al. reported a loss of FAK PY^{397} staining at adhesion sites and a decrease in the amount of phosphorylated FAK for fibroblasts cultured on cell-free three-dimensional matrices derived either from detergent-extracted mouse embryo sections or from naturally deposited ECMs of NIH 3T3 fibroblasts denuded of cells (Cukierman et al., 2001, Science, 294:1708-1712).

Fibroblasts displayed a classic pattern of fibrils after 24 hr of culture on glass (Fig. 5G). In contrast, fibroblasts grown on the nanofibrillar growth surface formed a thicker network of more randomly deposited apically localized fibrils (Fig. 5H). Isolated cells were also observed to produce an intensely stained network of fibronectin fibrils after 24 hr (Fig. 5H, insert), a condition not observed after 24 hr of growth on glass (data not shown). Cells cultured on the nanofibrillar growth surface exhibited an enhanced rate of fibrillar matrix formation similar to that observed for fibroblasts cultured on naturally deposited ECMs of NIH 3T3 fibroblasts denuded of cells (Cukierman et al., 2001, Science, 294:1708-1712).

NIH 3T3 fibroblasts cultured on glass for 2 and 3 days showed increases in apical deposition of fibronectin containing fibrils while still maintaining arrays of fibrillar basal adhesions (Figs. 6A and 6C, see arrows). In contrast, fibroblasts growth on the nanofibrillar growth surface exhibited a more extensive fiber matrix secreted on the apical surface of the cells (Figs. 6B and 6D, see double arrows) with significantly fewer fibrillar adhesions on the basal surface (Figs. 6B and 6D). Staining of NIH 3T3 fibroblasts grown for 2 days on glass with anti-vinculin demonstrated a pattern of focal adhesions normally observed for cells grown on two-dimensional substrates (Fig. 6E).

In contrast, fibroblasts grown on the nanofibrillar surface demonstrated a vinculin labeling pattern that occurs predominantly in lamellipodia (Fig. 6F). This data suggests that even after 48 hrs of culture on the nanofibrillar growth surface, classical focal adhesions had not formed even in the presence of an apically localized fibrillar matrix composed of fibronectin (Figs. 6B and 6D). Similarly, an extensive network of fibronectin containing fibrils on the apical surface of the cells and the absence of classical focal adhesions was also observed when fibroblasts were cultured on naturally deposited ECMs of NIH 3T3 fibroblasts denuded of cells (Cukierman et al., 2001, Science, 294:1708-1712).

The distribution pattern of actin (Figs. 7A and 7B), vinculin (Figs. 7C and 7D), and FAK PY^{397} (Figs. 7E and 7F) for NRK cells grown on glass or the nanofibrillar growth surface closely paralleled the observations for fibroblasts. The extent of fibronectin staining appeared to be less for NRK cells than for fibroblasts on both glass and the nanofibrillar growth surface (compare Figs. 5G and 5H with Figs. 7G and 7H). Distribution of fibronectin on the NRK cell surface was distinctly different from that on fibroblasts, with a more peripheral localization for NRK cells on both glass and nanofibers (Figs. 7G and 7H). However, as with fibroblasts, there appeared to be more fibril formation for NRK cells grown on the nanofibrillar growth surface (Fig. 7H).

Example 3

Nanotopography of Nanofibrillar Growth Surface Induces Proliferation of Fibroblasts and NRK Cells

The proliferation rate of fibroblasts on naturally deposited ECMs of NIH 3T3 fibroblasts denuded of cells has been shown to be at least double the relative growth rate of fibroblasts on fibronectin (Cukierman et al., 2001, Science, 294:1708-1712) after one day of culture. To determine if the nanofibrillar growth surface induces or enhances proliferation, we cultured fibroblasts and NRK cells on glass and the nanofibrillar growth surface.

Methods

NIH 3T3 fibroblasts and NRK cells were seeded on glass coverslips (18mm, No. 1; Fisher Scientific) or glass coverslips coated with nanofibers (ULTRAWEB™ Synthetic ECM, Part No. P607532, Donaldson, Bloomington, MN) at a density of 1 x 10^5 cells/ml. Cells were incubated in DMEM containing 10% calf serum (Invitrogen, Carlsbad, CA) in an atmosphere of 95% air and 5% CO_2 at 37° C. Cell growth was evaluated using a modification of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Mosmann, 1983, J. Immunol. Methods, 65:55-63). Briefly, a sterile solution of 0.1 mg/m MTT in DMEM was added to each well in a multi-well plate and incubated for 4 hr. The MTT color complex was then solubilized in dimethyl sulfoxide (DMSO), the solution was transferred to a 96-well plate, and transmission was evaluated at 570 nm.

In order to analyze the distribution of β-1 integrins on the surface of NRK cells, the NRK cells were stained with hamster anti-rat/mouse CD29 (integrin beta 1; Flanders, NJ) IgM diluted 1:100 as described in Example 2. Imaging was performed with a Zeiss Axioplan Epi-Fluorescent Microscope. Confocal imaging was performed with a Zeiss LSM-410 microscope.

Results

Compared to the growth of fibroblasts on glass, we found that growth of fibroblasts (Fig. 8A) and NRK cells (Fig. 8B) was approximately 1.5 and 1.9 times faster on the nanofibrillar growth surface during the first 24 hr. of culture. This difference decreased after longer periods of culture and provided evidence that the nanofibrillar growth surface did not induce uncontrolled cellular proliferation.

Proliferation of human fibroblasts and endothelial cells was known to be correlated with the amount of fibrillar fibronectin that is assembled on the apical surface of cells (Bourdoulous et al., 1998, J. Cell Biol., 143:267-276). This fibrillar assembly is believed to be mediated primarily through interactions with the $\alpha5\beta1$ integrin (Schwarzbauer, 1991, J. Cell Biol., 113:1463-1473), which is the predominant $\beta1$ integrin in NRK cells (Chrzanowska-Wodnicka et al., 2001, J. Cell Sci., 114:2553-2560) and was demonstrated to be the integrin in fibroblasts that is most involved in the formation of tissue derived 3D matrices (Cukierman et al., 2001, Science, 294:1708-1712). $\beta1$ integrin was localized at classical focal adhesions in cells cultured on two-dimensional substrates and at the morphologically distinct, long and slender matrix adhesions for cells cultured on three-dimensional matrices (Cukierman et al., 2001, Science, 294:1708-1712; Cukierman et al., 2002, Curr. Opin. Cell Biol., 14:633-6300).

In the current study, staining for $\beta1$ integrin was punctate in NRK cells cultured on glass (Fig. 7I) and organized in long, slender aggregates for cells cultured on the nanofibrillar growth surface (Fig. 7J). This may reflect localization of $\beta1$ integrin in focal adhesions and matrix adhesions, respectively. There is a rapid assembly of apically localized fibronectin matrix on fibroblast cells cultured on nanofibrillar growth matrix (Fig. 5H) that is minimal for cells cultured on glass (Fig. 5G). This rapid accumulation of apical fibronectin during the first 24 hr of growth on the nanofibrillar growth matrix may trigger the larger initial proliferative response. Accordingly, our results suggest that the nanofibrillar growth surface is permissive for integrin mediated fibrillogenesis on the apical surface of cells and that apically localized $\alpha5\beta1$ integrin-fibronectin complexes may provide a signal for proliferation in the absence of classical focal adhesion complexes and stress fiber formation.

The results in Examples 2 and 3 show the nanofibrillar growth surface promotes cellular growth that is similar to growth on naturally deposited ECMs. Cukierman et al. (2001, Science, 294:1708-1712) reported observing similar cell morphology, cytoskeletal organization, focal adhesion assembly, and integrin localization for cells cultured on naturally deposited ECMs of NIH 3T3 fibroblasts denuded of cells.

Example 4

Morphogenesis of T47D Breast Epithelial Cells Cultured on the Nanofibrillar Growth Surface

T47D breast epithelial cells form duct-like tubular structures and spheroids under conditions that promote three-dimensional interactions with collagen or Matrigel™ (Schmeichel et al., 2003, J. Cell Biol., 116:2377-2388; Wozniak et al., 2003, J. Cell Biol., 163:583-595; Deroanne et al., 2001, Cardiovasc. Res., 49:647-658; Ingber et al., 1989, J. Cell Biol., 109:317-330). To determine whether the three-dimensional structure and geometry of the nanofibrillar growth surface promotes morphogenesis, we cultured T47D breast epithelial cells on glass or the nanofibrillar growth surface.

Methods

T47D breast epithelial cells were seeded at $5x10^4$ cells/ml on glass coverslips (18mm, No. 1; Fisher Scientific, Hampton, NH) or glass coverslips coated with nanofibers (ULTRAWEB™ Synthetic ECM, Part No. P607532, Donaldson, Bloomington, MN) in 12 well cell culture plates (Corning, Corning, NY) and cultured in DMEM (4.5 g/l glucose) in the presence of 10% fetal calf serum at 5% CO_2 and 37° C. At 5, 8 and 10 days post seeding, cultures were stained for F-actin. The breast epithelial cells were rinsed once with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde in PBS (15 min), washed with PBS, treated with 0.5% Triton X-100 (5 min), washed with PBS, incubated with phalloidin-Alexa Fluor 488 (Sigma, St. Louis, MO) diluted 1:100 in PBS containing 0.3% Tween-20 for 1 hr, washed with PBS (3X, 5 min per wash), and then mounted on a slide with

GelMount. Imaging was performed with a Zeiss Axioplan Epi-Fluorescent Microscope. Confocal imaging was performed with a Zeiss LSM-410 microscope.

Results

Cultures were stained with phalloidin-Alexa Fluor 488 and examined at 5, 8, and 10 days. After 5 days of growth on the nanofibrillar growth surface, we observed a mixed population of multicellular structures comprised of tubules and spheroids. A decrease in the number of tubules was observed after 8 days (data not shown). Following 10 days in culture, the multicellular spheroids were dominant (Fig. 9A-9D), although some tubules persisted (Fig. 9E). Fig. 9A-9D show a confocal series through a multicellular spheroid demonstrating a lumen. The lowest portion of the spheroid, in contact with the nanofibers (Fig. 9D), appears to have an opening. A multicellular tubule with an elongated lumen is shown in Fig. 9E. In contrast, growth of T-47D breast epithelial cells on glass demonstrated a monolayer with groups of F-actin fibers (Fig. 9F). Interestingly, while MCF-7 cells (a human breast tumor line) proliferated as a monolayer on glass (Fig. 9G), these cells grew as more complex multilayers on nanofibers (Fig. 9H). These data demonstrate that epithelial cells undergo morphogenesis when cultured on the nanofibrillar growth surface.

Example 5

Nanotopography of Nanofibrillar Growth Surface Preferentially Enhances Activation of Rac GTPase in Fibroblasts and Kidney Cells

Rho, Rac, and Cdc42 in conjunction with their downstream effectors regulate cell adhesion, cell migration, cell polarity, endocytosis, vesicle trafficking, cell cycle progression, differentiation, oncogenesis, and gene transcription (Etienne-Manneville and Hall, 2002, *Nature*, 420:629-635). Two-dimensional growth surfaces predominantly activate Rho, which induces the assembly of focal adhesions and stress fibers through its effector Rho kinase (ROCK) (Geiger *et al.*, 2001, *Nature Reviews*, 2:793-805; Burridge and Wennerberg, 2004, *Cell*, 116:167-179).

Rac signaling plays a pivotal role in initiating cellular transformations such as morphogenesis and differentiation. Global changes in cell physiology and cell-cell interactions are believed to be initiated by activation of Rac, with a concomitant decrease in the activation state of either Rho and/or ROCK (Sander et al., 1999, *J.*

Cell Biol., 147:1009-1021; Connolly *et al.*, 2002, 13:2474-2485; Tsuji *et al.*, 2002, *J. Cell. Biol.*, 157:819-830). This example illustrates preferential induction of Rac activation in cells grown on a nanofibrillar growth surface. Importantly, activation of Rac in cells grown on the nanofibrillar growth surface is sustained and the cells do not default to the Rho/ROCK regulated pathway observed in cells grown on two-dimensional growth surfaces.

Methods

NIH 3T3 fibroblasts and normal rat kidney (NRK) cells were grown on glass coverslips 18mm, No. 1; Fisher Scientific) or glass coverslips coated with nanofibers (ULTRAWEB™ Synthetic ECM, Part No. P607532, Donaldson, Bloomington, MN). The coverslips were placed in a 12-well cell culture plate (Corning, Corning, NY) and seeded with 5×10^4 NIH 3T3 cells or NRK cells per well. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA) in the presence of 10% calf serum (Invitrogen, Carlsbad, CA) at 5% CO_2 and 37°C for the times indicated.

Activation of Rho (Cat. # BK036) and Rac/Cdc42 (Cat. # BK035) in cells plated on glass or the nanofibrillar growth surface after 1 hr or 6 hr of culture was determined using a commercially available kit (Cytoskeleton, Denver, CO). Briefly, cells were washed with PBS and lysed using lysis buffer according to the manufacturer's instructions. Aliquots were taken from each cell lysate to determine total Rho, Rac and Cdc42 present in the lysate. The GTP-bound (activated) form of Rho, Rac, and Cdc42 was pulled down from the reaction mixture using the instructions and reagents provided by the manufacturer. Proteins present in total cell lysate and the Rho/Rac/Cdc42 pulled down samples were separated by SDS-PAGE (12%) and transferred onto nylon membrane. Western blotting was performed according to the ECL™ western blotting detection kit protocol (Amersham Biosciences, Piscataway, NJ) using Rac, Rho or Cdc42 antibodies (Cytoskeleton, Denver, CO). Rho, Rac and Cdc42 bands were quantified by using the Kodak Imaging Station 2000R (Eastman Kodak Co, Rochester, NY). All samples were normalized to the amount of protein in each sample as determined by a protein assay kit (Bio-Rad, Hercules, CA, Cat No. 500-0006). Densitometric analysis of the ECL exposed films was performed for NIH 3T3 and NRK cells.

Results

Growth of NIH 3T3 and normal rat kidney (NRK) cells on the nanofibrillar growth surface resulted in the preferential and sustained activation of Rac. A large increase in the amount of GTP-Rac (activated) observed in both NIH 3T3 fibroblasts and NRK cells grown on the nanofibrillar growth surface when compared to cells grown on glass only (Figs. 10, 11A, and 11B). Activation of Rac was observed after 1 hour of culture and persisted with similar intensity for at least 6 hours (Fig. 10, results for cells grown on glass are shown in the column labeled G and results for cells cultured on the nanofibrillar growth surface are shown in the column labeled N). Importantly, a two-dimensional sheet of polyamide did not support cell attachment or growth (data not shown). Unlike cells cultured on flexible two-dimensional substrates, the proliferation rate of cells cultured on the nanofibrillar growth surface was enhanced (data not shown). Therefore the three-dimensional geometry of the nanofibrillar growth surface is a prerequisite for the observed changes in Rac activation.

In contrast, the extent of Rho activation was approximately the same for NRK cells cultured on nanofibrillar and glass surfaces and only slightly elevated for NIH 3T3 fibroblasts cultured on nanofibrillar surfaces after 1 and 6 hours (Figs. 10, 11A, 11B). These results demonstrate the capacity of the nanofibrillar growth surface to preferentially enhance Rac activation (Figs. 10, 11A, 11B). Moreover, the nanofibrillar growth surface induced sustained Rac activation in the cells and the cells did not default to the Rho/ROCK regulated pathway observed for two-dimensional growth surfaces.

Example 6

Nanotopography of Nanofibrillar Growth Surface Preferentially Enhances Activation of Rac GTPase in Mouse Embryonic Stem Cells

Embryonic stem (ES) cells derived from the inner cell mass of preimplantation embryos can be grown in cell culture under conditions that maintain pluripotency and the ability to self-renew (Birdon et al., 2002, Trends Cell Biol., 12:432-438). An important component of this self-renewal signaling *in vitro* has been suggested to be the ECM produced by feeder cells (Li et al., 2002, J. Cell Biol., 157:1279-1290; Xu et al., 2001, Nat. Biotechnol., 19:971-974). In mouse embryonic stem (MES) cells, the expression of laminin and associated matrix components and their assembly into basement membrane affect the differentiation of primitive endodermal and epiblast cells (Li et al., 2004, Develop., 131:5277-5286). The expression of laminin-1 and rho kinase (ROCK) in stem cells has been associated with their differentiation into epiblasts demonstrating a connection between stem cell ECM and activation of Rho GTPases (Li et al., 2004, Develop., 131:5277-5286).

To determine the effect of the nanotopography of a growth surface on the activation states of Rho GTPases in stem cells, mouse embryonic stem (MES) cells were cultured on a synthetic nanofibrillar growth surface that mimicked the nanotopography of the ECM/basement membrane. We compared growth of MES cells cultured on a two-dimensional surface (coverslip) or three-dimensional surface (nanofibrillar growth surface) to determine whether three-dimensional structure and geometry of the growth surface have an effect on stem cell fate through the regulation of GTPase activity.

Methods

Cell culture of mouse embryonic stem (MES) cell culture. Gelatin (1%) in phosphate buffered saline (PBS) was poured onto culture plates or dishes and incubated for 30 min. Excess gelatin solution was removed from the culture plates or dishes by aspiration and rinsing with PBS. Primary mouse embryonic fibroblasts (MEF) were plated and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum in 95% air and 5% CO_2 at 37°C overnight. MES cells were suspended at a density of 10^5/ml in stem cell medium (DMEM containing fetal calf serum (5%), 2-mercaptoethanol (10 mM), nonessential amino acids (10 mM), nucleotides (2.5 mM), and LIF (10 ng/ml)) and plated onto the MEF feeder layer. The cells were incubated under conditions described above with change of media every 48 hr until the MES cells were about 80% confluent. The cells were then trypsinized (0.05% trypsin) and suspended at a density of 10^5 cells/ml (0.5 ml/well) in the stem cell medium. Glass coverslips (18mm, No. 1; Fisher Scientific) or glass coverslips coated with nanofibers (ULTRAWEB™ Synthetic ECM, Part No. P607532, Donaldson, Bloomington, MN) were placed into individual wells of a 12 well cell culture plate (Corning, Corning, NY), sterilized under ultraviolet (UV) light for 15 min, and coated with gelatin as described above. The trypsinized MES cells were seeded on feeder layer-free coverslips with and without nanofibers at a density of 1 x 10^5 cells/ml. A small amount of feeder cells (approximately 5% of the feeder layer) were transferred with the trypsinized MES cells.

Assay for pluripotency of MES cells. Pluripotency of MES cells was determined by alkaline phosphatase assay. MES cells were cultured for 3-5 days in stem cell medium to spheroidal colonies. Cells were washed with PBS and fixed with paraformaldehyde (in PBS) for 5 minutes. After washing with PBS, the fixed cells were incubated in alkaline phosphatase (AP) staining mixture (mixture of fast red violet solution with naphthol AS-BI phosphate solution and water in a 2:1:1 ratio) for 15 minutes in the dark at room temperature. The cells were than washed with PBS and pluripotent colonies were counted under a microscope (Zeiss Axioplan Epi-Fluorescent Microscope, Thornwood, NY). Pluripotent colonies appeared red while differentiated colonies were colorless.

Proliferation assay. MES cells were seeded on glass coverslips (18mm, No. 1; Fisher Scientific) or glass coverslips coated with nanofibers (ULTRAWEB™ Synthetic ECM, Part No. P607532, Donaldson, Bloomington, MN) at a density of 10^5 cells/ml. Cells were incubated in DMEM containing 5% fetal calf serum (Invitrogen, Carlsbad, CA), 2-mercaptoethanol (1X), nonessential amino acids (1X), nucleotides (1X) and lymphocyte inhibitory factor (LIF; 10 ng/ml) in an atmosphere of 95% air and 5% CO_2 at 37°C. Cell growth was evaluated by two independent methods: a) MES cells were trypsinized and the number of viable cells were counted by trypan blue exclusion method or b) MES cells were fixed and stained for alkaline phosphatase as described above. The number of colonies was counted using fluorescent microscopy. Colony areas were determined for 50 colonies using the NIH Image J program.

Rho/Rac/Cdc42 pull down assay. Separate cultures of MES cells and MEF cells were trypsinized and suspended at a density of 5×10^5 cells per milliliter in the stem cell medium described above. 1×10^6 cells were poured onto glass or nanofiber coated glass cover slips placed in 12 well culture plate (Corning, Corning, NY). The cells were allowed to attach to the cover slips by incubating for 30 min. Stem cell medium was then added to each well of 12 well culture plate and incubated under standard cell culture conditions at 37° C.

Pull-down assays to determine GTPase activation were performed after 16 hours of culture. Activation of Rho and Rac/Cdc42 was determined as described in Example 4.

Western blotting. MES cells were seeded on glass or the nanofibrillar growth surface at a density of 10^5 cells/ml. Cells were incubated in DMEM containing 5% fetal calf serum, 2-mercaptoethanol (1X), nonessential amino acids (1X), nucleotides (1X) and LIF (10 ng/ml) in an atmosphere of 95% air and 5% CO_2 at 37°C for 24 hrs. The cells were then lysed in Lammeli SDS sample buffer and proteins were separated by SDS-PAGE (12%) and transferred onto a nylon membrane. Western blotting was performed according to the ECL protocol provided by the suppliers Amersham Biosciences (Buckinghamshire, UK) using specific antibodies.

Results

Growth of MES cells on the nanofibrillar growth surface resulted in the preferential activation of Rac. Compared to MES cells grown on glass, Rac activity was significantly higher in MES cells cultured on the nanofibrillar growth surface (Figs. 12 and 13; results for cells cultured on glass are shown in the column labeled "2D" and results for cells cultured on the nanofibrillar growth surface are shown in the column labeled "3D"). In contrast to MES cells grown on glass, there was only a mild activation of Rho and Cdc42 for cells cultured on 3D surfaces (Figs. 12 and 13).

We attempted to determine the activation of Rac, Rho, and Cdc42 in MEF cells at a concentration approximately equivalent to the number of MEF cells (approximately 5% of the feeder layer) transferred with the MES cells plated on the nanofibrillar growth surface. The activity of Rac, Rho, and Cdc42 I in the MEF cells was below the levels of detection for the pull down assay (data not shown). This suggests that the observed activation of Rac was a result of activation within the stem cells rather than activation within the transferred feeder fibroblasts.

Growth of MES cells on the nanofibrillar growth surface correlated with enhanced proliferation and self-renewal. MES cells were cultured for 1-3 days on glass coverslips or nanofiber coated coverslips and examined for expression of alkaline phosphatase, a marker for undifferentiated stem cells. MES cells cultured on glass had small colonies of undifferentiated cells (Fig. 14). In contrast, MES cells cultured on the nanofibrillar growth surface had significantly larger colonies

(Fig 15). Measurements of colony area for 50 colonies after three days of culture showed the mean colony area of cells grown on glass was 0.383±0.192 units, while the mean colony area for cells grown on the nanofibrillar growth surface was 1.056±0.352 units. These numbers are consistent with measurements of cell number shown in Fig. 16.

It is important to emphasize the MES cells proliferated in the presence of less than 5% of the dense layer of mouse embryonic fibroblasts normally used as a feeder layer. These results suggest that the nanofibrillar growth surface can, for at least 3 days, compensate for the absence of these feeder cells. Growth of the MES cells on glass, a two-dimensional growth surface, did not perform the same synergistic or replacement function. The activation of Rac in MES cells and the rapid proliferation and self renewal of MES cells on the nanofibrillar growth surface suggests a role for Rac in maintaining the undifferentiated state of MES cells during cell proliferation.

Example 7

Increased Rac Activation Enhances the Growth of MES Cells On the Nanofibrillar Growth Surface

In order to determine whether the increase in Rac activation was causally related to the enhanced rate of proliferation and self-renewal in MES cells, we transfected the MES cells with a dominant negative Rac mutant (dnRac) or a constitutively active Rac mutant (caRac) and cultured the transfected MES cells on the nanofibrillar growth surface.

Methods

Transfection of Rac mutants. MES cells were seeded onto glass coverslips with or without the nanofibrillar growth surface as described in Example 6 and incubated overnight in 5% CO_2 at 37°C. The MES cells were transfected with a plasmid containing a dominant negative Rac mutant (dnRacN17) or a constitutively active Rac mutant (caRacQL) (Sachdev et al., 2001, *J. Biol. Chem.*, 276:26461-26471). Each vector was complexed with 2 μg of Cellfectin (Invitrogen, Carlsbad, CA) and the MES cells were transfected according to the manufacturer's instructions. Medium was replaced after 16 hr and incubation was continued for another 72 hr. The MES cells were then fixed with 4% paraformaldehyde and stained for alkaline phosphatase as described above. The number of alkaline phosphatase positive colonies was counted in 25 random fields using a Zeiss Axioplan Epi-Fluorescent Microscope.

Results

As shown in Fig. 18, the number of colonies for MES cells transfected with a control plasmid vector was greater on the nanofibrillar growth surface compared to glass only. Compared to the control, the number of colonies growing on the nanofibrillar growth surface was reduced for MES cells transfected with dominant negative Rac (dnRac) and slightly enhanced for MES cells transfected with constitutively active Rac (caRac). Both dnRac and caRac had little effect when expressed in MES cells grown on glass only. These results indicate an essential role for Rac in the proliferation of MES cells cultured on the nanofibrillar growth surface.

Example 8

Nanog Expression is Enhanced in MES Cells Cultured on the Nanofibrillar Growth Surface and is Dependent on the PI3K Signaling Pathway

Several investigations have demonstrated a role for phosphoinositide 3-kinase (PI3K) as a downstream effector in the LIF/gp130 pathway for self-renewal in MES cells (Paling et al., 2004, *J. Biol. Chem.*, 279:48063-48070; Jirmanova et al., 2002, *Oncogene*, 21:5515-5528). In particular, both the regulation of STAT3 and ERKs by PI3K has been implicated in the control of pluripotency and self-renewal (Paling et al., 2004, *J. Biol. Chem.*, 279:48063-48070; Jirmanova et al., 2002, *Oncogene*, 21:5515-5528). Activation of PI3K/AKT is a necessary step for activating the phosphoinositide 3-kinase pathway. In this example, we examined whether the nanofibrillar growth surface activates PI3K/AKT; whether the nanofibrillar growth surface induces expression of Nanog, a homeoprotein whose expression is required for stem cell self-renewal and maintenance of pluripotency; and whether inhibitors of PI3K reduced the expression of Nanog.

Methods

Inhibitor experiments. MES cells or MEF cells were seeded onto glass coverslips with or without the nanofibrillar growth surface at a density of 10^5 cells/ml as described in Example 6 and incubated in stem cell medium at 37° C for

24 hr. Wortmannin (0, 50, or 100 nM), caffeine (0, 0.25, 0.5, or 1 mM), or retinoic acid (0, 17, 35, or 70 nM) was added to the medium, and the cells were incubated for 24 hr. The cells were then lysed in Lammeli SDS sample buffer. Proteins were separated by SDS-PAGE (12%) and transferred onto a nylon membrane. Western blotting was performed as described in Example 6.

Induction of differentiation. MES cells were seeded onto glass coverslips with or without the nanofibrillar growth surface at a density of 10^5 cells/ml and incubated for 24 hr as described above. The cells were treated with dimethylsulfoxide (DMSO), Wortmannin (100 nM), caffeine (1 mM), or retinoic acid (70 nM) for another 72 hr. Induction of differentiation-inducible gene expression was determined by the semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Cells were trypsinized and collected by centrifugation at 5,000 rpm for 5 min. The cell pellet was lysed, and total RNAs were isolated using a TRIzol kit (Invitrogen Life Technologies, Carlsbad, CA), followed by cleanup with an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Expression of mRNA was determined using the Superscript^{TM} III First-Strand Synthesis System with platinum^{R} Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA) according to the supplier's instructions. For semi-quantitation, RT-PCR was programmed for 28 cycles of amplification. The following primers were used for RT-PCR: GFAP (forward, 5' tct ccc tgt ctc gaa tga ct 3' (SEQ ID NO:9), and reverse, 5' gcc ggg cac tgt tgg ccg ta 3' (SEQ ID NO:10)); nestin (forward, 5' atg ggg acg agg atc aag 3' (SEQ ID NO:11), and reverse, 5' gtg agc cac aga aga aag 3' (SEQ ID NO:12)); nanog (forward, 5' ctc aag gac agg ttt cag a 3' (SEQ ID NO:13), and reverse, 5'ggt gct gag ccc ttc tga a 3' (SEQ ID NO:14), and actin (forward, 5'cgg ctt cgc ggg cga cga tg 3' (SEQ ID NO:15), and reverse, 5' tct tgc tct ggg cct cgt c 3' (SEQ ID NO:16)). The PCR products were characterized by 1.5% agarose gel electrophoresis.

Results

As shown in Figure 19A and B, culture of MES cells on the nanofibrillar growth surface enhanced phosphorylation of AKT, the downstream kinase substrate of PI3K (Fig. 19A), and enhanced expression of Nanog (Fig. 19B) when compared to MES cells cultured on uncoated glass surfaces. Expression levels of P-AKT (Fig. 19A) and Nanog (Fig. 19B) were normalized to β-actin expression levels.

Importantly, enhanced phosphorylation of AKT was not observed for feeder fibroblasts cultured on the nanofibrillar growth surface in the absence of stem cells (Fig. 19A). The PI3K inhibitors Wortmannin and caffeine decreased the expression of Nanog (Figs. 19C and D, respectively).

Retinoic acid has been shown to induce differentiation of embryonic stem cells (Anneren et al., 2004, *J. Biol Chem.*, 279: 31590-31598). We found that retinoic acid inhibited expression of Nanog in MES cells cultured on the nanofibrillar growth surface (Fig. 19E). Using RT-PCR, it was found that the PI3K inhibitors Wortmannin and caffeine as well as retinoic acid inhibited transcription of the Nanog gene (Fig. 19F). These data suggest a link between Rac activation, PI3K activity, and the expression level of Nanog in MES cells cultured on the nanofibrillar growth surface

Although Nanog expression is essential for maintaining the undifferentiated state of stem cells, it apparently is not involved in the proliferation of stem cells with self-renewal (Chambers et al., 2003, *Cell*, 113: 643-655). c-Fos is an important regulator of cell growth and proliferation (Ransone and Verma, 1990, *Annu. Rev. Cell Biol.*, 6: 539-557). To examine a putative signaling target of the PI3K and Rac pathways that could influence proliferation, we examined the expression of c-Fos in MES cells grown on glass or the nanofibrillar growth surface. It has previously been shown that differentiation of MES cells inhibits c-Fos expression (Smith et al., 2004, *J. Cell Biol.*, 164:689-699). We therefore hypothesized that the level of c-Fos would be higher for cells grown on the nanofibrillar growth surface compared to cells grown on standard glass tissue culture surfaces. As shown in Figure 19G, c-Fos expression was indeed enhanced in MES cells cultured on the nanofibrillar growth surface, whereas enhancement of c-Fos expression was not observed for feeder fibroblasts alone. Expression levels of c-Fos were normalized to β-actin expression levels.

Example 9

MES Cells Cultured on the Nanofibrillar Growth Surface Retain the Ability to Differentiate

Previous studies have shown that treatment of MES cells with retinoic acid induces differentiation of embryonic stem cells into neuronal precursor cells as evidenced by up-regulation of glial fibrillary acidic protein (GFAP) and nestin (Geschwind et al., 2001, *Neuron*, 29:325-339; Bibel et al., 2004, *Nat. Neurosci.*, 7:1003-1009; Zhou et al., 2005, *J. Huazhong Univ. Sci. Technol. Med. Sci.*, 25:13-16). Utilizing semi-quantitative RT-PCR, we studied the expression of GFAP and nestin in MES cells to determine whether MES cells cultured on the nanofibrillar growth surface retain the ability to differentiate when exposed to differentiating factors.

Methods

Induction of differentiation. MES cells were seeded onto glass coverslips with or without the nanofibrillar growth surface at a density of 10^5 cells/ml and incubated for 24 hr as described above. The cells were treated with retinoic acid (70 nM) for another 72 hr. Induction of differentiation-inducible gene expression was determined by the semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Cells were trypsinized and collected by centrifugation at 5,000 rpm for 5 min. The cell pellet was lysed, and total RNAs were isolated using aTRIzol kit (Invitrogen Life Technologies, Carlsbad, CA), followed by cleanup with a RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Expression of mRNA was determined using the Superscript^{TM} III First-Strand Synthesis System with platinum^{R} Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA) according to the supplier's instructions. For semi-quantitation, RT-PCR was programmed for 28 cycles of amplification. The following primers were used for RT-PCR: GFAP (forward, 5' tct ccc tgt ctc gaa tga ct 3' (SEQ ID NO:9), and reverse, 5' gcc ggg cac tgt tgg ccg ta 3' (SEQ ID NO:10)); nestin (forward, 5' atg ggg acg agg atc aag 3' (SEQ ID NO:11), and reverse, 5' gtg agc cac aga aga aag 3' (SEQ ID NO:12)); nanog (forward, 5' ctc aag gac agg ttt cag a 3' (SEQ ID NO:13), and reverse, 5'ggt gct gag ccc ttc tga a 3' (SEQ ID NO:14)), and actin (forward, 5'cgg ctt cgc ggg cga cga tg 3' (SEQ ID NO:15), and reverse, 5' tct tgc tct ggg cct cgt c 3' (SEQ ID NO:16)). The PCR products were characterized by 1.5% agarose gel electrophoresis.

Results

Treatment with retinoic acid induced the expression of GFAP and nestin (Fig. 20) in MES cells cultured on glass or the nanofibrillar growth surface. Levels of GFAP and nestin transcripts were normalized to β-actin transcript levels. Importantly, GFAP and nestin were not detected in MES cells not treated with retinoic acid (Fig. 20). These results indicate MES cells cultured on the nanofibrillar growth surface maintain their ability to differentiate in the presence of differentiating factors.

Example 10

Nanofibrillar Growth Surface Coated with Laminin-1 Induced Differentiation of MES Cells

Up-regulation of nestin has been correlated with differentiation of embryonic stem cells into neuronal precursor cells (Bibel et al., 2004, *Nat. Neurosci.*, 7:1003-1009; Zhou et al., 2005, *J. Huazhong Univ. Sci. Technol. Med. Sci.*, 25:13-16). Utilizing semi-quantitative RT-PCR, we studied the expression of nestin in MES cells grown on a nanofibrillar growth surface coated with laminin-1, an extracellular matrix (ECM) molecule.

Methods

Laminin-1 was dissolved in Hank's balanced salt solution (HBSS) at a concentration of 10 μg per ml. Five hundred μl of the laminin-1 solution was added to glass coverslips with the nanofibrillar growth surface (500 μl in each well of a 24 well plate) and incubated overnight at 37 °C. MES cells were than seeded onto glass coverslips with a coated or uncoated nanofibrillar growth surface at a density of 10^5 cells/ml and incubated for 24 hr as described above. Nestin expression in MES cells cultured on laminin-1 coated nanofibrillar growth surfaces or uncoated nanofibrillar growth surfaces were determined by RT-PCR as described in Example 9.

Results

Laminin-1 coated nanofibrillar growth surfaces induced the differentiation of MES cells. Nestin expression was up regulated in MES cells grown on nanofibrillar growth surfaces coated with laminin-1 (Fig. 21). Nestin expression was not detected in MES cells grown on uncoated nanofibrillar growth surfaces (Fig. 21). These results indicate that ECM molecules can be attached to the nanofibrillar growth surface by adsorption and that the attached ECM molecules can modulate the differentiation and/or proliferation of MES cells cultured on the nanofibrillar growth surface.

All publications and patent applications in this specification are indicative of the level of ordinary skill in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference.

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications can be made while remaining within the spirit and scope of the invention.

WHAT IS CLAIMED IS:

1. A system, comprising one or more cells and a growth surface, wherein the growth surface comprises an average surface roughness of about 13 nm to about 1200 nm and preferentially enhances activation of Rac GTPase in the cells.

2. The system of claim 1, wherein activation of Rac GTPase is sustained.

3. The system of claim 1 or 2, wherein activation is sustained for at least about 6 hr.

4. The system of any one of claims 1-3, wherein Rac GTPase activation is at least 2 fold greater than Rho GTPase activation.

5. The system of any one of claims 1-4, wherein the Rac GTPase is Rac1, Rac2, and/or Rac3.

6. The system of any one of claims 1-5, wherein the system is implantable.

7. The system of any one of claims 1-6, wherein the growth surface is biodegradable.

8. The system of any one of claims 1-7, wherein the cells are embryonic cells, epithelial cells, endothelial cells, mesenchymal cells, neurons, muscle cells, stem cells, or lymphoid cells.

9. The system of claim 8, wherein the stem cells comprise embryonic stem cells.

10. The system of any one of claims 1-9, wherein the growth surface comprises one or more of the following:

(a) a maximum peak height (Rp) of between about 13 nm and 1200 nm;

(b) average spacing between peaks (S) of about 20 nm to about 700 nm;

(c) a peak count (Pc) of about 1 to about 30 peaks per μm; or

(d) elongation of about 25 percent to about 500 percent.

11. The system of claim 1, wherein the Ra is about 50 nm to about 400 nm.

12. The system of claim 1, wherein the Ra is about 100 nm to about 300 nm.

13. The system of claim 10, wherein the Rp is between about 50 nm and about 600 nm.

14. The system of claim 10, wherein S is about 100 nm to about 600 nm.

15. The system of claim 10, wherein Pc is about 1 to about 10 peaks per μm.

16. The system of claim 10, wherein elongation is about 100 percent to about 500 percent.

17. The system of any one of claims 1-16, wherein the growth surface comprises an etched surface, micropatterned surface, hydrogel, peptide scaffold, a polymer scaffold, or fiber surface.

18. The system of claim 17, wherein the growth surface is glass, silicon, or plastic.

19. The system of claim 17 or 18, wherein the surface is micropatterned with one or more extracellular matrix ligands.

20. The system of claim 17, wherein the peptide scaffold comprises a self-assembling peptide hydrogel.

21. The system of claim 17, wherein the polymer scaffold comprises a sponge.

22. The system of claim 17, wherein the fiber surface comprises a network of one or more nanofibers.

23. The system of claim 22, wherein the network comprises a fiber diameter of about 30 nm to about 1200 nm, an average interfiber spacing of about 100 nm to about 600 nm, and solidity of about 70% or less.

24. The system of claim 23, wherein the fiber comprises Ra of about 0.01 nm to about 5 nm.

25. The system of any one of claims 1-16, wherein the growth surface comprises a nanofibrillar structure having one or more nanofibers.

26. The system of claim 25, wherein the nanofibrillar structure comprises a fiber diameter of about 30 nm to about 1200 nm, an average interfiber spacing of about 100 nm to about 600 nm, and a solidity of about 70 percent or less.

27. The system of claim 25 or 26, wherein the nanofibrillar structure comprises a solidity of about 50 percent or less, about 30 percent or less, about 10 percent or less, or about 5 percent or less.

28. The system of any one of claims 25-27, wherein the nanofibrillar structure comprises one or more growth factors, growth inhibitors, differentiation factors, differentiation inhibitors, adhesive compounds, deadhesive compounds, targeting compounds, or a combination thereof.

29. The system of any one of claims 25-27, wherein the growth surface comprises two or more nanofibrillar structures layered to form a multilayered assembly.

30. The system of 29, wherein the multilayered assembly comprises a spacer having a thickness and a first and second surface wherein the first surface of the spacer contacts a surface of a first nanofibrillar structure and a second surface of the spacer contacts a surface of a second nanofibrillar structure such that the first and second nanofibrillar structures are separated by the thickness of the spacer.

31. The system of claim 29 or 30, wherein the growth factors, growth inhibitors, differentiation factors, differentiation inhibitors, adhesive compounds, deadhesive compounds, or targeting compounds of each individual nanofibrillar structure are selected to promote or inhibit the growth or differentiation of a specific cell type.

32. The system of any one of claims 28-31, wherein the nanofibrillar structure releases one or more growth factors, one or more differentiation factors, or combinations thereof.

33. The system of claim 32, wherein a rate of release is determined by a rate of dissolution or degradation of the nanofiber or substrate.

34. The system of any one of claims 28-33, wherein at least one of the growth factors is vascular endothelial growth factor, bone morphogenic factor β, epidermal growth factor, endothelial growth factor, platelet-derived growth factor, neural growth factor, fibroblast growth factor, insulin growth factor, or transforming growth factor.

35. The system of any one of claims 28-33, wherein at least one of the differentiation factors is neurotrophin, colony stimulating factor, or transforming growth factor.

36. The system of any one of claims 25-35, wherein the growth surface is porous plastic.

37. The system of any one of claims 25-35, wherein the growth surface is a film.

38. The system of claim 37, wherein the film is water soluble, biodegradable, biocompatible, porous, or a combination thereof.

39. The system of claim 37 or 38, wherein the film comprises polychlorotrifluoroethylene, polystyrene, polymethylpentene, or polycycloolefin.

40. The system of claim 37 or 38, wherein the film is a polyvinyl alcohol film.

41. The system of any one of claims 25-35, wherein the substrate is non-porous glass.

42. The system of any one of claims 22-41, wherein the nanofiber comprises a non-cytotoxic polymer.

43. The system of claim 42, wherein the polymer is biodegradable and/or water insoluble

44. The system of claim 42 or 43, wherein the polymer is polyester, polytetrafluoroethylene (PTFE), polyethylene, polypropylene, poly(4-methyl-1-pentene) (PMP), polyoxymethylene (POM), poly(vinyl fluoride) (PVDF), or polyamide.

45. The system of claim 44, wherein the polyester is polyglycolate or polylactate.

46. The system of claim 44, wherein the polyamide is a nylon.

47. The system of claim 46, wherein the nylon is poly epsilon caprolactone.

48. A mammal comprising the system of any one of claims 1-47.

49. A Rac activated cell isolated from the system of any of claims 1-47.

50. An isolated Rac activated cell, wherein Rac GTPase was preferentially enhanced by growing the cell on a growth surface as defined in any one of claims 10-47.

51. The cell of claim 50, wherein the cell is an embryonic cell, epithelial cell, endothelial cell, mesenchymal cell, neuron, muscle cell, stem cell, or lymphoid cell.

52. The cell of claim 51, wherein the stem cell comprises an embryonic stem cell.

53. A mammal comprising the cell of any one of claims 50-51.

54. A method of preferentially enhancing activation of Rac GTPase in a cell, comprising growing the cell on a growth surface as defined in any one of claims 10-47.

55. The method of claim 54, wherein the Rac GTPase is Rac1, Rac2, and/or Rac3.

56. The method of claim 54 or 55, wherein activation of Rac GTPase is sustained.

57. The method of claim 56, wherein activation is sustained for at least about 6 hr.

58. The method of any one of claims 54-57, wherein Rac GTPase activation is at least 2 fold greater than Rho GTPase activation.

59. The method of any one of claims 54-58, wherein the growth surface comprises a network of one or more fibers wherein fiber(s) forms pores and thickness, wherein the fibers have a diameter of about 13 nm to about 1200 nm, the pores have a diameter of about 100 nm to about 600 nm, and the network thickness is about 20 nm to about 2000 nm.

60. A dressing for a wound, comprising a growth surface as defined in any one of claims 10-47.

61. The dressing of claim 60, wherein the growth surface preferentially enhances Rac activation of a cell or tissue of the wound.

62. The dressing of claim 60 or 61, wherein the dressing is an adhesive dressing.

63. The dressing of any one of claims 60-62, wherein the growth surface comprises a nanofibrillar growth surface comprising a fiber diameter of about 30 nm

to about 1200 nm, an average interfiber spacing of about 100 nm to about 600 nm, and solidity of about 70% or less.

64. A method for treating a wound, comprising applying a dressing of any one of claims 60-63 directly to the wound.

65. The method of claim 64, wherein the wound comprises a cut, laceration, incision, abrasion, burn, dermal ulcer, or penetration of a tissue.

66. The method of claim 65, wherein the tissue is vascular tissue.

67. The method of claim 65, wherein the tissue is skin.

68. The method of claim 65, wherein the incision is a surgical incision.

69. The method of claim 65, wherein the dermal ulcer comprises a pressure sore, venous ulcer, hemophiliac ulcer, or diabetic ulcer.

70. A method of inducing proliferation of undifferentiated stem cells, comprising culturing the stem cells on a synthetic growth surface as defined in any one of claims 10-47, wherein the proliferating stem cells maintain an undifferentiated state.

71. The method of claim 70, wherein the stem cells maintain the ability to differentiate.

72. The method of claim 71, wherein a differentiation factor induces differentiation of the stem cells.

73. The method of claim 72, wherein the differentiation factor comprises retinoic acid.

74. The method of any one of claims 70-73, wherein the stem cells comprise embryonic stem cells.

75. The method of any one of claims 70-74, wherein activation of Rac GTPase is preferentially enhanced in the stem cells.

76. The method of claim 75, wherein Rac GTPase activation is sustained.

77. The method of any one of claims 70-76, wherein the growth surface comprises a nanofibrillar growth surface comprising fiber diameter of about 30 nm to about 1200 nm, an average interfiber spacing of about 100 nm to about 600 nm, and solidity of about 70% or less.

Fig. 1

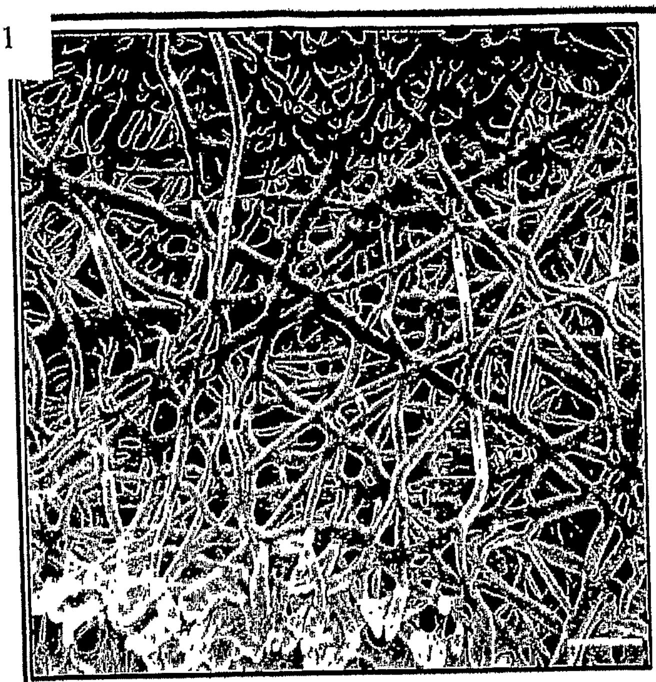


Fig. 2A

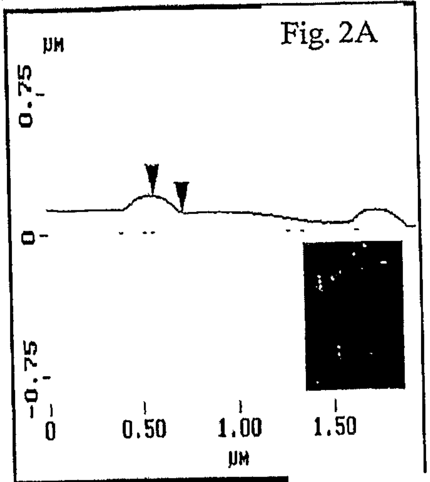


Fig. 2B

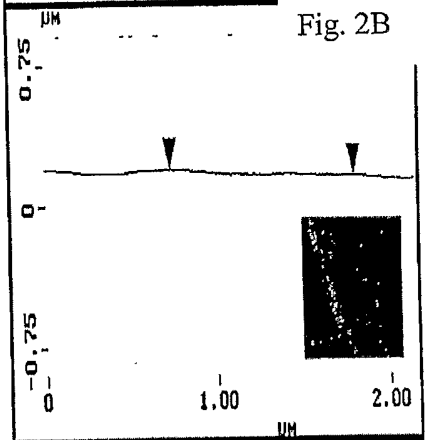


Fig. 2C

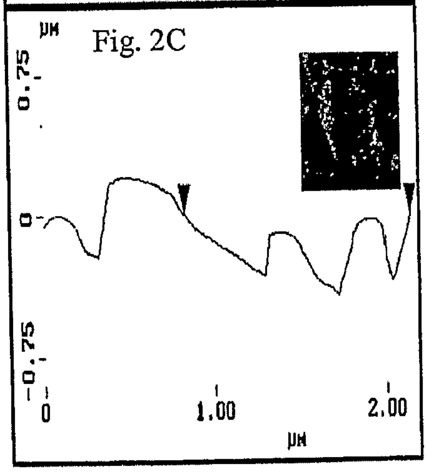


Fig. 3

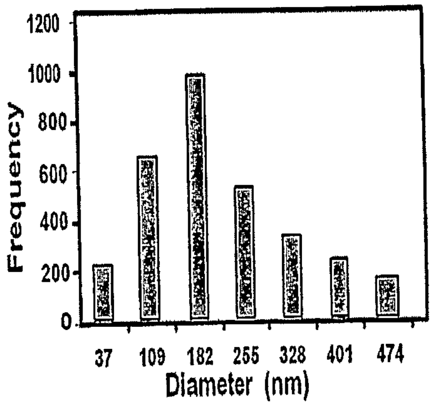


Fig. 4

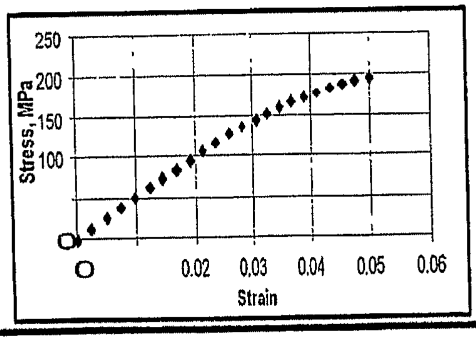



Fig. 5A


Fig. 5B

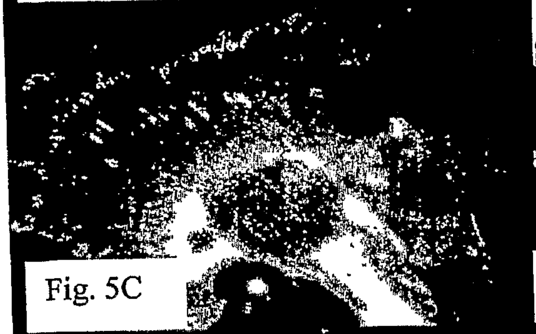


Fig. 5C


Fig. 5D

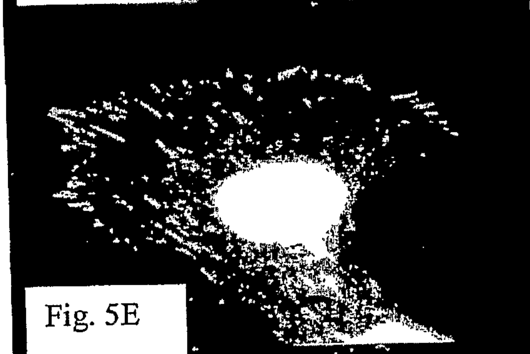


Fig. 5E

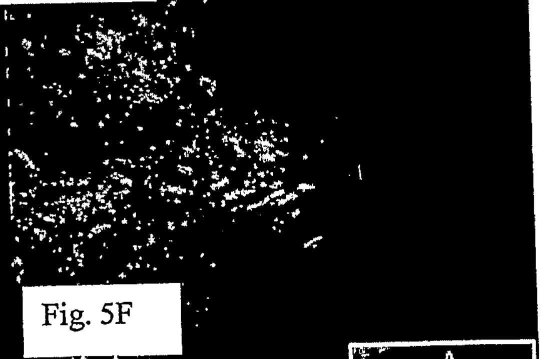


Fig. 5F

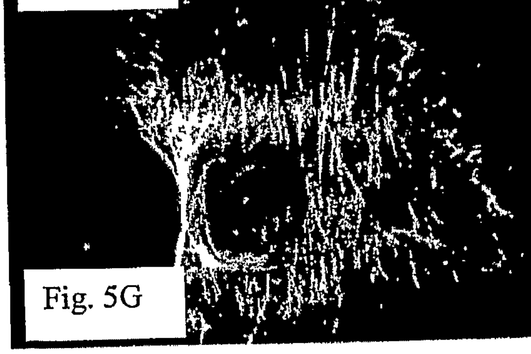


Fig. 5G

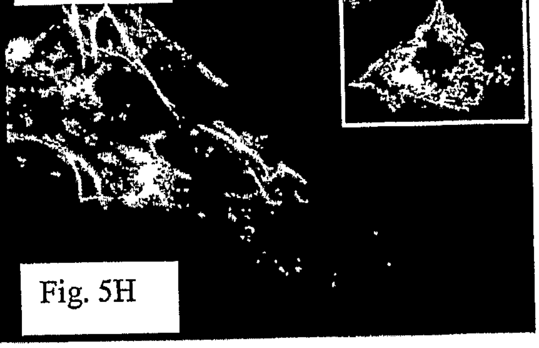


Fig. 5H

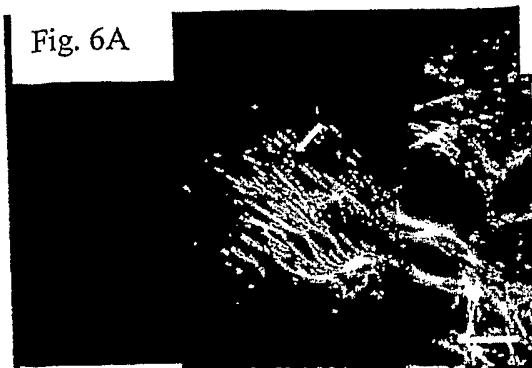
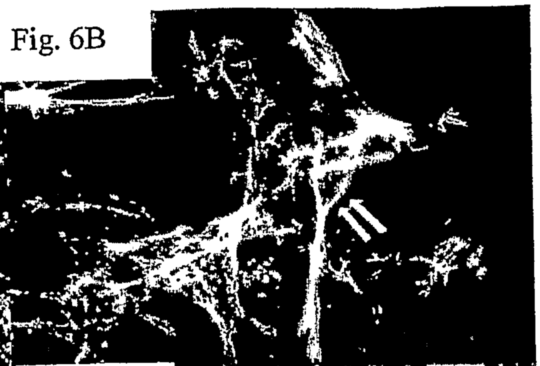


Fig. 6A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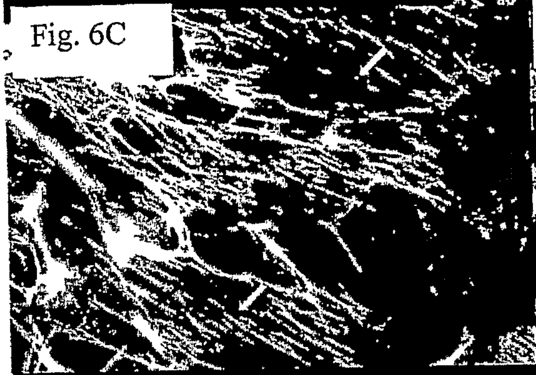


Fig. 6C

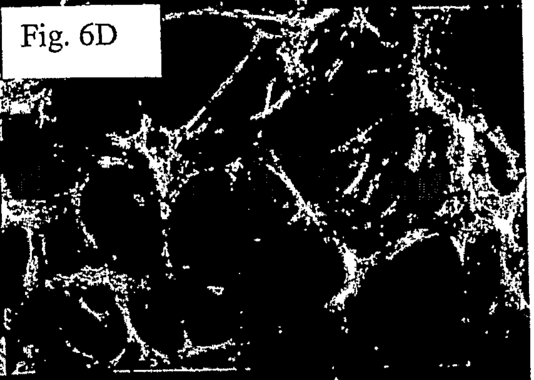


Fig. 6D


Fig. 6E

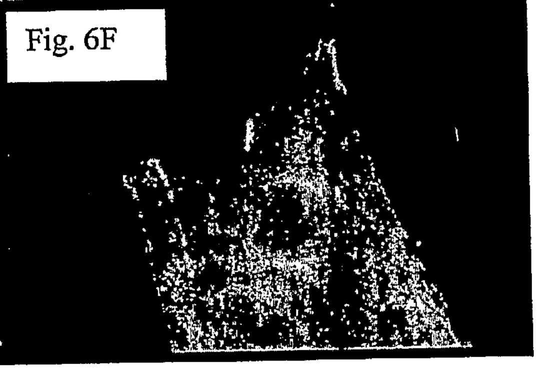


Fig. 6F

Fig. 7A

Fig. 7B

Fig. 7C

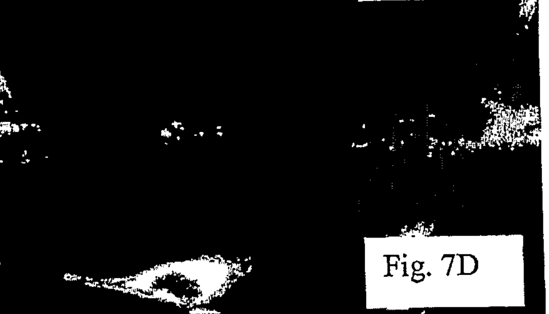


Fig. 7D

Fig. 7E

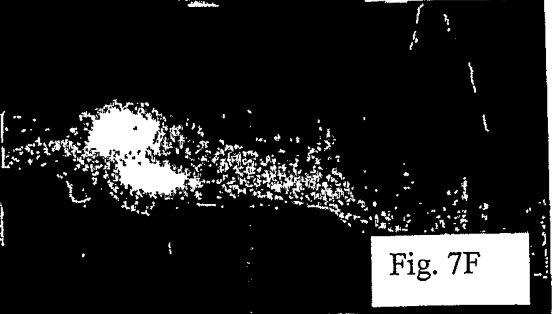


Fig. 7F

Fig. 7G

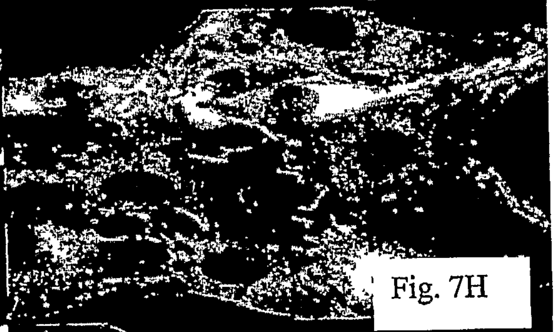


Fig. 7H

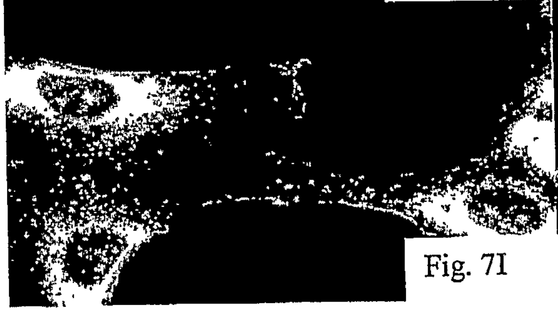


Fig. 7I

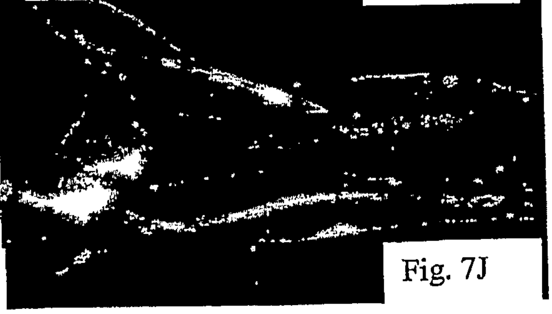


Fig. 7J

Figure 8A

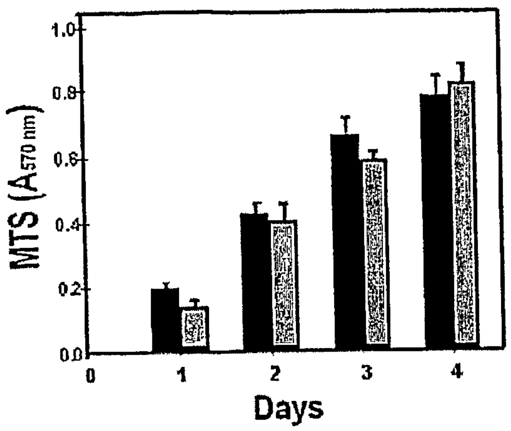
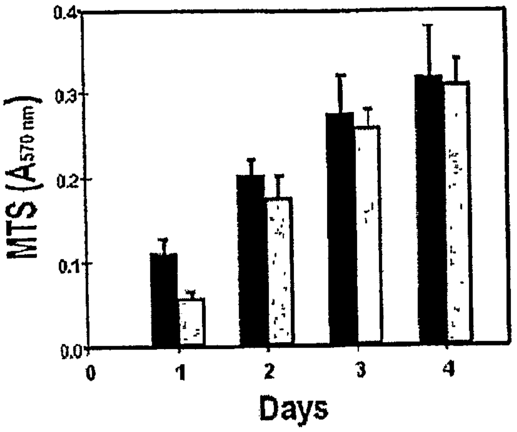


Figure 8B



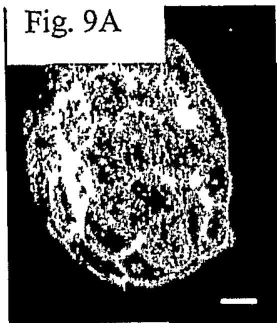


Fig. 9A

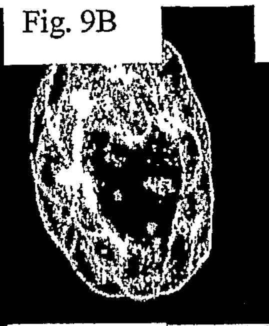


Fig. 9B

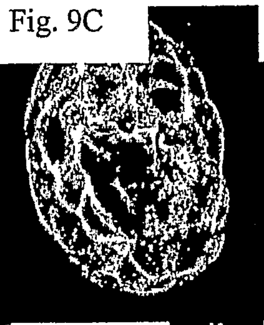


Fig. 9C

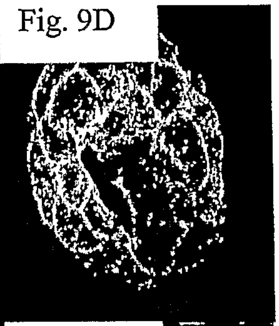


Fig. 9D

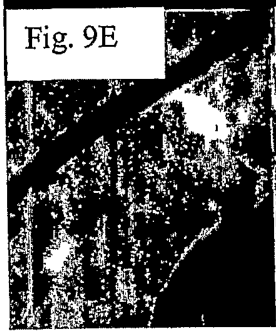


Fig. 9E

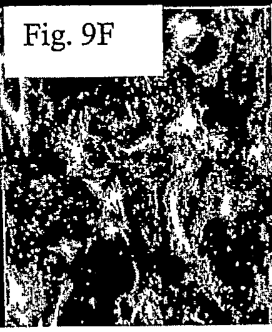


Fig. 9F

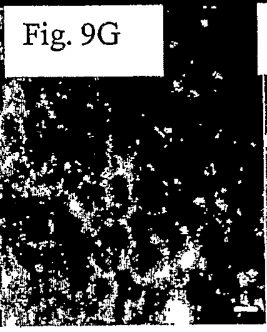


Fig. 9G

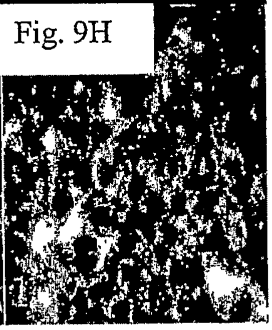
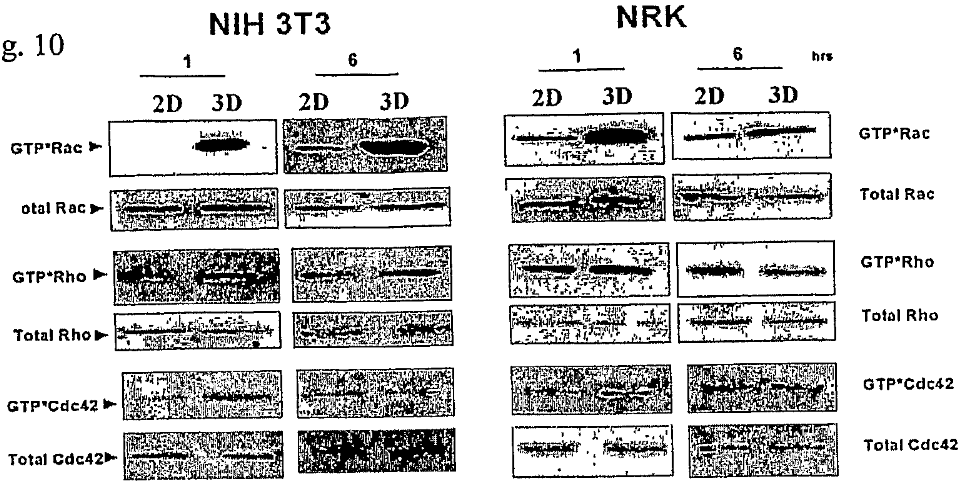
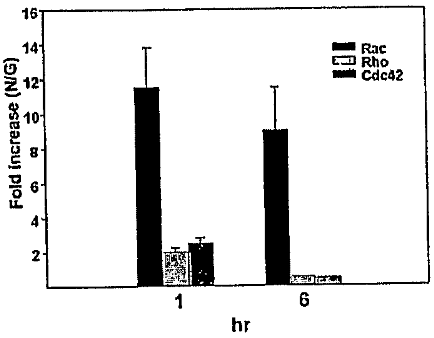


Fig. 9H

Fig. 10





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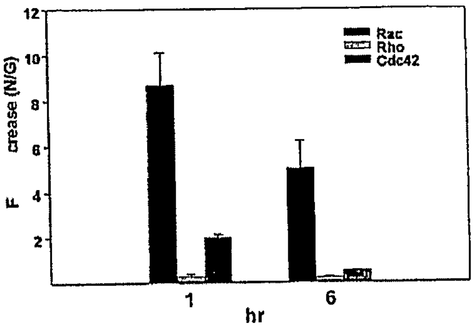
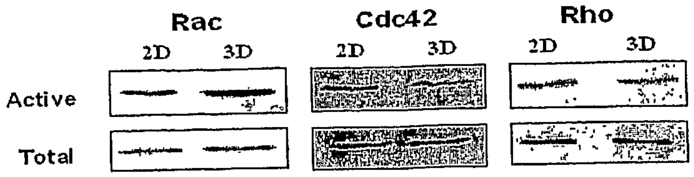


Fig. 11B

Fig. 12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Cdc42
Rho
2D
3D
2D
3D
2D
3D
Active
Total

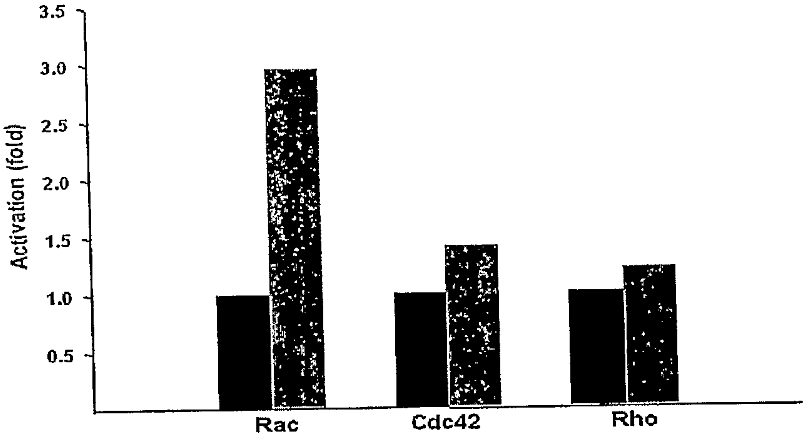

Activation (fold)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Rac
Cdc42
Rho

Fig. 13

Fig 14

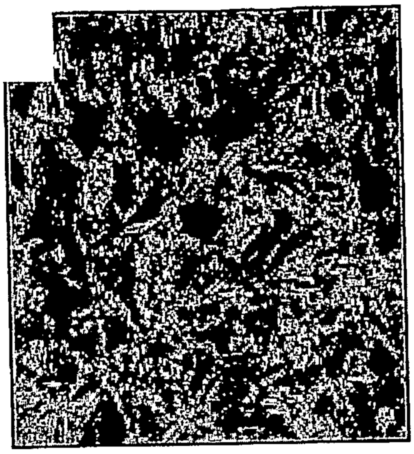
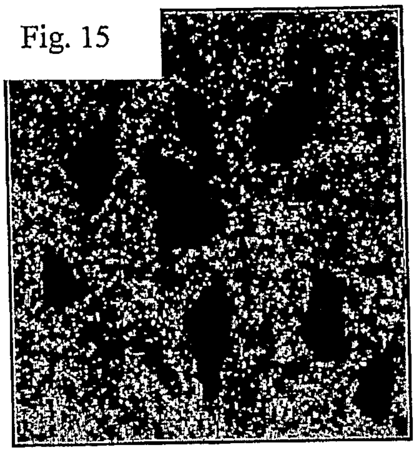


Fig. 15



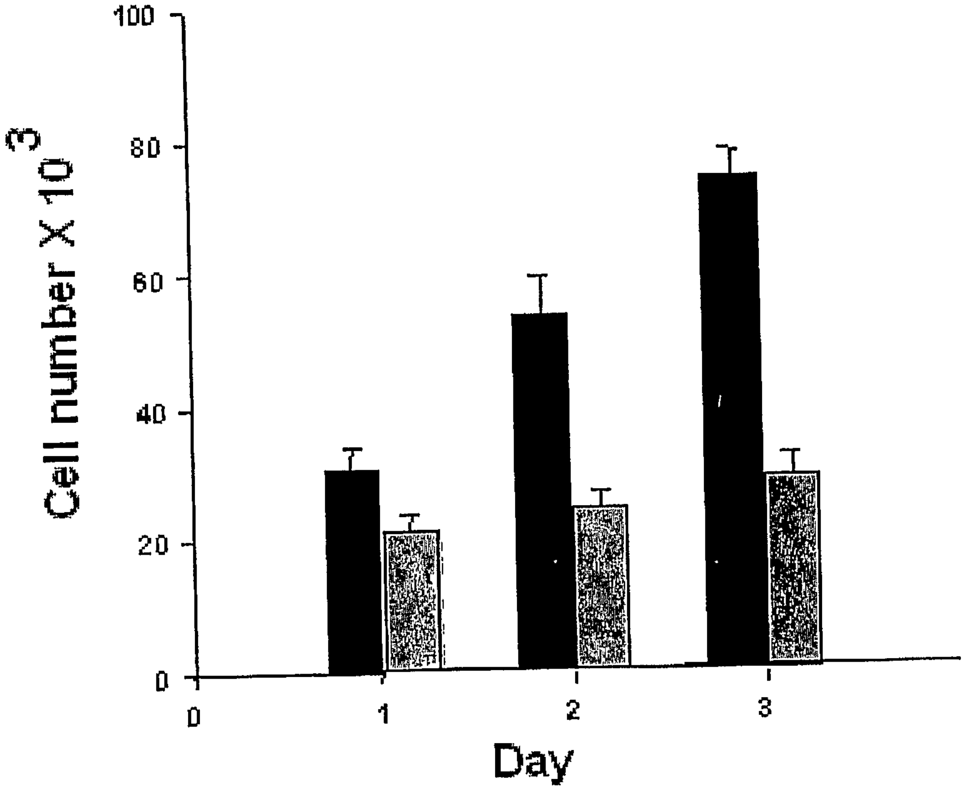


Fig. 16

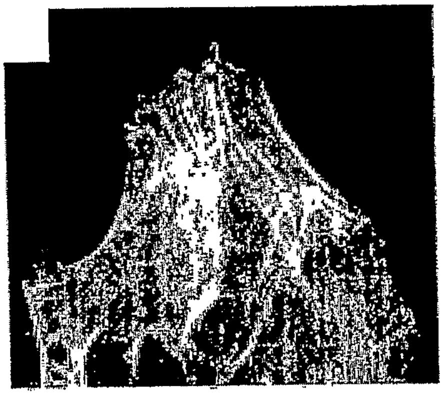
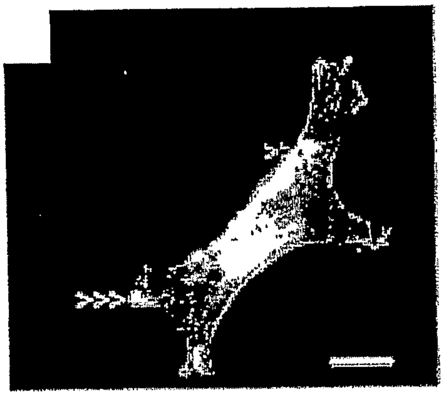


Fig. 17A



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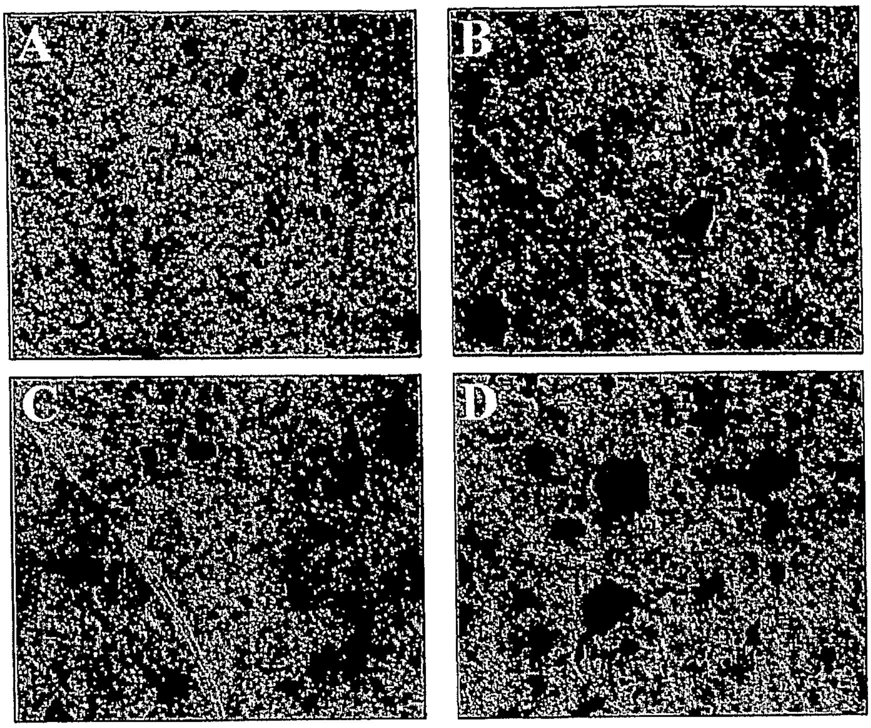


Figure 18

Fig. 19A

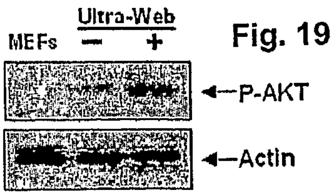


Fig. 19B

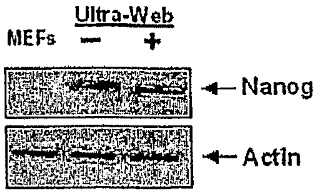


Fig.19C

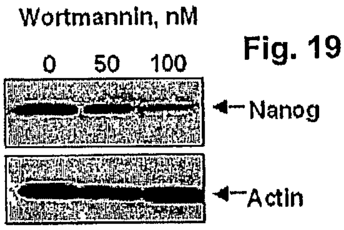
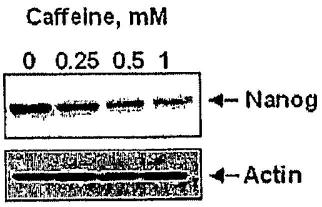


Fig. 19D



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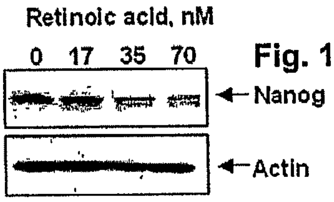

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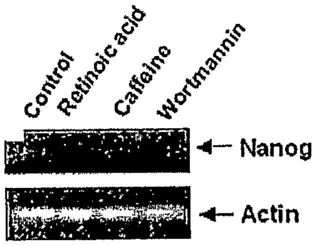
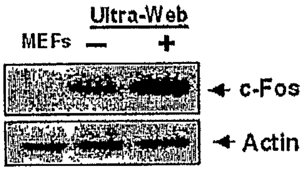
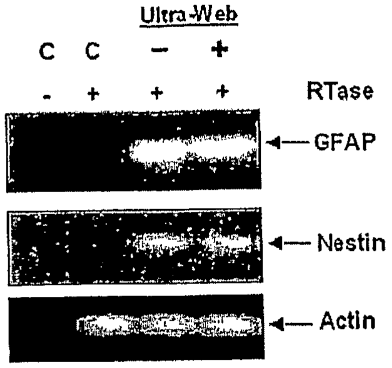


Fig. 19G





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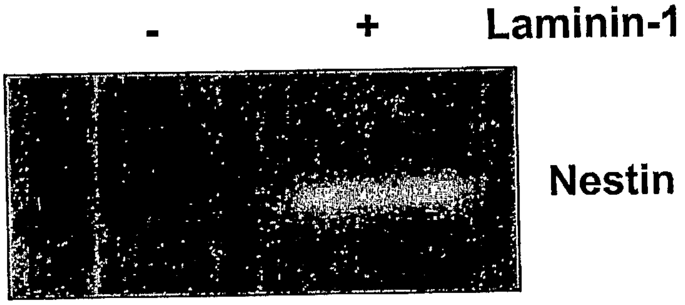

Figure 21

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       Meiners, Sally A.
       Nur-E-Kamal, Mohammed Shah Alam
       Chung, Hoo Y.
       Weik, Thomas M.
       Grafe, Timothy

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<130>  00758.1821WOU1

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