Abstract: Compositions and methods are provided for cell growth on an oriented collagen film. The cells may be grown in vitro, e.g. the culture of cells, including regenerative cells such as stem cells, lineage committed progenitors, etc. Cells may also be grown in vivo, e.g. where an oriented collagen thin film provides a substrate for cell growth, e.g. as a wound covering or artificial skin construct.
INTRODUCTION

[0001] Collagen is one of the most important and abundant proteins in the human body, and is present in skin, cartilage, and bone. It is the major protein comprising the ECM. Collagen consists of three left-handed polyproline II-like chains supercoiled in a parallel direction with one-residue shift, resulting in a right-handed superhelix. This packing requires glycine (Gly) as the every third residue. As a result, each chain consists of a constant amino acid sequence of Gly-X-Y, where X and Y can be any amino acid residues. About 20% of the amino acids in the collagen structure are the imino acids proline and hydroxyproline. This stiff, filament shaped molecule derives its notable tensile strength from its triple helical structure and its ability to assemble into fibrous bundles. The collagen molecule is itself a rod of about 300 nm in length and 1.5 nm in diameter. The three polypeptide strands from which it is formed are wound in a left-handed helix instead of the right-handed conformation that is typical of the alpha helix. This molecule is distinctive because of the regular pattern of amino acids that are arranged to form each strand. This architecture, which places glycine with its small size in the third position, accounts for the molecules ability to form its triple helix.

[0002] Collagen has the facility of organizing into a hierarchy of aggregated structures that ultimately arrive at fibrils with diameters measuring several hundred nanometers. These fibrils display a banded structure characterized by regular 67 nm spacings. Recent atomic force microscopy has produced detailed images of these fibers that suggest the fibrils have an inhomogeneous cross-section and react mechanically as tubes instead of rods.

[0003] The uses of collagen include providing a substrate for cell growth, for example in wound dressings, and in tissue culture. For such purposes collagen can be deposited on a surface, or can be used as a three-dimensional gel. In other uses, the replacement of human tissues with human- or animal-derived tissues such as skin or bone grafts results in the improvement of the wound healing process because of the presence of collagen. The application of collagen-derived products as biomaterials has tremendous impact in biomedicine because of (i) the natural structure of these products as a biological support for cells and scaffold for tissue repair or regeneration, (ii) their biodegradability that obviates removal of implants, and (iii) their biocompatibility. Collagen has been used to design biomaterials such as wound dressings, artificial dermis, bone or tendon substitutes, tissue engineered devices, and injectable materials in plastic surgery.

[0004] Cell culture has become one of the major tools used in the life sciences today. In cell culture methods, animal or plant cells are placed in an artificial environment conducive to growth. This environment usually consists of a culture vessel containing a liquid or semisolid
medium that supplies the nutrients essential for survival and growth. Two basic culture systems are used for growing cells, based on the ability of the cells to grow attached to a substrate (monolayer culture), or in suspension. Many cell lines, especially those derived from normal tissues, are anchorage dependent, and only grow when attached to a suitable substrate. Transformed cells and some normal cells, particularly cells of the hematopoietic lineages, grow freely while floating in suspension.

[0005] Anchorage-dependent cells require a good substrate for attachment and growth. Glass and plastics treated to make the surface hydrophilic are the most commonly used substrates. However, attachment factors such as collagen, gelatin, fibronectin and laminin, can be used as substrate coatings to improve growth and function of normal cells derived from brain, blood vessels, kidney, liver, skin, etc. Normal anchorage dependent cells may also function better if they are grown on a permeable or porous surface, which allows them to polarize as they do in the body.

[0006] The effectiveness of a substrate for culturing a particular cell type may be monitored by a visual assessment of poor or unusual patterns of cell attachment or growth. Growth rate is also an indication of optimized conditions. Plating efficiency can be used, where small numbers of cells are placed in a culture vessel and measuring the number of colonies they form. The percentage of cells forming colonies is a measure of survival, while the colony size is a measure of growth rate. Cells may also be monitored for the retention or acquisition of desired attributes, for example the retention of pluripotency in stem cells; or the differentiation of stem cells into desired progeny.

[0007] For many purposes, there is an interest in being able to expand cells, particularly including stem and progenitor cells, in culture. However, in many cases it is not simply a matter of maintaining cell viability for the cells, but also of ensuring that the cells increase in numbers without losing their distinctive phenotype, e.g. with stem cells. Stem cells have also been grown in co-culture with stromal cells. However, it is particularly desirable to expand stem cells in a culture of known composition, rather than relying upon the presence of other cells for their maintenance.

[0008] The healing of wounds in skin in the absence of scarring is also affected by orientational processes. A scar is most often characterized by highly oriented collagen fibers that are aligned parallel to the wound. The result is a visible scar that lacks many of the attributes of the surrounding skin (hair follicles and the ability to tan, for example). It is well documented that fetal skin, as long as the fetus is younger than the second trimester, it has a remarkable ability to repair wounds without scar formation, a result that adult skin cannot achieve. The propensity of the collagen to crosslink increases with the age of the fetus. Fetal skin also deposits collagen in the region of a wound with a reticular, basket weave pattern. It would be desirable to have the means of recreating this particular collagen
[0009] There continues to be a strong demand for improvements substrates for cell growth, which find use in wound healing, and in the in vitro culture of cells, particularly regenerative cells such as stem cells and progenitor cells. The present invention addresses this need.

SUMMARY OF THE INVENTION

[0010] Compositions and methods are provided for cell growth, including growth of regenerative cells, on an oriented collagen film. The cells may be grown in vitro, e.g. the culture of cells, including regenerative cells such as stem cells, lineage committed progenitors, etc. The cells will usually be contact oriented cells. Cells may also be grown in vivo, e.g. where an oriented collagen film provides a substrate for regenerative cell growth, e.g. as a wound covering or artificial skin construct.

[0011] The substrate for cell growth in the methods of the present invention is an oriented collagen film. The oriented collagen films may be nematic, usually a nematic thin film; or cholesteric, where the cholesteric film may be a thin film or a thicker structure. The orientation of the protein in the thin film can be evaluated by viewing through cross-polarizing microscopy, atomic force microscopy and optical techniques, or orientation can be verified directly at the air/water interface by measuring dichroism, e.g. arising from sirius red dye molecules that are intercalated within the collagen chains.

[0012] In one embodiment of the invention, an oriented collagen thin film is created by the method of layering a concentrated acidic collagen solution over an aqueous high salt solution. The thin film is generated by compression of the layered collagen solution, which provides for a nematic orientation of the collagen. The oriented collagen is deposited by Langmuir-Blodgett methods onto a solid substrate, resulting in a thin film coating of uniaxially oriented protein.

[0013] In another embodiment, a cholesteric collagen solution is deposited on a substrate to generate a cholesteric oriented film. The collagen is brought to a high concentration in an acidic solution, usually greater than about 20 mg/ml. The solution at 20mg/ml is isotropic. The solution is directly deposited onto a substrate. The collagen molecules are oriented as a result of the flow processing. The film is then dessicated, which increases the concentration and produces the banding structures. As the the concentration increases the structure becomes cholesteric. In some embodiments, a reticular pattern is obtained, in others a banded pattern.

[0014] In one embodiment of the invention a system is provided for cell growth, comprising at least one oriented collagen film as a substrate for cell growth. The film is optionally sterile. The system may further comprise a vessel suitable for cell growth, e.g. a flask, multi-
well plate, etc., where the oriented collagen film is present within the vessel. In an alternative embodiment, the system further comprises a dressing suitable for wound repair, *e.g.* an inner surface of an oriented collagen thin film, and an outer surface structure that protects the wound, *e.g.* a porous outer layer.

[0015] In another embodiment of the invention, methods are provided for regenerative growth of tissues *in vivo*, the method comprising contacting a tissue surface, *e.g.* a dermal surface, with an oriented collagen film, for a period of time sufficient to allow cell growth on the collagen.

[0016] In another embodiment of the invention, methods are provided for growth of cells in culture, the method comprising introducing cells to culture medium comprising an oriented collagen film, and maintaining the cells under conditions and for a period of time sufficient to allow cell growth on the collagen.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0017] Figure 1: Surface pressure as a function of area per molecule for various concentrations of salt in phosphate buffered saline solutions.

[0018] Figure 2: Dichroism-induced extinction of light sent through a layer of collagen at the air/water interface. The sample has been subjected to a sequence of compressions and expansions of the layer on a Langmuir trough.

[0019] Figure 3: Brewster angle microscope image of collagen fibrils aligned at the air-water interface by compression of Langmuir barriers. The fibrils are oriented parallel to the barriers.

[0020] Figure 4: Human fibroblast cells growing on unoriented collagen (left) and oriented collagen (right). The red dye shows actin, the blue dye reveals the nuclei, and the green dye indicates the presence of green fluorescent protein.

[0021] Figure 5: Microscope images of a stripe of collagen extruded from the cholesteric phase onto glass. The image on the right was taken using a polarizing microscope and shows a banding structure running parallel to the flow direction. The AFM image on the right indicates that the collagen has initiated fibril formation with a distinctive basket weave pattern. The AFM image measures 10 by 10 microns.

[0022] Figure 6: Human fibroblast cells growing on a stripe of collagen deposited from a cholesteric state. The image on the left shows a portion of the stripe running roughly from right to left. The image on the right shows the initial deposition spot where the extrusion syringe was first dropped down onto the substrate. The cells are observed to be elongated and oriented parallel to the banding structure of the collagen.

[0023] Figure 7. A schematic of cholesteric banding.
Figure 8. Light Microscopy of dried films, showing the result of collagen deposition and air drying at room temperature.

Figure 9. Contact guidance and banding structure.

Figure 10. Surface topology of cholesteric film.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Compositions and methods are provided for cell growth, including growth of regenerative cells, on an oriented collagen film. The cells may be grown in vitro or in vivo. The oriented collagen films are characterized as having oriented collagen, as evidenced, for example, by dichroism extinction, polarizing light microscopy, etc. The films are generally supported during initial production, but after drying may be removed from the support. In vitro cultures utilizing the oriented collagen films employ conventional media for the growth of cells, optionally in the absence of serum using only defined protein factors and in the absence of feeder cells.

Interfacial and bulk flow processing methods are used to create substrates derived from collagen for the purpose of controlling their morphology. The formulation of scaffolds and substrates provides a physical environment for the attachment and proliferation of cells to improve or replace biological functions. In addition to the molecular composition of the matrix, its morphology and the orientation distributions of its constituents influence the growth and attachment of cells.

The present invention utilizes interfacial flow and liquid crystal flow processing to create orientational and morphological patterns in thin films and higher dimensional structures of collagen. These films are distinguished from previous work by the microstructures that are produced and in the manner in which cells grow on them. Characterization methods, including, without limitation, optical polarimetry such as birefringence and dichroism; microscopies such as atomic force microscopy, Brewster angle microscopy; and x-ray diffraction are useful in determining the details of the microstructures, and to define oriented films. Collagen films of the present invention are made with a high degree of control of morphology and orientation at both the molecular and mesoscale levels. This is accomplished through a combination of hydrodynamic forces and manipulation of molecular forces.

In one embodiment, the creation of cholesteric collagen solutions allows production of orientational patterns of the protein that are useful in creating films useful as growth scaffolds. In another embodiment, constraining to collagen solution to a fluid interface facilitates taking it to a high concentration in a controlled fashion, inducing liquid crystalline order.
Previous methods have utilized electrospinning of collagen and synthetic materials to generate a desired texture, which methods normally produce a non-woven mat with an absence of orientation. However, because native ECMs extracted from tissue and organs have an oriented morphology, it is desirable to design compositions and methods that provide a specific orientational order. In addition to controlling morphology and texture, many applications in regenerative medicine require scaffolds characterized by specific orientational order. Bone, muscle, and nervous tissue are examples of systems where the orientation of cells and extracellular matrix material directly influences the ultimate physical properties of the resulting tissue, or is essential for proper performance of that tissue. The healing of wounds in skin in the absence of scarring is also affected by orientational processes.

DEFINITIONS

It is to be understood that this invention is not limited to the particular methodology, protocols, cell lines, animal species or genera, and reagents described, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

As used herein the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the culture" includes reference to one or more cultures and equivalents thereof known to those skilled in the art, and so forth. All technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs unless clearly indicated otherwise.

Collagen. As used herein the term "collagen" refers to compositions in which at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95% or more of the protein present is collagen in a triple helical configuration. Collagens are widely found in vertebrate species, and have been sequenced for many different species. Due to the high degree of sequence similarity between species, collagen from different species can be used for biomedical purposes, e.g. between mammalian species. Typical commercial animal sources include the bovine Achilles tendon, calfskin and the bones of cattle. In some embodiments the collagen used in the preparation of the oriented thin film is Type I, Type II or Type III collagen, and is derived from any convenient source, e.g. bovine, porcine, etc., usually a mammalian source.

Collagen has a triple-stranded ropelike coiled structure. The major collagen of skin, tendon, and bone is collagen I, containing 2 alpha-1 polypeptide chains and 1 alpha-2 chain. The collagen of cartilage contains only 1 type of polypeptide chain, alpha-1. The fetus also
contains collagen of distinctive structure. The genes for types I, II, and III collagens, the interstitial collagens, exhibit an unusual and characteristic structure of a large number of relatively small exons (54 and 108 bp) at evolutionarily conserved positions along the length of the triple helical gly-X-Y portion.

[0036] Types of collagen include I (COL1A1, COL1A2); II (COL2A1); III (COL3A1); IV (COL4A1, COL4A2, COL4A3, COL4A4, COL4A5, COL4A6); V (COL5A1, COL5A2, COL5A3); VI (COL6A1, COL6A2, COL6A3); VII (COL7A1); VIII (COL8A1, COL8A2); IX (COL9A1, COL9A2, COL9A3); X (COL10A1); XI (COL11A1, COL11A2); XII (COL12A1); XIII (COL13A1); XIV (COL14A1); XV (COL15A1); XVI (COL16A1); XVII (COL17A1); XVIII (COL18A1); XIX (COL19A1); XX (COL20A1); XXI (COL21A1); XXII (COL22A1); XXIII (COL23A1); XXIV (COL24A1); XXV (COL25A1); XXVI (COL26A1); XXVII (COL27A1); XXVIII (COL28A1). As a non-limiting example, the following are references to the known human collagens. It will be understood by one of skill in the art that other collagens, including mammalian collagens, e.g. bovine, porcine, equine, etc. collagen, are equally suitable for the methods of the invention.

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Where the oriented thin film comprises proteins other than collagen, the additional proteins may be naturally occurring matrix proteins, e.g. laminin, fibronectin, entactin, etc. Alternatively a wide variety of materials, including bioactive proteins, may be oriented with the collagen or incorporated into the film by adsorption to the collagen, as desired for a particular cell culture system. These include, but are not limited to antibodies, enzymes, receptors, growth factors, additional components of the extracellular matrix, cytokines, hormones, etc.

Collagen Film. The subject oriented collagen thin films can be prepared using any convenient means. The film comprises at least about 50% collagen, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95% or more of the protein present as collagen, which collagen is typically a fibrous collagen, e.g. Type I, II, III, etc. The collagen is oriented, as assessed by any one of optical polarimetry such as birefringence and dichroism; microscopies such as atomic force microscopy, Brewster angle microscopy; and x-ray diffraction.

In some embodiments the dichroism of the oriented collagen, for example as measured with Sirius red dye and wavelengths generated by an argon ion laser, is greater than zero, and may be greater than 1, greater than 2, greater than 4, greater than 6, greater than 8 and may be greater than 10. Figure 2 shows the dichroism from a layer of collagen following a sequence of compression and expansion experiments.

In one embodiment, the film is a nematic thin film. Such a film consists of a thin film, which may have a thickness of less then about 1 mm thick, usually less than about 0.5 mm thick, and may be about or less than about 100 µm thick. In such an embodiment, the thin film is prepared by layering a concentrated acidic collagen solution, where the collagen is present at a concentration of at least about 1 mg/ml, at least about 2.5 mg/ml, and may be at least about 5 mg/ml. The pH of the solution may range from about pH 2.5 to about pH 4.5, usually ranging from around pH 3 to about pH 4. The solution is layered over a high salt solution, where the salt is present at a concentration of at least about 0.5 M, at least about 0.75 M, at least about 1.25 M, and may be higher, up to saturation. The salt solution may comprise NaCl, KCl, etc., e.g. 5-10X PBS is exemplary.

The layered solution is compressed along one dimension, for example by compressing the troughs of a Langmuir-Blodgett trough. The compression may be
performed at a high speed, e.g. of from about 30-40 cm²/minute. The pressure is taken to at least about 5 Newtons, and may be taken to at least about 7.5 N, at least about 9 N or more. Compression of the layer of collagen molecules orients the molecules in direction that is perpendicular to the compression direction. Once the molecules are oriented in the directional flow and during compression, a film is generated by Langmuir-Blodgett deposition of the layers onto a solid support, resulting in a thin film coating of uniaxially oriented protein.

This enhancement in the presence of collagen at the interface with increasing salt concentration in the subphase is particularly noticeable at lower surface pressures. This procedure effectively forces the fibril formation to occur in two dimensions. The action of compressing the monolayers of collagen by bringing the barriers of a Langmuir trough together impresses hydrodynamic forces onto the layer, because reducing the area of the collagen film by bringing the barriers together induces a combination of dilational and shearing deformations on the layer. These forces act on the rodlike collagen and the resulting fibrils to orient the microstructure. This flow-induced orientation can be observed by measuring dichroism in the layer. Conversely, it is also possible to generate purely extensional interfacial flows using a four roll mill and a simple shear surface flow using a parallel band apparatus.

[0042] The connection between the strength of the compressive deformations and the flow-induced orientation of the collagen can be monitored using dichroism measurements, allowing effective control of the rate of strain in the surface at constant area and the independent influence of velocity gradient and surface pressure.

[0043] In an alternative embodiment, the films have a cholesteric configuration. In such a configuration the collagen may be reticulated or banded. Where the collagen is banded, it forms bands that relate to the pitch of collagen. The bands, which are visible under cross-polarizing filters, are spaced at about 5 to about 7 µm apart, and may be spaced at about 6 µm apart. Using atomic force microscopy, the bands have a topographic rise of about 50 nm in height. The thickness of the cholesteric film can vary from at least about 500 µm to greater than 1 mm, greater than 5 mm, usually less than about 10 mm, but in some embodiments may be thicker and will form substantially 3 dimensional structures. As shown in the Figures, bands are formed parallel to the direction of flow during deposition.

[0044] Cholesteric collagen films are prepared by directly depositing a concentrated acidic collagen solution on a substrate, where the collagen is present at a concentration sufficient to provide the collagen in a cholesteric state, e.g. of at least about 20 mg/ml, at least about 25 mg/ml, or higher, while providing for a flowable solution. The pH of the solution may range from about pH 2.5 to about pH 4.5, usually ranging from around pH 3 to about pH 4.

[0045] In some embodiments, the flowable solution is deposited onto a substrate through an
aperture, where the aperture may be of any convenient geometry, e.g. elliptical, round, rectangular, etc. In some embodiments an elliptical tip is used. Various sizes of aperture also find use, and will be selected based on the specific thickness of film that is desired. In some embodiments, the the height or diameter of the aperture is at least about 1 mm, at least about 2 mm, at least about 5 mm, and not more than about 10 mm, where the width may be selected to achieve the desired width of band, e.g. from around about 2 mm to around about 100 mm. Sufficient pressure is applied to generate a flow rate, usually a flow rate of at least about 0.1 ml/min/mm², at least about 0.5 ml/min/mm², at least about 10 ml/min/mm², or more. To generate directionality, the aperture is moving relative to the substrate, where either the substrate or the aperture may be moved, as is convenient. Usually the movement will be from around 1 mm/s to around about 100 mm/s, or faster.

The deposited collagen is then allowed to dessicate by exposure to air, humidified air, etc., and usually is not covered with a solid material until the dessication is complete, and the cholestaeric structure has formed.

This strategy was found to create strips of collagen that are accompanied by a shear banding structure that is qualitatively different than patterns that are normally generated in nematic polymer liquid crystals. Instead of the typical result where bands run perpendicular to the flow direction, the bands are oriented parallel to the flow. Alternative methods for generating the oriented collagen film include the use of high rate shear coaters to produce thin, oriented films.

Supports. A variety of solid supports or substrates may be used with the collagen films, including both deformable and rigid substrates. By deformable is meant that the support is capable of being damaged by contact with a rigid instrument. Examples of deformable solid supports include hydrogels, polyacrylamide, nylon, nitrocellulose, polypropylene, polyester films, such as polyethylene terephthalate; PDMS (polydimethylsiloxane); etc. Also included are gels, microfabricated or bioengineered surfaces, microchannels, microfluidics, chambers, and patterned surfaces. Rigid supports do not readily bend, and include glass, fused silica, nanowires, quartz, plastics, e.g. polytetrafluoroethylene, polypropylene, polystyrene, polycarbonate, and blends thereof, and the like; metals, e.g. gold, platinum, silver, and the like; etc.

Derivatized and coated slides are commercially available, or may be produced using conventional methods. For example, SuperAldehyde™ substrates contain primary aldehyde groups attached covalently to a glass surface. Coated-slides include films of nitrocellulose (FastSlides™, Schleicher & Schuell), positively-charged nylon membranes (CastSlides™, Schleicher & Schuell), hydrogel matrix (HydroGel™, Packard Bioscience, CodeLink, Amersham), and simulated biologic surfaces (SurfaceLogix) etc.

The solid supports can take a variety of configurations, including filters, fibers,
membranes, beads, blood collection devices, particles, dipsticks, sheets, rods, capillaries, etc., usually a planar or planar three-dimensional geometry is preferred.

[0051] Cells. The oriented collagen films of the invention provide a substrate for cell growth, usually contact guided cells, which may be vertebrate cells, e.g. mammalian cells, where the term refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, laboratory, sports, or pet animals, such as dogs, horses, cats, cows, mice, rats, rabbits, etc. Preferably, the mammal is human. The cells which are employed may be fresh, frozen, or have been subject to prior culture. They may be fetal, neonate, adult. The cells may be primary cell cultures, cell lines, cells present in an animal, etc. It is shown herein that cells will orient themselves along the direction of the collagen orientation, as shown in Figure 4.

[0052] The invention is suitable for use with any cell type, including primary cells, normal and transformed cell lines, transduced cells and cultured cells, which can be single cell types or cell lines; or combinations thereof. In assays, cultured cells may maintain the ability to respond to stimuli that elicit a response in their naturally occurring counterparts. Cultured cells may have gone through up to five passages or more, sometimes 10 passages or more.

[0053] In some embodiments, the cells are regenerative, that is they give rise to new cells and tissues, e.g. as stem cells, progenitor cells, lineage committed progenitor cells, and the like. The term stem cell is used herein to refer to a cell that has the ability both to self-renew, and to generate differentiated progeny (see Morrison et al. (1997) Cell 88:287-298). Generally, stem cells also have one or more of the following properties: an ability to undergo asynchronous, or symmetric replication, that is where the two daughter cells after division can have different phenotypes; extensive self-renewal capacity; capacity for existence in a mitotically quiescent form; and clonal regeneration of all the tissue in which they exist, for example the ability of hematopoietic stem cells to reconstitute all hematopoietic lineages. "Progenitor cells" differ from stem cells in that they typically do not have the extensive self-renewal capacity, and often can only regenerate a subset of the lineages in the tissue from which they derive, for example only lymphoid, or erythroid lineages in a hematopoietic setting.

[0054] Stem cells may be embryonic or fetal stem cells, which can be pluripotent, totipotent, or lineage committed. Pluripotent stem cells are cells derived from any kind of tissue (usually embryonic tissue such as fetal or pre-fetal tissue), which stem cells have the characteristic of being capable under appropriate conditions of producing progeny of different cell types that are derivatives of all of the 3 germinal layers (endoderm, mesoderm, and ectoderm). These cell types may be provided in the form of an established cell line, or they may be obtained directly from primary embryonic tissue and used immediately for differentiation. Included are cells listed in the NIH Human Embryonic Stem Cell Registry, e.g. hESBGN-01,
hESBGN-02, hESBGN-03, hESBGN-04 (BresaGen, Inc.); HES-1, HES-2, HES-3, HES-4, HES-5, HES-6 (ES Cell International); Miz-hES1 (MizMedi Hospital-Seoul National University); HSF-1, HSF-6 (University of California at San Francisco); and M, H7, H9, H13, H14 (Wisconsin Alumni Research Foundation (WiCell Research Institute)).

[0055] Stem cells of interest also include embryonic cells of various types, exemplified by human embryonic stem (hES) cells, described by Thomson et al. (1998) Science 282:1 145; embryonic stem cells from other primates, such as Rhesus stem cells (Thomson et al. (1995) Proc. Natl. Acad. Sci USA 92:7844); marmoset stem cells (Thomson et al. (1996) Biol. Reprod. 55:254); and human embryonic germ (hEG) cells (Shamblott et al., Proc. Natl. Acad. Sci. USA 95:13726, 1998). Also of interest are lineage committed stem cells, such as mesodermal stem cells and other early cardiogenic cells (see Reyes et al. (2001) Blood 98:2615-2625; Eisenberg & Bader (1996) Circ Res. 78(2):205-16; etc.) The stem cells may be obtained from any mammalian species, e.g. human, equine, bovine, porcine, canine, feline, rodent, e.g. mice, rats, hamster, primate, etc.

[0056] Stem cells of interest also include adult stem cells, which include mesodermal stem and progenitor cells, neural crest stem and progenitor cells, liver stem and progenitor cells, pancreatic stem and progenitor cells, mesenchymal stem and progenitor cells, epidermal skin and progenitor cells, etc.

[0057] Culture medium: In embodiments where cells are grown on the oriented collagen thin film in vitro, the thin film is placed in a suitable vessel for culture, e.g. a flask, plate, multiwell plate, etc. The cells are grown in vitro in an appropriate liquid nutrient medium. Generally, the seeding level will be at least about 10 cells/ml, more usually at least about 100 cells/ml and generally not more than about 10^5 cells/ml, usually not more than about 10^4 cells/ml. Any cell culture medium appropriate for growth and differentiation of cells may be used in cell cultures employing the present collagen cell culture substrates. These include, but are not limited to, DMEM, MEM, M-199 and RPMI. Supplements, as are known in the art, may be added to the culture medium and include serum (e.g., FBS or calf serum), serum-containing supplements (N IU-SERUM), and serum-free supplements (MITO+).

[0058] Various media are commercially available and may be used, including Ex vivo serum free medium; Dulbecco's Modified Eagle Medium (DMEM), RPMI, Iscove's medium, etc. The medium may be supplemented with serum or with defined additives. Appropriate antibiotics to prevent bacterial growth and other additives, such as pyruvate (0.1-5 mM), glutamine (0.5-5 mM), 2-mercaptoethanol (1-10x10^-5 M) may also be included.

[0059] Culture in serum-free medium is also of interest. The medium may be any conventional culture medium, generally supplemented with additives such as iron-saturated transferrin, human serum albumin, soy bean lipids, linoleic acid, cholesterol, alpha thioglycerol, crystalline bovine hemin, etc., that allow for the growth of stem cells.
Wound dressing. Oriented collagen films find use as a wound dressing, or artificial skin, by providing an improved substrate that minimizes scarring. An effective bioactive wound dressing can facilitate the repair of wounds that may require restoration of both the epidermis and dermis. An oriented collagen thin film is placed onto, and accepted by, the debrided wound of the recipient and provide a means for the permanent re-establishment of the dermal and epidermal components of skin. The graft suppresses the formation of granulation tissue which causes scarring. The collagen substrates also find use in other situations where it is desirable to orient cell growth in vivo.

Additional criteria for biologically active wound dressings include: rapid adherence to the wound soon after placement; proper vapor transmission to control evaporative fluid loss from the wound and to avoid the collection of exudate between the wound and the dressing material. Skin substitutes should act as barrier to microorganisms, limit the growth of microorganisms already present in the wound, be flexible, durable and resistant to tearing. The substitute should exhibit tissue compatibility, that is, it should not provoke inflammation or foreign body reaction in the wound which may lead to the formation of granulation tissue. An inner surface structure of an oriented collagen thin film is provided that permits ingrowth of fibro-vascular tissue. An outer surface structure may be provided to minimize fluid transmission and promote epithelialization.

Typical bioabsorbable materials for use in the fabrication of porous wound dressings, skin substitutes and the like, include synthetic bioabsorbable polymers such as polyactic acid or polyglycolic acid, and also, biopolymers such as the structural proteins and polysaccharides. The finished dressing is packaged and preferably radiation sterilized.

Such biologically active products can be used in many different applications that require the regeneration of dermal tissues, including the repair of injured skin and difficult-to-heal wounds, such as burn wounds, venous stasis ulcers, diabetic ulcers, etc.

CELL GROWTH

A population of cells, which may comprise progenitor and/or stem cells is contacted with an oriented collagen film under conditions permissive for growth of the cell. The population of cells is present in an in vitro culture, or in a living mammal.

In an in vitro culture, after seeding the culture medium, the culture medium is maintained under conventional conditions for growth of mammalian cells, generally about 37°C and 5% CO₂ in 100% humidified atmosphere. Fresh media may be conveniently replaced, in part, by removing a portion of the media and replacing it with fresh media. Various commercially available systems have been developed for the growth of mammalian cells to provide for removal of adverse metabolic products, replenishment of nutrients, and
maintenance of oxygen. By employing these systems, the medium may be maintained as a continuous medium, so that the concentrations of the various ingredients are maintained relatively constant or within a predescribed range. Such systems can provide for enhanced maintenance and growth of the subject cells using the designated media and additives.

[0066] These cells may find various applications for a wide variety of purposes. The cell populations may be used for screening various additives for their effect on growth and the mature differentiation of the cells. In this manner, compounds which are complementary, agonistic, antagonistic or inactive may be screened, determining the effect of the compound in relationship with one or more of the different cytokines.

[0067] The populations may be employed for transplantation. For example, mesenchymal cells are used to regenerate bone, collagagenous and muscle defects. Neural stem cells find us in the repair of neurological defects. Pancreatic progenitor cells find use in the regeneration of islet beta or alpha cells. Epithelial, e.g. dermal and epidermal progenitors find use in the regeneration of skin.

[0068] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

[0069] All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

[0070] The present invention has been described in terms of particular embodiments found or proposed by the present inventor to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. For example, due to codon redundancy, changes can be made in the underlying DNA sequence without affecting the protein sequence. Moreover, due to biological functional equivalency considerations, changes can be made in protein structure without affecting the biological action in kind or amount. All such modifications are intended to be included within the scope of the appended claims.
EXPERIMENTAL

Materials and Methods

[0071] Nematic thin film. A collagen solution is prepared of Rat Tail Type I Collagen dissolved in acetic acid at a concentration of 1-5 mg/mL collagen at pH 3-4. The collagen is layered over a PBS solution, and compressed at a speed of 36 cm²/min along one axis at a pressure of 9-10 N to orient the collagen. Once a highly oriented layer of collagen fibrils is been produced, it is transferred onto a substrate using standard Langmuir-Blodgett deposition techniques, resulting in a thin film coating of uniaxially oriented protein. The supported film is washed once with water to remove excess salt, and misted with ethanol and dried to sterilize.

[0072] Cholesteric film. A collagen solution is prepared of Rat Tail Type I Collagen dissolved in acetic acid with an initial concentration of 3-10 mg/mL collagen at pH 3-4. The solution is placed inside of dialysis tubing (Molecular Weight cutoff of 12,000) and the ends sealed. This tubing is then surrounded by polyethylene glycol (PEG) with Molecular weight 15,000-20,000 (any weight can be used as long as it is above the cutoff of dialysis tubing). This is then placed in cold room (4 degrees C) for 45 minutes. The PEG removes water from the dialysis bag, leaving behind a concentrated collagen solution. The pH can then be adjusted if desired, then the solution is harvested and transferred to scintillation vials stored in the refrigerator. The concentrated solution is deposited at room temperature onto a glass or PDMS substrate through a 21-26 guage needle, which is gently crushed to create a more elliptical aperture. The solution is pumped at a speed of 0.1-0.2 ml/minute flow, with a substrate moving at a speed of 0-100 mm/second. The deposited film is dessicated at ambient conditions in air.

[0073] Cell culture investigations were performed with fibroblasts and adipose stem cells. Adipose stem cells were extracted from inguinal fat of C57BL/6 mice, seeded at a low density of 5-25,000 cells/mL, depending on the length of the experiment. Experiments that run for 6-12 hours used a much higher cell density so that more cells can be observed. The most commonly used amount is roughly 4,000 cells per cm² of plate. This is for experiments that are run for 24 hours.

[0074] The duration of cells in medium ranged from 6 hours to one week depending on the experiment. The cells are observed to orient on the substrate as soon as they have settled to the surface. The most common amount of time is 24 hours. The medium is used for cell culture was 50OmL DMEM (high glucose) without dye; 50mL FBS; 5mL 100x Penicillin-Streptomycin; 5 mL 100x glutamine; 5 mL MEM Non-Essential Amino Acids; Sodium pyruvate 5mL of 100x; Filter and Store at 4°C, for up to 4 weeks.
Results

Dissolving collagen in 0.1 M acetic acid inhibits fiber formation and leads to molecular solutions of the triple helix molecule. Molecular solutions of this rodlike protein offer the possibility of creating liquid crystalline liquids with collagen. Nematic and cholesteric phases can be produced at concentrations above approximately c > 20 mg/ml. Routes can be used to transition a collagen solution to a cholesteric phase or a fibril suspension by adjusting concentration and pH.

Because collagen spontaneously aggregates to form fibers, the resulting networks with physical and covalent bonds can be very difficult to process in order to systematically control orientation. The distribution of fiber size can also be challenging to control. Constraining complex materials to a fluid interface can facilitate taking them to a high concentration in a controlled fashion. In the case of a rodlike chain, this can induce liquid crystalline order.

In one strategy, a layer of collagen is floated at the surface of air and water. This is accomplished by dissolving the collagen in acetic acid (pH 3.5) since this prevents the formation of fibrils prior to spreading. By using a phosphate buffered saline solution (PBS, where 1X PBS is 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) as the subphase, the collagen is maintained at the interface and effectively spreads. This is demonstrated in Figure 1 where the surface pressure is plotted against the surface area per molecule for several concentrations of salt in PBS.

This enhancement in the presence of collagen at the interface with increasing salt concentration in the subphase is particularly noticeable at lower surface pressures. This procedure effectively forces the fibril formation to occur in two dimensions. The action of compressing the monolayers of collagen by bringing the barriers of a Langmuir trough together impresses hydrodynamic forces onto the layer. This is because reducing the area of the collagen film by bringing the barriers together induces a combination of dilational and shearing deformations on the layer. These forces can act on the rodlike collagen and the resulting fibrils to orient the microstructure.

This flow-induced orientation can be observed by measuring dichroism in the layer. This optical effect refers to the polarization-dependent absorption of light and can produce a measure of the order parameter in the layer. For this purpose, experiments were conducted by placing the dye molecule, Sirius red, into the subphase. This dye molecule has been used successfully in the past to transform collagen into a birefringent and dichroic material for wavelengths generated by an argon ion laser. We have available a polarization modulated dichroism/birefringence experiment and Figure 2 shows the dichroism from a layer of collagen following a sequence of compression and expansion experiments. Also shown are the cycles of surface pressure. Compressing the layer causes an increase in
dichroism in concert with the surface pressure indicating alignment of the collagen layer parallel to the barriers. Re-expanding the layer by withdrawing the barriers causes the dichroism to drop back towards zero.

[0080] Using Brewster angle microscopy, the morphology of the layer at the air/water interface can be imaged. One such image is shown in Figure 3, which reveals an oriented array of collagen fibrils running parallel to the compression barriers. Once a highly oriented layer of collagen fibrils has been produced, it can be successfully transferred onto a substrate using standard Langmuir-Blodgett deposition techniques.

[0081] Cells are cultured on oriented substrates as described above to investigate the influence of orientation of the collagen. Figure 4 shows the result of growing human fibroblast cells onto glass coated with unoriented collagen (left photomicrograph) and onto a layer of highly oriented collagen (right photomicrograph). In these pictures, dyes were incorporated into the cells to reveal actin (red), the nuclei (blue), and green fluorescent protein. This experiment indicates that the cells align themselves parallel to the collagen fibrils onto which they are attached. It is important to emphasize that these experiments are the first to demonstrate a method whereby the order parameter of collagen fibrils can be controlled and quantified.

[0082] An alternative route to processing collagen to create highly ordered structures provides for three dimensional structures as opposed to the largely two dimensional formats possible with the Langmuir-Blodgett method described above. This approach employs collagen solutions of sufficient concentration to induce the formation of cholesteric phases. In these liquid crystalline states, flow-induced alignment is made more efficient as long as rotational components in the flow are properly attenuated. Polymer liquid crystals are also well-known to produce remarkable orientational instabilities and textures. This phenomenon, often called shear banding is very noticeable during the process of relaxation of orientation following cessation of flow and normally leads to bands oriented perpendicular to the flow direction.

[0083] We have developed a robot that is capable of extruding collagen solutions with very good spatial control to create elaborate patterns of deposited material. Figure 5 shows two microscope images of a stripe of collagen deposited onto glass. The image on the left was taken using a polarizing microscope at 40x magnification. Interestingly, the banding that is evident in the image runs parallel to the flow direction. The image on the right was taken using an atomic force microscope and shows a distinctive basket weave pattern within the collagen. The reticulated basket weave is from an area of the film that does not show the banding structure.

[0084] Human fibroblast cells grown on these robotically extruded stripes were found to grow with an orientation that is parallel to the bands. This is shown in Figure 6 where two
images are shown. On the left, a phase contrast microscope picture is shown of a stripe that roughly runs from right to left. Within the stripe, the cells are highly oriented along the extrusion direction. The image on the right shows the initial deposition spot where the syringe tip used to extrude the stripe was first dropped down onto the glass substrate. At this location, the banding of the cholesteric collagen solution has a hairpin shape and the cells are observed to precisely track this pattern.

[0085] Figure 7 illustrates the orientation of the collagen fibrils in the cholesteric banding. The surface topology is shown in Figure 8. The contact guidance of cells is shown in Figure 9. The band spacing is shown in Figure 10.
WHAT IS CLAIMED IS:

1. A method for growth of mammalian cells, the method comprising:
   contacting a population of cells with an oriented collagen film under conditions
   permissive for growth of the cells.

2. The method of Claim 1, wherein the oriented collagen film is an oriented
   monomolecular film of at least 50% fibrous collagen.

3. The method of Claim 2, wherein the film is a nematic collagen thin film.

4. The method of Claim 3, wherein the film is created by the method of:
   layering a concentrated acidic collagen solution over a high salt solution;
   compressed the layered solution along one dimension at a pressure sufficient to
   orient the collagen, and
   depositing the compressed solution on a substrate to generate an oriented thin film.

5. The method of Claim 1, wherein the oriented collagen film is a cholesteric
   film.

6. The method of Claim 5, wherein the film is generated by the method of:
   depositing a cholesteric collagen solution onto a substrate under flow conditions
   sufficient to generate collagen banding after dessication.

7. The method of Claim 2, wherein the cells are stem or progenitor cells.

8. The method of Claim 1, wherein the cells are contact guided cells.

9. The method of Claim 1, wherein the conditions permissive for growth are in
   vitro culture conditions.

10. The method of Claim 1, wherein the conditions permissive for growth are in
    vivo.

11. The method of Claim 5, wherein the cells are skin cells.

12. A system for cell growth, comprising at least one oriented collagen film as a
    substrate for cell growth.
13. The system of Claim 12, wherein the film is a nematic collagen thin film.

14. The system of Claim 13, wherein the film is created by the method of:
layering a concentrated acidic collagen solution over a high salt solution;
compressed the layered solution along one dimension at a pressure sufficient to
orient the collagen, and
depositing the compressed solution on a substrate to generate an oriented thin film.

15. The system of Claim 12, wherein the oriented collagen film is a cholesteric film.

16. The system of Claim 15, wherein the film is generated by the method of:
depositing a cholesteric collagen solution onto a substrate under flow conditions
sufficient to generate collagen banding after dessication.

17. The system of Claim 12, further comprising a vessel suitable for cell growth,
where the oriented collagen thin film is present within the vessel.

18. The system of Claim 12, further comprising a dressing suitable for wound repair.
FIG. 1
FIG. 2
FIG. 3
INTERNATIONAL SEARCH REPORT

International application No
PCT/US 08/12744

A CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 38/17, C09H 1/00 (2009 01)
USPC - 530/356

According to International Patent Classification (IPC) or to both national classification and IPC

B FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC - 530/356, 602/50, 602/42, 602/43, 424/443, 424/574, 435/297 5, 435/273

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 530/356, 602/50, 602/42, 602/43, 424/443, 424/574, 435/297 5, 435/273

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST - GPB, USPT, USOC, EPO, JPAB, Dialog Classic Files 6354, 652, 351, 349, 315, 6, 35, 65, 155, Google Scholar; USPTO Web Page, Search terms - stem cells, growth, α2β1 collagen, in vitro, in vivo, nematic, choo σ, low pH, hyperosmotic, depositing, film, wound dressing, skin cells, substrate

C DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>US 2004/007813</td>
<td>A1 (SIMPSON, et al)</td>
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<td>05 April 2007 (05 04 2007), pg 4, ln 5-6, ln 8-32, pg 5, ln 3-4, ln 13-17, pg 19, ln 30-31, pg 22, ln 11-20, pg 23, ln 20-29</td>
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15 JAN 2009

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