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(54) Title: PRODUCTION OF GRAFT TISSUE FROM EXTRACELLULAR MATRIX		
(57) Abstract Using connective tissues as starting materials, a method is described for producing matrix particulates. The invention includes an embodiment wherein the matrix particulates are seeded with living human cells or with cells of other species. Further, the invention encompasses fusing the particulates to constitute composites of various shapes, or holding them together in a porous container made of membranous biopolymers. The particulates or composites can be used as tissues for grafting or as model systems for research and testing. The invention also encompasses the spinning of threads in which the matrix particulates are components.		

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PRODUCTION OF GRAFT TISSUE
FROM EXTRACELLULAR MATRIX

Background of the Invention

5 The use of synthetic materials such as polyester
fiber (Dacron™) or polytetrafluoroethylene
(PTFE) (Teflon™) as implants designed to replace diseased
or damaged body parts has been extensive. These materials
have however, enjoyed limited success. This has been due
10 to the poor biocompatibility of these materials which
among other problems, frequently initiate persistent
inflammatory reactions. Additionally, the failure of the
body to integrate these materials, because they do not
break down and do not lend themselves to remodeling by
15 tissue cells that may come into contact with them, causes
further problems.

 Efforts to use animal or human materials have also
been unsatisfactory when these materials are cross-linked
by formaldehyde or glutaraldehyde, for example. The
20 process of generalized aldehydic cross-linking renders
biomaterials sufficiently unrecognizable to tissue cells
so that normal remodeling and integration are not
promoted. Similarly, other types of chemical processing
of animal or human biomaterials, such as extraction with
25 detergents, or hypertonic buffers or hypotonic buffers can
alter them to the degree that they are ineffective in
promoting angiogenesis and in stimulating repair and
remodeling processes needed for the conversion of an
implant into a functional substitute for the tissue or
30 organ being replaced.

 A third approach has been that of reconstituting
tissue and organ equivalents from structural matrix

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components, such as collagen, for example, that have been extracted and purified and combined with specialized cells. The process depends upon interactions between the cells and matrix proteins that the cells condense and
5 organize. While tissue-like constructs have been fabricated and been shown to somewhat resemble their natural counterparts, they do not readily develop the matrix complexity characteristic of the actual tissues they are meant to imitate. See, for example, U.S. Patent
10 Nos. 4,485,096 and 4,485,097, E. Bell, 1984.

Summary of the Invention

The present invention relates to a method of producing graft tissue by obtaining a desired connective
15 tissue source and processing it to remove living cells without removing factors necessary for cell growth. The tissue is frozen or freeze dried and then fragmented, and the living cells removed to produce connective tissue matrix particulates. In the alternative, the tissue can
20 be fragmented before freezing.

The invention also includes methods of producing graft tissue which comprise the above method with the additional steps, alone or in combination, of 1) again
25 processing the connective tissue particulates to remove the cytoplasmic and nucleic acid components without removing the factors necessary for cell growth and differentiation; and 2) seeding the connective tissue particulates with cultivated cells under such conditions that the cells adhere to or populate the connective tissue
30 particulates.

In one embodiment of the invention, the matrix particulates are seeded with selected cultured human or other cells. The resulting cell populated material can be
35 fused into composites of various shapes, with or without the addition of growth and differentiation factors.

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The present invention embodies a new method for preparing animal tissues as transplants designed to substitute for tissues or organs that are damaged or diseased. This is accomplished in such a way that their native complexity is preserved and thus allows them to be remodeled and integrated by the specialized cells with which they are brought into contact. In other words, because the matrix particulates of the present invention contain cell growth and differentiation stimulatory molecules, they provide the biomolecular signals needed for in vivo tissue repair. In contrast to methods previously described, the method of the present invention avoids the use of harsh reagents such as high salt, or delipidation reagents such as butanol/ether or detergents. The use of such reagents in methods previously described is responsible for removing from the connective tissue a great many factors whose presence we consider essential for stimulating repair and remodeling processes needed for the conversion of an implant into a functional substitute for the tissue or organ being replaced.

Advanced methods in in vitro cell cultivation permit expansion of small numbers of differentiated cells into large cell banks. Tissue matrix vehicles that can direct the organization and differentiation of these specialized cells for delivery to the human recipient in the clinic are needed. The present invention can be used to bring a disease such as diabetes under control by engineering and implanting reconstituted tissues or composites populated by cells with the appropriate functional capacity to overcome the problems caused by absent or insufficient insulin biosynthesis or its regulated delivery. For example, particulates of the present invention can be combined in vitro with cells from the islets of Langerhans, or with undissociated islets, to form a composite which is then implanted under the kidney

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capsule. Further, cell-populated matrix composites may be implanted directly, for example by using a hypodermic syringe or similar device to deliver the composite to the desired site. This invention similarly may be used to
5 treat Parkinson's disease by providing the patient with a tissue containing dopamine producing cells. One can also plate nerve cells on the particulates and allow a composite to form. This would be useful in the repair of neural tissue defects.

10 The method of this invention also has broad applications in the rebuilding of damaged or aberrant body structures such as skin, various tubular structures and skeletal structures such as bone, tendon and ligament. The invention may also be used to create connective tissue
15 matrices to be used as tissue scaffolds which are populated by circulating cells after transplantation into the desired site. In one embodiment, the matrix particulates are mixed with a substance which can form porous membranous polymers containing the particulates,
20 and then shaped. Matrix particulate-containing threads are one example of the many shapes which can be formed using particulates of the present invention. For example, the particulates can be mixed with collagen, or another bioabsorbable or nonbioabsorbable polymer, and extruded
25 into a dehydration or coagulation bath containing absolute ethanol or acetone, or another dehydrating solution. The thread can also be subjected to dehydration by some other means, for example vacuum-drying. The particulates can also be coextruded with collagen or another polymer
30 serving as a hollow fiber. Together, the particulates and the hollow fiber serve as a loaded hollow fiber with the particulates filling the core of the hollow polymer fiber. The threads produced in this manner can then be braided or woven to form fabrics or other complex constructs for
35 implantation. In addition to the advantage provided by

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the high strength, flexibility and increased surface area of braided or woven threads, sleeves of the loaded hollow fiber threads can be made porous, thus allowing colonizing cells to occupy the surface of the thread and thereby
5 contact the tissue matrix particles within the thread.

In a further embodiment, the method of the present invention is used to produce matrix particulates from fetal skeletal elements derived from animal sources, prior to mineralization. These fetal matrix particulates can be
10 combined with osteogenic cells in cylindrically shaped collagenous containers to form skeletal constructs. The collagenous external surface of the containers can be plated with a second cell type associated with periosteal tissue. In a similar manner, a composite derived from
15 dermal matrix particulates, or from a sheet of previously frozen matrix that has been perforated, can be over-plated with keratinocytes to form a skin-like construct.

Detailed Description of The Invention

20 The invention includes a method of producing graft tissue which comprises obtaining a desired connective tissue source, then processing it to remove living cells without removing factors necessary for cell growth and differentiation. The cell-free connective tissue source
25 is frozen or freeze-dried, and fragmented, either before or after freezing or freeze-drying, to produce connective tissue particulates. In the alternative, the tissue can be frozen or freeze-dried after fragmentation. The cell-free connective tissue particulates can be used as an
30 implant for host cells to populate. The freeze-dried connective tissue can also be used without fragmentation, but perforated before use. According to this embodiment, the cell-free tissue can be used in the form of perforated sheets or strips without fragmentation.

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The invention includes another method for producing graft tissue which comprises obtaining a desired connective tissue source and processing as above to remove living cells without removing factors necessary for cell growth and differentiation. The connective tissue source is frozen or freeze-dried, then fragmented to produce connective tissue particulates. The connective tissue particulates are seeded with cultivated cells under such conditions that the cells adhere to and populate the connective tissue particulates. In one embodiment of the present invention, the connective tissue particulates are seeded with cultivated cells and then packed into a preformed porous polymeric container. This method can also include the optional step of further processing the connective tissue particulates before they are seeded, in order to remove nucleic acids without removing other factors necessary for cell growth and differentiation. Further processing can include contacting the connective tissue particulates with nuclease preparations.

The invention includes another method for producing graft tissue which comprises obtaining a desired connective tissue source and processing as above to remove living cells without removing factors necessary for cell growth and differentiation. The connective tissue source is then frozen or freeze-dried and fragmented to produce connective tissue particulates. In the alternative, the cells can be removed prior to fragmentation. The particulates are mixed with a porous polymer, such as collagen or other bioabsorbable or nonbioabsorbable polymers, then extruded to form threads composed of the polymer and the connective tissue particulates. The threads of the invention can be either mixed fiber (in which the thread is comprised of a mixture of the polymer and the cell matrix particulate), or loaded hollow fiber (in which the thread is comprised of a core of cell matrix

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particulates surrounded by a porous polymer sleeve). These threads are then braided or woven to form fabrics or other complex constructs for implantation. The sleeve of the loaded hollow fiber threads is porous, thus allowing
5 colonizing cells to occupy the surface of the thread and thereby contact the tissue matrix particles within the thread.

The invention encompasses another method of producing graft tissue which comprises obtaining a desired
10 connective tissue source and processing to remove living cells without removing factors necessary for cell growth and differentiation. According to this method, the connective tissue source is frozen or freeze-dried, but not fragmented. Instead, the tissue is processed in the
15 form of sheets or strips. These sheets or strips are frozen or freeze-dried, then thawed and perforated to allow cells to infiltrate the tissue. In one embodiment, the connective tissue sheets or strips are seeded with
20 cultivated cells under such conditions that the cells adhere to and populate the sheets or strips. The seeded sheets or strips can be further shaped or molded to form useful graft tissue. In another embodiment of the subject invention, the sheets or strips can be implanted into a host without first seeding with cultivated cells.
25 According to this embodiment, the sheets or strips would provide a site for host cells to populate.

Fragmentation of the desired tissue can be achieved by freezing or freeze-drying, and then mechanically
blending the frozen or freeze-dried tissue or mechanically
30 crushing it between rollers. The desired tissue can also be fragmented by blending the tissue without first freezing it. An optional nuclease treatment is carried out for the purpose of digesting the nucleic acids released when the cells and cell nuclei are ruptured. The
35 connective tissue particulates are then washed and

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collected by the serial steps of centrifugation, resuspension, recentrifugation and resuspension.

The invention further encompasses a method wherein the connective tissue particulates obtained by the above methods are layered onto a synthetic porous membrane to a thickness which allows diffusion of nutrients to all the connective tissue particulates. One embodiment of this method includes an additional step of fusing the connective tissue particulates to form a composite using a fusing agent. The type of fusing agents which can be selected are known to one skilled in the art. In a preferred embodiment, in vitro cultivated cells are plated onto the connective tissue composite in a volume which will just cover the composite and allow transportation of nutrients. The cells are plated under conditions that allow the cells to populate or adhere to the connective tissue particulates. One or more than one cell type can be plated onto the composite.

The invention further includes a construct which is produced by any of the above methods, including a multi-cell construct.

The connective tissue source can be derived from bovine, ovine, porcine or marine animal connective tissues, as well as other species of vertebrates of invertebrates. The invention further includes connective tissues which are derived from a variety of body parts or tissues. In a preferred embodiment of the invention connective tissue is derived from embryonic or fetal tissues. Embryonic and fetal tissues are advantageous because they include various biomolecular factors which are present in normal tissue at different stages of cell development. The factors present in fetal tissues include, for example, factors necessary for cell division, differentiation and tissue morphogenesis.

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The connective tissue matrix particulates derived through the above methods can be seeded with selected cultured human or other cells. The resulting cell-populated matrix can be fused into composites of various shapes, with or without the addition of growth and differentiation factors.

In a preferred embodiment of the invention the matrix particulates are selected to match the tissue being fabricated. For example if a skin replacement is the objective, preferably dermal matrix would be particularized and a composite with dermal cells constructed. If neural tissue replacement is the goal, preferably embryonic or fetal central nervous system connective tissue matrix would be chosen for combination with the appropriate cells to form a composite.

The processing steps of the above methods are performed in order to remove cells from the selected animal tissues, thereby producing connective tissue matrix particulates. There are many methods known in the art for removing cells from connective tissue. The following are examples of some of these methods. These examples are not intended to be an exhaustive list, but are merely examples of some of the known methods. The choice of method and the sequence of treatments depend on the type of tissue being processed.

In one method for removing cells from the connective tissue, the tissue is scraped with a blade or blade-like instrument. The tissue can also be passed successively between parallel rollers, where the rollers are brought closer together in successive steps thereby squeezing out soft components including cells. The tissue can also be treated with enzymes, particularly nucleases. Additionally, the tissue is passed through a freeze-thaw, or freeze-dry-thaw cycle in order to disrupt the living cells. These methods can also be employed to remove

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remaining cytoplasmic and nuclear components in connective tissue particulates produced by fragmentation of the connective tissue source.

The freezing and/or freeze-drying, and fragmentation
5 steps are done to produce particulates and to further free the tissue of undesirable components. Methods for sterilizing processed tissues are known to one skilled in the art and include contacting the tissue with a peracetic acid solution, preferably about a 0.5% solution, and
10 washing the tissue in a sterile buffer, preferably sterile phosphate buffered saline. The tissue can then be freeze-dried by being brought to the temperature of liquid nitrogen, then fragmented under sterile conditions. Fragmentation at low temperature can be carried out in
15 many ways known to those skilled in the art. Fragmentation methods could include a shearing device, such as a mechanical blender, or mechanically crushing the tissue between rollers to yield particulates of such a size so as to allow for vascularization upon implantation.
20 In a preferred embodiment of the invention, the particulates are between about 50 and about 300 micrometers in diameter, but for some applications the particulates can be as small as 5-10 microns. Depending on the starting tissue type, further nuclease cleaning and
25 washes followed by centrifugation and resuspension in fresh phosphate buffered saline can be carried out in an effort to minimize the presence of components which are immunogenic.

In another embodiment of the invention the tissue can
30 be dehydrated chemically with acetone, absolute alcohol or hydrophilic polymers such as carbowax, and then fragmented. In a preferred embodiment of the invention the particulates are further treated by seeding the matrix with cells. Preferably one would use human cells for
35 populating the matrix, but the invention is not limited to

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human cells. In one embodiment the seeding process is carried out by rehydrating the matrix particulates using tissue culture medium, with or without growth and differentiation supplements added, and allowing the
5 particulates to swell to equilibrium with their fluid environment.

In another embodiment of the invention the matrix particulates are populated with cells of one, or more than one phenotype in a bioreactor, spinner flask or other
10 similar device. The number of cells used and the time of residence in the device depend on cell attachment time and the desired degree of coverage of the particulate by the cells. These parameters will vary depending on the type of cell used and the cell density required for the final
15 product. The particular parameters for each cell type will be apparent to one skilled in the art. In a preferred embodiment of the invention the starting cell density is on the order of about 10^4 to about 10^6 cell per ml.

20 In a further embodiment of the invention the particulates are fused into composites of various shapes with or without the addition of growth and differentiation factors. This step can be carried out on particulates which have been seeded with cells, or on particulates
25 which do not contain cells. In the latter case the fused matrix composite can be seeded with cells after the particulates are fused into composites. The matrix composite, with or without cells, is then layered onto a synthetic porous membrane. Examples of a synthetic porous
30 membrane, for illustrative purpose only and not to limit the invention in any way, include membranes made of polycarbonate, for example in a transwell arrangement, or a bioabsorbable polymer, such as a porous collagen sheet that lines a mold having a porous membrane for a bottom to

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hold the collagen sheet and permit passage of medium from below. The membrane may also be made of other bioabsorbable polymers such as poly-l-lactate. In one embodiment of the invention the particulates are layered
5 in a single layer. In a preferred embodiment the layer is less than about 0.5 millimeters in thickness.

If the matrix has been seeded with cells, the thickness of the layer must be controlled in such a way so as to ensure that the necessary nutrients may flow to
10 the cells. The seeding of the matrix is carried out by applying cells in suspension directly onto the particulate composite in a volume that will just cover the composite but not run off it, under such conditions that the cells populate the matrix.

15 Non-limiting examples of a few of the ways in which the porosity of the composites can be controlled include: limiting cell growth, limiting crosslinking, and mechanically, by placing a brush-like device with spaced Teflon™ "hairs" onto the substrate prior to delivering
20 the particulates to it. If the last expedient is used the sheet will form around the microposts or "hairs", thereby creating holes when the "hairs" are removed.

If the matrix is seeded with cells and then incubated for a period of time in tissue culture medium, the
25 construct will be in the form of a coherent sheet that can be handled alone or, for example, by the porous collagen membrane on which it formed. In a preferred embodiment of the invention the incubation period is about 7-14 days. The collagen membrane can also be used to wrap the
30 composite. The collagen membrane can be fixed to the composite, for example by suturing, so that a flat package is formed. The sheet can also be rolled around a shaft to form a tube at any stage in its development after it becomes coherent.

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A further embodiment of the invention includes the use of multiple cell types combined with the composite by plating cells on either side of the composite, or by plating cells on the porous collagen membrane used to wrap the composite. Further, a layer of one cell type can be plated over a layer containing another cell type, or cells of different types may be plated together.

Still further the invention encompasses the case where the cell-laden particulates are packaged in a preformed porous collagen or other polymeric container of any desired shape. The container can be a rectangular box where, in a preferred embodiment, the box is not thicker than about 1.0mm. Additionally the invention discloses the use of concentric tubes of differing radii where, in a preferred embodiment, the radii would differ by only by about 1.0mm or less. It is also envisioned that other shapes can be used as well.

The containers can be reinforced by struts or other means to maintain uniform thickness. The frequency of the struts is preferably about 1-10 per mm square. The container can be made out of a collagen sheet with pores which are too small to allow particulates to escape. In a preferred embodiment, the pores measure about 30 to about 60 microns. After introduction of particulates through an end opening, the container can be sealed by suturing or other means.

In another embodiment of the present invention, the connective tissue particulates are formed into threads suitable for weaving or braiding. The threads can be woven into fabrics or other complex constructs for implantation. the braided or woven fabrics would provide a scaffold of matrix material for host cells to adhere to. In addition, the thread or fabrics produced from the threads, can be seeded with cells in vitro, as above, and then transplanted into the appropriate site in the host.

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The threads of this invention can be either of the mixed fiber, or the loaded hollow type. Mixed fiber threads are made by mixing connective tissue particulates with a bioabsorbable or nonbioabsorbable polymer, then
5 extruding the mixture, using, for example, a spinnaret, to form a mixed thread. The hollow fiber thread comprises a core of connective tissue particles of this invention surrounded by a porous polymeric sleeve. The polymer used for producing the threads can be either bioabsorbable or
10 nonbioabsorbable. In a preferred embodiment the polymer is collagen. After extrusion, threads of this invention are dehydrated and coagulated, for example by extrusion into a bath containing absolute alcohol, carbowax or acetone. The threads can then be annealed, for example by
15 pulling the threads over rollers from the coagulation bath into an annealing bath containing a saline solution. The threads can then be dried and spooled, for example by pulling the threads over more rollers to dry, and then winding onto spools.

20

Exemplification

Animal sources are obtained for connective tissue matrix materials, such as organs, from porcine, bovine, ovine, marine or other animals. For example, to
25 reconstitute an endocrine pancreas equivalent at least three organs can be used. The first is the pancreas itself, which would provide the tissue specificity of the matrix. The second is the duodenum or jejunum of the gut and the third is the skin.

30 The organs are packed on ice in sterile containers immediately after animal slaughter and transported to the laboratory. The pancreas is treated using the protocol for islet removal, that is, the pancreatic duct is cannulated and Hank's solution containing 2mg/ml
35 collagenase (Type X, Sigma) and 2% fetal calf serum is

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injected. In addition the two arteries that supply the pancreas, the celiac and the superior mesenteric, are similarly perfused with a collagenase solution containing heparin to remove endothelium from the circulatory tree of the gland. Adequate cleaning of the pancreas may require heparinizing animals before slaughter. A compression device is used to gently knead the organ as it is perfused and reperfused in a sterile chamber in the cold.

In the course of recirculation the collagenase solution is gradually diluted. A fibrous network of ducts and vessels remains after collagenase digestion. After washing in phosphate buffered saline, the fibrous material is freeze-dried and mechanically fractured in an Omnimixer™ shearing tube. After thawing, the particulates are washed again and sterilized in 5.0% peracetic acid.

The objective of the freezing and fracturing procedure is to provide matrix particulates of between 100 and 200 μm in diameter. The islet of Langerhans of the pancreas, responsible for secreting insulin and other hormones, itself measures between 100 and 200 μm . The size of the particulates is also determined by the maximum allowable thickness of a tissue graft made to a host. The thickness must be consistent with survival after implantation in vivo. A diffusion distance greater than 0.5mm is not desirable, because the graft must depend on diffusing tissue fluids for gas exchange and nutrients until it is vascularized. The goal is to produce particulates of relatively uniform size from the frozen tissue by a mechanical process that will not melt the material by frictional heating. Blending, grinding, crushing, and/or percussion in the cold at -30°C or below are methods which can be used.

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The particulates are then combined into aggregates with small clusters of cultivated cells, or are used as microbeads in a cell bioreactor in which cells from islet cell cultures are seeded. The cells attach to surfaces of the matrix microbeads in the bioreactor. The last mentioned strategy is useful as a means of expanding islet cell cultures; it can also serve as a means for constituting pseudo-islets.

The second example of an organ processed for its matrix is the jejunum or duodenum which is mechanically scraped after opening. As described by Badylak, *et al.*, U.S. Patent Serial No. 4,902,508 (1990), the stratum compactum, about 100 μm thick, lying between luminal layers on one side and the muscular layers on the other, is delaminated from them, leaving an acellular collagenous connective tissue. The washed stratum compactum is particularized after freeze-drying as described above.

Similarly the third organ, skin, is stripped of epidermis and underlying adipose material and the dermis is sectioned into 100-200 μm thick sheets parallel to the dermal surface with a dermatome. It is then freeze-dried, particularized and washed in preparation for combination with cells or transplantation. Alternatively the sheets derived from the jejunum and the dermis can be made porous or perforated with a meshing machine or similar device so that they can be seeded with cells or pseudo-islets or both.

Aggregates can also be formed when suspensions of cultured cells are combined with matrix particulates in a transwell chamber, such as that produced by CostarTM. The fluid in which the cells and particulates are suspended drains through the porous membrane that serves as the floor of the chamber and the cells are brought into close proximity with the particulates whose surfaces they attach

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to. Secreted cell products provide the additional matrix that gives the mass of particulates coherence.

Two other approaches to giving the particulate-cell, or particulate-pseudo-islet combinations coherence to facilitate grafting as sheets are the following: A flat porous collagen container, or one made with a poly-l-lactate or other biopolymer, is fabricated and filled with the matrix particulates and adherent cells constituted in a bioreactor or other device. The biopolymer container and its contents can then be maintained in a chamber whose floor is a transwell membrane in contact with nutrient medium.

The other method consists of crosslinking the particulates non-toxically with transglutaminase in the presence of dermatan sulfate and fibronectin thereby forming three dimensional reconstituted tissues. Because this method is not damaging to cells, crosslinking can occur in the presence of cells. The assembled constructs, that is three dimensional reconstituted tissues, are incubated at 37°C in a 5% CO₂ incubator and allowed to undergo differentiation in vitro in preparation for transplantation.

Equivalents

Those skilled in the art will know, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

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CLAIMS

1. A method of producing graft tissue which comprises:
- 5 a) freezing a connective tissue source;
- b) processing the connective tissue source to
remove living cells without removing factors
necessary for cell growth; and
- 10 c) fragmenting the connective tissue to produce
connective tissue particulates.
2. A method of Claim 1, which further comprises the
additional step of:
- 15 d) seeding the connective tissue particulates with
cultivated cells under such conditions that the
cells adhere to the connective tissue
particulates.
3. A method of producing graft tissue which comprises:
- 20 a) freezing a connective tissue source;
- b) processing the connective tissue source to
remove living cells without removing factors
necessary for cell growth;
- 25 c) fragmenting the connective tissue to produce
connective tissue particulates;
- d) processing the connective tissue particulates
further to remove the cytoplasmic and nuclear
components without removing the factors
necessary for cell growth; and
- 30 e) seeding the connective tissue particulates with
cultivated cells under such conditions that the
cells adhere to the connective tissue
particulates.

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4. A method of Claim 1, wherein the fragmentation of the connective tissue in step b comprises mechanically blending the frozen connective tissue.
- 5 5. A method of Claim 1, wherein the fragmentation of the connective tissue in step b comprises mechanical crushing between rollers.
6. A method of Claim 1, wherein the fragmentation of the
10 connective tissue in step b is followed by enzymatic treatment to remove nucleic acids.
7. A method of Claim 1, wherein the connective tissue
15 particulates are layered onto a synthetic porous membrane to a thickness which allows diffusion of nutrients to all the connective tissue particulates.
8. A method of Claim 7, wherein the connective tissue
20 particulates are fused by a fusing agent to form a composite.
9. A method of Claim 2, wherein the connective tissue
25 particulates are layered onto a synthetic porous membrane to a thickness which allows diffusion of nutrients to all the connective tissue particulates.
10. A method of Claim 9, wherein the connective tissue
30 particulates are fused by a fusing agent to form a composite.
11. A method of Claim 10, wherein the cultivated cells are suspended and plated onto the composite in a volume which will just cover the composite and allow transportation of nutrients, under such conditions

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that the cells populate the connective tissue particulates.

- 5 12. A method of Claim 11, wherein more than one cell type is plated onto the composite.
- 10 13. A method of Claim 3, wherein the connective tissue particulates are layered onto a synthetic porous membrane to a thickness which allows diffusion of nutrients to all the connective tissue particulates.
- 15 14. A method of Claim 13, wherein the connective tissue particulates are fused by a fusing agent to form a composite.
- 20 15. A method of Claim 14, wherein the cultivated cells are suspended and plated onto the composite in a volume which will just cover the composite and allow transportation of nutrients, under such conditions that the cells populate the connective tissue particulates.
- 25 16. A method of Claim 15, wherein more than one cell type is plated onto the composite.
- 30 17. A method of Claim 2, wherein the connective tissue particulates upon being seeded with cultivated cells are packed into a preformed porous polymeric container.
- 35 18. A method of Claim 3, wherein the connective tissue particulates upon being seeded with cultivated cells are packed into a preformed porous polymeric container.

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19. A construct produced by the method of Claim 1.
20. A construct produced by the method of Claim 2.
- 5 21. A construct produced by the method of Claim 3.
22. A construct produced by the method of Claim 7.
23. A construct produced by the method of Claim 8.
- 10 24. A construct produced by the method of Claim 9.
25. A construct produced by the method of Claim 10.
- 15 26. A construct produced by the method of Claim 11.
27. A construct produced by the method of Claim 13.
28. A construct produced by the method of Claim 14.
- 20 29. A construct produced by the method of Claim 15.
30. A multi cell construct produced by the method of
Claim 12.
- 25 31. A multi cell construct produced by the method of
Claim 16.
- 30 32. A method of producing a mixed particulate and polymer
thread for use as a graft tissue which comprises:
a) freezing a connective tissue source;
b) processing the connective tissue source to
remove living cells without removing factors
necessary for cell growth;

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- 5 c) fragmenting the connective tissue to produce connective tissue particulates; and
- d) mixing the particulates with a polymer and extruding the mixture so as to form a mixed particulate and polymer thread.
33. A method of Claim 32, wherein the polymer is bioabsorbable.
- 10 34. A method of Claim 32, wherein the biopolymer is collagen.
35. A method of producing a loaded hollow porous polymer thread for use as graft tissue which comprises:
- 15 a) freezing a connective tissue source;
- b) processing the connective tissue source to remove living cells without removing factors necessary for cell growth;
- 20 c) fragmenting the connective tissue to produce connective tissue particulates;
- d) extruding the particulates inside a porous polymer sleeve so as to form a thread comprised of a core of connective tissue particulates surrounded by a porous polymer sleeve.
- 25 36. A method of Claim 35, wherein the polymer is bioabsorbable.
37. A method of Claim 35, wherein the polymer is
- 30 collagen.
38. A thread comprised of connective tissue particulates and a porous polymer.

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39. A thread of Claim 38 wherein the polymer is bioabsorbable.
40. A thread of Claim 38 wherein the polymer is collagen.
5
41. A thread comprised of a central core and an outer sleeve, wherein the central core is comprised of connective tissue particulates and the sleeve is comprised of a porous polymer.
10
42. A thread of Claim 41 wherein the polymer is bioabsorbable.
43. A thread of Claim 41 wherein the polymer is collagen.
15
44. A fabric produced by weaving threads comprised of connective tissue matrix and a porous polymer.
45. A fabric of Claim 44 wherein the polymer is bioabsorbable.
20
46. A fabric of Claim 44 wherein the polymer is collagen.
47. A method of producing sheets of acellular tissue matrix for use as graft tissue which comprises:
25
- a) freezing a connective tissue source;
 - b) processing the connective tissue source to remove living cells without removing factors necessary for cell growth, thereby producing a sheet of acellular connective tissue matrix; and
30
 - c) perforating the sheets produced in step b), thereby producing a sheet of acellular connective tissue matrix for use as graft tissue.

AMENDED CLAIMS

[received by the International Bureau on 13 January 1994 (13.01.94);
original claims 1,3-6,32,34 and 35 amended; other claims
unchanged (7 pages)]

1. A method for producing graft tissue, which comprises the steps of:
 - 5 a) freezing a connective tissue source having living cells, whereby the living cells are disrupted to form cell remnants;
 - b) processing the connective tissue source to remove the cell remnants from the connective tissue source without removing factors necessary for cell growth
10 to form a processed connective tissue source; and
 - c) fragmenting the processed connective tissue source to produce connective tissue particulates, thereby producing graft tissue.

2. A method of Claim 1, which further comprises the
15 additional step of:
 - d) seeding the connective tissue particulates with cultivated cells under such conditions that the cells adhere to the connective tissue particulates.

3. A method for producing graft tissue, which comprises the
20 steps of:
 - a) freezing a connective tissue source having living cells, whereby the living cells are disrupted to form cell remnants;
 - b) processing the connective tissue source to remove
25 the cell remnants from the connective tissue source without removing factors necessary for cell growth to form processed connective tissue source;
 - c) fragmenting the processed connective tissue source;
 - d) processing the connective tissue source further to
30 remove cytoplasmic and nuclear components without

- removing factors necessary for cell growth to form connective tissue particulates; and
- 5 e) exposing the connective tissue particulates to cultivated cells under such conditions that the cells adhere to the connective tissue particulates, thereby producing graft tissue.
4. A method of Claim 1, wherein the fragmentation of the connective tissue in step c comprises mechanically cutting up the frozen connective tissue.
- 10 5. A method of Claim 1, wherein the fragmentation of the connective tissue in step c comprises mechanical crushing between rollers.
6. A method of Claim 1, wherein the fragmentation of the connective tissue in step c is followed by enzymatic treatment to remove nucleic acids.
- 15 7. A method of Claim 1, wherein the connective tissue particulates are layered onto a synthetic porous membrane to a thickness which allows diffusion of nutrients to all the connective tissue particulates.
- 20 8. A method of Claim 7, wherein the connective tissue particulates are fused by a fusing agent to form a composite.
9. A method of Claim 2, wherein the connective tissue particulates are layered onto a synthetic porous membrane to a thickness which allows diffusion of nutrients to all the connective tissue particulates.
- 25

10. A method of Claim 9, wherein the connective tissue particulates are fused by a fusing agent to form a composite.
- 5 11. A method of Claim 10, wherein the cultivated cells are suspended and plated onto the composite in a volume which will just cover the composite and allow transportation of nutrients, under such conditions that the cells populate the connective tissue particulates.
- 10 12. A method of Claim 11, wherein more than one cell type is plated onto the composite.
13. A method of Claim 3, wherein the connective tissue particulates are layered onto a synthetic porous membrane to a thickness which allows diffusion of nutrients to all the connective tissue particulates.
- 15 14. A method of Claim 13, wherein the connective tissue particulates are fused by a fusing agent to form a composite.
- 20 15. A method of Claim 14, wherein the cultivated cells are suspended and plated onto the composite in a volume which will just cover the composite and allow transportation of nutrients, under such conditions that the cells populate the connective tissue particulates.
16. A method of Claim 15, wherein more than one cell type is plated onto the composite.

17. A method of Claim 2, wherein the connective tissue particulates upon being seeded with cultivated cells are packed into a preformed porous polymeric container.
18. A method of Claim 3, wherein the connective tissue
5 particulates upon being seeded with cultivated cells are packed into a preformed porous polymeric container.
19. A construct produced by the method of Claim 1.
20. A construct produced by the method of Claim 2.
21. A construct produced by the method of Claim 3.
- 10 22. A construct produced by the method of Claim 7.
23. A construct produced by the method of Claim 8.
24. A construct produced by the method of Claim 9.
25. A construct produced by the method of Claim 10.
26. A construct produced by the method of Claim 11.
- 15 27. A construct produced by the method of Claim 13.
28. A construct produced by the method of Claim 14.
29. A construct produced by the method of Claim 15.
30. A multi-cell construct produced by the method of Claim
12.

31. A multi-cell construct produced by the method of Claim 16.
32. A method for producing a mixed particulate and polymer thread for use as a graft tissue, which comprises the steps of:
- 5
- a) freezing a connective tissue source having living cells, whereby said living cells are disrupted to form cell remnants;
 - b) processing the connective tissue source to remove the cell remnants from the connective tissue source without removing factors necessary for cell growth to form processed connective tissue source;
 - c) fragmenting the processed connective tissue source to form connective tissue particulates;
 - 10 d) mixing the connective tissue particulates with a polymer, whereby a mixture is formed; and
 - e) extruding the mixture so as to form a mixed particulate and polymer thread, thereby producing the thread for use as graft tissue.
- 20 33. A method of Claim 32, wherein the polymer is bioabsorbable.
34. A method of Claim 32, wherein the polymer is collagen.
35. A method for producing a loaded hollow porous polymer thread for use as a graft tissue, which comprises the steps of:
- 25
- a) freezing a connective tissue source having living cells, whereby the living cells are disrupted to form cell remnants;

- b) processing the connective tissue source to remove the cell remnants from the connective tissue source without removing factors necessary for cell growth;
- 5 c) fragmenting the connective tissue to produce connective tissue particulates; and
- d) extruding the connective tissue particulates into a porous polymer sleeve so as to form a thread comprised of a core of connective tissue particulates surrounded by a porous polymer sleeve, thereby producing the thread for use as graft tissue.
- 10
36. A method of Claim 35, wherein the polymer is bioabsorbable.
37. A method of Claim 35, wherein the polymer is collagen.
- 15 38. A thread comprised of connective tissue particulates and a porous polymer.
39. A thread of Claim 38 wherein the polymer is bioabsorbable.
40. A thread of Claim 38 wherein the polymer is collagen.
- 20 41. A thread comprised of a central core and an outer sleeve, wherein the central core is comprised of connective tissue particulates and the sleeve is comprised of a porous polymer.
- 25 42. A thread of Claim 41 wherein the polymer is bioabsorbable.

43. A thread of Claim 41 wherein the polymer is collagen.
44. A fabric produced by weaving threads comprised of connective tissue matrix and a porous polymer.
- 5 45. A fabric of Claim 44 wherein the polymer is bioabsorbable.
46. A fabric of Claim 44 wherein the polymer is collagen.
47. A method of producing sheets of acellular tissue matrix for use as graft tissue which comprises:
- 10 a) freezing a connective tissue source;
- b) processing the connective tissue source to remove living cells without removing factors necessary for cell growth, thereby producing a sheet of acellular connective tissue matrix; and
- 15 c) perforating the sheets produced in step b), thereby producing a sheet of acellular connective tissue matrix for use as graft tissue.

INTERNATIONAL SEARCH REPORT

Intern. Application No
PCT/US 93/07364

A. CLASSIFICATION OF SUBJECT MATTER IPC 5 C12N5/00 A61L27/00		
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) IPC 5 A61L C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 358 506 (MARROW-TECH INCORPORATED) 14 March 1990 see page 6, line 40 - page 11, line 7 ---	1,2
Y	IN VITRO vol. 16, no. 9, September 1980 pages 797 - 808 SHERWOOD GITHENS III, ET AL. 'DUCTS OF THE RAT PANCREAS IN AGAROSE MATRIX CULTURE' see the whole document -----	1,2
<input type="checkbox"/> Further documents are listed in the continuation of box C.		
<input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents :		
A document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. *A* document member of the same patent family	
Date of the actual completion of the international search <div style="text-align: center; font-weight: bold;">15 November 1993</div>	Date of mailing of the international search report <div style="text-align: center; font-weight: bold;">10 -12- 1993</div>	
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer <div style="text-align: center; font-weight: bold;">REMP, G</div>	

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. Application No
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