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**BARКСBY, H.E. et al., Arthritis and Rheumatism, 2006, vol. 54, no. 2, pages 540-550**  
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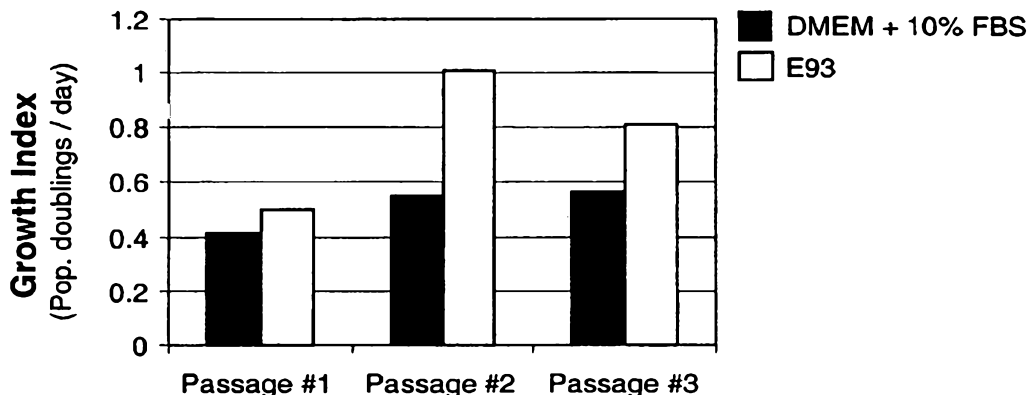
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(54) Title: SERUM-FREE MEDIA AND THEIR USES FOR CHONDROCYTE EXPANSION



(57) Abstract: The present invention provides defined serum-free cell culture media useful in culturing fibroblasts, especially articular chondrocytes, that avoid problems inherent in the use of serum-containing media. The defined media comprise platelet-derived growth factor (PDGF), chemically defined lipids, oncostatin M (OSM), interleukin-6 (IL-6), leukemia inhibitory factor (LIF), or combinations of these compounds. In another aspect, the present invention also provides tissue culture methods that comprise incubating chondrocytes in the defined serum-free media. The methods enhance attachment and proliferative expansion of chondrocytes seeded at low density while maintaining their redifferentiation potential.

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## **SERUM-FREE MEDIA AND THEIR USES FOR CHONDROCYTE EXPANSION**

[0001] This application claims priority to United States application No. 60/805,307, filed on June 20, 2006, which is incorporated herein by reference in its entirety.

### **Field of the Invention**

[0002] The present invention relates to the field of cell and tissue culture. More specifically, the invention relates to methods and compositions for ex vivo propagation of cells capable of forming cartilaginous tissue intended for treatment or repair of cartilage defects.

### **Background of the Invention**

[0003] Articular cartilage is composed of chondrocytes encased within the complex extracellular matrix produced by those chondrocytes. The unique biochemical composition of this matrix provides for the smooth, nearly frictionless motion of articulating surfaces of the joints. With age, tensile properties of human articular cartilage change as a result of biochemical changes. After the third decade of life, the tensile strength of articular cartilage decreases markedly. Damage to cartilage produced by trauma or disease, e.g., rheumatoid and osteoarthritis, can lead to serious physical debilitation.

[0004] The inability of cartilage to repair itself has led to the development of several surgical strategies to alleviate clinical symptoms associated with cartilage damage. More than 500,000 arthroplastic procedures and joint replacements are performed annually in the United States alone. Autologous chondrocyte implantation is a procedure that has been approved for treatment of articular cartilage defects. The procedure involves harvesting a piece of cartilage from a non-weight bearing part of the femoral condyle and propagating the isolated chondrocytes ex vivo for subsequent implantation back into the same patient. Brittberg et al., *New Engl. J. Med.* 331:889-895 (1994).

[0005] Articular chondrocytes express articular cartilage-specific extracellular matrix components. Once articular chondrocytes are harvested and

separated from the tissue by enzymatic digestion, they can be cultured in monolayers for proliferative expansion. However, during tissue culture, these cells adopt a fibroblastic morphology and cease to produce type II collagen and proteoglycans characteristic of hyaline-like articular cartilage. Such "dedifferentiated" cells proliferate rapidly and produce type I collagen, which is characteristic of fibrous tissue. Nevertheless, when placed in an appropriate environment such as suspension culture medium in vitro (Aulthouse et al., *In Vitro Cell. & Devel. Biology* 25:659-668 (1989)) or in the environment of a cartilage defect in vivo (Shortkroff et al., *Biomaterials* 17:147-154 (1996)), the cells redifferentiate, i.e., express articular cartilage-specific matrix molecules again. The reversibility of dedifferentiation is key to the successful repair of articular cartilage using cultured autologous chondrocytes.

[0006] Human chondrocytes are typically cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS). Aulthouse et al., *In Vitro Cell. & Devel. Biology*, 25:659-668 (1989); Bonaventure et al., *Exp. Cell Res.*, 212:97-104 (1994). However, even though serum is widely used for mammalian cell culture, there are several problems associated with its use: (1) serum contains many unidentified or non-quantified components and therefore is not "defined;" (2) the composition of serum varies from lot to lot, making standardization difficult for experimentation or other uses of cell culture; (3) many of the serum components affect cell attachment, proliferation, and differentiation making it difficult to control these parameters; (4) some components of serum are inhibitory to the proliferation of specific cell types and to some degree may counteract its proliferative effect, resulting in sub-optimal growth; and (5) serum may contain viruses and other pathogens which may affect the outcome of experiments or provide a potential health hazard if the cultured cells are intended for implantation in humans. Freshney (1994) Serum-free media. In: *Culture of Animal Cells*, John Wiley & Sons, New York, 91-99.

[0007] Thus, the use of defined serum-free media is particularly advantageous in the ex vivo expansion of chondrocytes for treatment of cartilage defects. However, such defined serum-free media must be sufficient for attachment of adult human articular chondrocytes seeded at low density, sustain

proliferation until confluent cultures are attained, and maintain the capacity of chondrocytes to re-express the articular cartilage phenotype.

[0008] There has been some effort to develop biochemically defined media (DM) for cell culture. DM generally includes nutrients, growth factors, hormones, attachment factors, and lipids. The precise composition must be tailored for the specific cell type for which the medium is designed. Successful growth of some cell types, including fibroblasts, keratinocytes, and epithelial cells, has been achieved in various DM. Freshney, 1994 and Butler M. et al., Appl. Microbiol. Biotechnol. 68:283-91 (2005).

[0009] The amounts of starting cell material available for autologous chondrocyte implantation are generally limited. Therefore, it is desirable to seed articular chondrocytes at a minimal subconfluent density. Attempts to culture articular chondrocytes at subconfluent densities in DM have been only partially successful. Although DM that can sustain the proliferative capacity of the chondrocytes seeded at low density have been developed, the use of these media still requires serum for the initial attachment of cells to the tissue culture vessel after seeding. Adolphe et al., Exp. Cell Res. 155:527-536 (1984), and U.S. Patent No. 6,150,163.

[0010] Consequently, a need exists to optimize, standardize, and control conditions for attachment, proliferation and maintenance of redifferentiation-capable chondrocytes for use in medical applications, especially, in humans.

### **SUMMARY OF THE INVENTION**

[0011] This invention provides compositions of chemically defined culture media (DMs), methods of making such media, and methods of using such media, e.g., for culturing cells, in particular, human articular chondrocytes for repair of cartilage defects. One of the distinguishing features of the DM of the invention is the presence of one or more substantially pure cytokines of the IL-6 family, such as, e.g., oncostatin M (OSM), interleukin-6 (IL-6), and leukemia inhibitory factor (LIF).

[0012] Among other advantages, the invention allows one to avoid the use of serum in chondrocyte cultures, enhance cell attachment and proliferation under serum-free conditions, and/or to maintain the capacity of chondrocytes to re-express cartilage-specific phenotype. In one aspect, the invention provides DM that is sufficient for the initial attachment of cells to a culture substratum, thereby eliminating a need for a serum-containing medium in the initial stage of cell culture. Another aspect of the invention provides defined serum-free cell culture media that promote proliferation of cells such as chondrocytes without use of serum at any stage during cell culture. Yet another aspect of the invention provides cell culture media that may be used to prime chondrocytes prior to implantation into a subject or included as a redifferentiation-sustaining medium to chondrocytes embedded in a matrix intended for implantation into cartilage defects. Another aspect of the invention provides a method of culturing a chondrocyte to a state that is suitable for treating a patient suffering from a cartilage defect. Additional advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention.

[0013] The DM of the invention comprises a basal medium supplemented with one or more supplements, including one or more cytokines of the IL-6 family, such as, e.g., OSM, IL-6, and LIF.

[0014] The basal medium may be any suitable medium. In preferred embodiments, the basal medium is cDRF (Table 3) or cDRFm (Table 4). cDRF and cDRFm, are made by mixing DMEM, RPMI-1640, and Ham's F-12 at a 1:1:1 ratio or by appropriately combining pre-mixed media and adding certain growth supplements to arrive at the basal media as defined in Tables 3 and 4 respectively.

[0015] In further preferred embodiments, the basal medium is additionally supplemented with platelet-derived growth factor (PDGF) and/or one or more lipids. In some embodiments, the lipids are a chemically defined lipid mixture (CDLM; Table 5) or one or more lipids from CDLM (e.g., stearic acid, myristic acid, oleic acid, linoleic acid, palmitic acid, palmitoleic acid, arachidonic acid, linolenic acid, cholesterol, and alpha-tocopherol acetate). In some

embodiments, a DM of the invention may include a basal medium (e.g., cDRF or cDRFm) supplemented with:

- (a) one or both of substantially pure PDGF and CDLM; and
- (b) one or more of substantially pure OSM, substantially pure IL-6, and substantially pure LIF.

For example, in preferred embodiments, DM of the invention may include: (a) a basal medium; (b) 0.1-100 ng/ml PDGF; (c) 0.05-5% CDLM; (d) 0.01-10 ng/ml OSM; and/or (e) 0.01-10 ng/ml IL-6.

[0016] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

[0016a] Definitions of the specific embodiments of the invention as claimed herein follow.

[0016b] According to a first embodiment of the invention, there is provided a method for propagating chondrocytes, wherein the method comprises a step of incubating the chondrocytes with a culture medium comprising a basal medium supplemented with oncostatin M (OSM).

[0016c] According to a second embodiment of the invention, there is provided a method of treating a cartilage defect in a subject, comprising propagating the chondrocytes according to the method of the first embodiment.

[0016d] According to a third embodiment of the invention, there is provided use of chondrocytes for the manufacture of a medicament for the treatment of a cartilage defect in a subject, wherein the manufacture comprises propagating the chondrocytes according to the method of the first embodiment.

[0016e] According to a fourth embodiment of the invention, there is provided a method for manufacturing a medicament intended for the treatment of a cartilage defect in a subject, wherein the method comprises propagating chondrocytes according to the method of the first embodiment and the use of said chondrocytes for the manufacture of said medicament.

[0016f] According to a fifth embodiment of the invention, there is provided a culture medium for propagating cells, wherein the culture medium comprises a basal medium that is supplemented with substantially pure oncostatin M (OSM), and wherein the OSM is present at a concentration of from 0.01 ng/ml to 5 ng/ml.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

[0017] Figure 1 depicts a comparison in growth of primary human chondrocytes cultured in (1) DMEM + 10% FBS or (2) the E93 medium (cDRFm, as defined in Table 4, supplemented with CDLM, PDGF, IL-6, and OSM) over three passages.

[0018] Figure 2 depicts a comparison in cell yield of primary human chondrocytes cultured in (1) DMEM + 10% FBS or (2) the E93 medium (cDRFm, as defined in Table 4, supplemented with CDLM, PDGF, IL-6, and OSM) over three passages.

[0019] Figure 3 shows an RPA of cell lysate from chondrocytes grown in E93 (lanes 2, 3, 4) or DMEM+ 10% FBS (lanes 5, 6, 7). The cartilage markers, collagen 2 and aggrecan, are expressed in all samples.

#### **DETAILED DESCRIPTION OF THE INVENTION.**

[0020] This invention provides compositions of chemically defined culture media (DMs), methods of making such media, and methods of using such media, e.g., for culturing cells, such as human articular chondrocytes, for repair of

[Text continues on page 6]

cartilage defects. The invention is based, at least in part, on the discovery that the basal medium referred to as cDRFm supplemented with PDGF and CDLM and one or more cytokines of the IL-6 family is sufficient for attachment, proliferation and maintenance of redifferentiation-capable chondrocytes in culture and can substitute for a serum-containing medium in all stages of cell culture. The cytokines of the IL-6 family of cytokines include, for example, OSM, IL-6, and LIF.

[0021] Accordingly, in one aspect, the invention provides a culture medium comprising a basal medium supplemented with one or more supplements, which include one or more cytokines of the IL-6 family, such as, e.g., OSM, IL-6, and LIF.

[0022] The term "supplemented with" indicates that a supplement has been added to a starting material to arrive at an ending material. Unless specifically indicated, the supplement or supplements need not be added at a specific time or in a specific order. The term "supplemented with" does not preclude the starting material from being additionally supplemented with other supplements, at any point in time, before or after being supplemented with the present supplement. Unless specifically indicated, supplements are added to the medium in a "substantially pure" form. The term "substantially pure" indicates that a supplement is substantially free of components with which it naturally occurs in nature. For example, a substantially pure cytokine could be a purified cytokine or a cytokine that is recombinantly produced.

#### **I. Preparation of Basal Medium**

[0023] The first step in preparing defined, serum-free media (DM) of the invention is to obtain a basal medium. The basal medium may be any suitable medium. In illustrative embodiments, the basal medium is cDRF as defined in Table 3. cDRF can be prepared from commercially available starting components as described below. cDRF is a modification of the DM developed by Adolphe et al. (Exp. Cell Res. 155:527-536 (1984)) and by McPherson et al. (U.S. Patent No. 6,150,163).

[0024] The three starting components of cDRF are DMEM, RPMI-1640, and Ham's F12 (Invitrogen; Carlsbad, CA). The starting components are

combined at a 1:1:1 ratio. All three media can be combined at once, or any two of the media can be premixed and then combined with an appropriate amount of a third medium. The precise composition of starting components is set forth in Table 1. The resulting medium (defined in Table 2 and referred to as DRF) is then supplemented with ITS (10 µg/ml insulin, 5.5 µg/ml transferrin, 7 ng/ml selenium, and, optionally, 2.0 µg/ml ethanolamine; Invitrogen, Carlsbad, CA), human fibronectin (BD Biosciences; San Jose, CA), human serum albumin (HSA) (Grifols; Los Angeles, CA; or Baxter; Westlake Village, CA), linoleic acid (Sigma-Aldrich; St. Louis, MO), human basic fetal growth factor (bFGF) (R&D Systems, Minneapolis, MN), gentamycin (Invitrogen; Carlsbad, CA), and hydrocortisone (Sigma-Aldrich; St. Louis, MO) to create cDRF. All materials are reconstituted, diluted, and stored as per the supplier's recommendations. The exact order of combining components to arrive at a final medium is not essential. The complete medium may be prepared using standard laboratory techniques and stored preferably at 2-8°C until use. In a preferred embodiment, the basal medium is prepared essentially as described above with adjustments to the amount of human serum albumin, linoleic acid, and hydrocortisone to arrive at modified cDRF (cDRFm) as defined in Table 4.

[0025] In some embodiments, the basal medium is a medium that comprises all essential components of cDRF listed in Table 3. A component or a subset of components listed in Table 3 is non-essential if, when its concentration is reduced, or the component is eliminated, the properties of the medium related to chondrocyte attachment, proliferation, and/or redifferentiation, remain substantially the same. The stated concentrations of individual components may be adjusted for specific cell culture conditions. Such adjustments can easily be made by a person skilled in the art using routine techniques.

[0026] Additional components may be added to the medium if such components are desirable and do not negatively impact on chondrocytes attachment, proliferation, and redifferentiation. Such components include, but are not limited to, growth factors, lipids, serum proteins, vitamins, minerals, and carbohydrates. For example, it may be advantageous to supplement the medium with growth factors or hormones that promote chondrocyte redifferentiation such as TGF-β (TGF-β1, -β2, -β3), IGF, and insulin, as described in U.S. Patent No.

6,150,163. Such growth factors and hormones are commercially available. Additional examples of supplements include, but are not limited to, bone morphogenetic proteins (BMPs), of which there are at least 15 structurally and functionally related proteins. BMPs have been shown to be involved in the growth, differentiation, chemotaxis, and apoptosis of various cell types. Recombinant BMP-4 and BMP-6, for example, can be purchased from R&D Systems (Minneapolis, MN). The concentration of various such supplements in DM of the invention can be determined with minimal experimentation. For example, the concentration of BMP in DM of the invention is chosen from 0.01-0.1 ng/ml, 0.1-1 ng/ml, 1-10 ng/ml, 100 ng/ml, 10-50 ng/ml, 50-100 ng/ml, and 0.1-1 µg/ml.

[0027] A skilled artisan will appreciate that DM of the invention have advantages in addition to avoiding the use of serum. However, it may be desirable to utilize the DM of the invention in applications where the use of undefined components is acceptable. Consequently, the DM of the invention may be supplemented with serum e.g., fetal calf serum, or other chemically undefined components such as, for example, animal or plant tissue extracts. Thus, in certain embodiments, the DM of the invention may be supplemented with 10% or less, for example, 8% or less, 6% or less, 4% or less, 2% or less, or 1% or less of serum.

[0028] A skilled artisan will also appreciate that equivalents of cDRF may be prepared from a variety of known media, e.g., Basal Medium Eagle medium (Eagle, Science, 122:501 (1955)), Minimum Essential medium (Dulbecco et al., Virology, 8:396 (1959)), Ham's medium (Ham, Exp. Cell Res. 29:515 (1963)), L-15 medium (Leibvitz, Amer. J. Hyg. 78:173 (1963)), McCoy 5A medium (McCoy et al., Proc. Exp. Biol. Med. 100:115 (1959)), RPMI medium (Moore et al., J. A. M. A. 199:519 (1967)), Williams' medium (Williams, Exp. Cell Res. 69:106-112 (1971)), NCTC 135 medium (Evans et al., Exp. Cell Res. 36:439 (1968)), Waymouth's medium MB752/1 (Waymouth, Nat. Cancer Inst. 22:1003 (1959)), etc. These media may be used singularly or as mixtures in suitable proportions to prepare a basal medium equivalent to cDRF. Alternatively, cDRF or its equivalent can be prepared from individual chemicals or from other media and growth supplements. The invention is not limited to media of any particular

consistency and encompasses the use of media ranging from liquid to semi-solid and includes solidified media and solid compositions suitable for reconstitution.

**Table 1. Compositions of Starting Medium**

	DMEM/F12	RPMI-1640
	1x Liquid, mg/L	1x Liquid, mg/L
<b>Inorganic Salts:</b>		
CaCl <sub>2</sub> (anhyd.)	116.6	--
Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O	--	100
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.0013	--
Fe(NO <sub>3</sub> ) <sub>2</sub> ·9H <sub>2</sub> O	0.05	--
FeSO <sub>4</sub> ·7H <sub>2</sub> O	0.417	--
KCl	311.8	400
MgSO <sub>4</sub> (anhyd.)	48.84	48.84
MgCl <sub>2</sub> (anhyd.)	28.64	--
NaCl	6995.5	6000
NaHCO <sub>3</sub>	1200	2000
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	62.5	--
Na <sub>2</sub> HPO <sub>4</sub> (anhyd.)	71.02	800
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.432	--
<b>Other Components:</b>		
D-Glucose	3151	2000
Glutathione (reduced)	--	1
Hypoxanthine Na	2.39	--
Linoleic Acid	0.42	--
Lipoic Acid	0.105	--
Phenol Red	8.1	5
Putrescine 2HCl	0.081	5
Sodium Pyruvate	55	--
Thymidine	0.365	--
HEPES	--	5300
<b>Amino Acids:</b>		
L-Alanine	4.45	--
L-Arginine	--	200
L-Arginine·HCl	147.5	--
L-Asparagine·H <sub>2</sub> O	7.5	--
L-Asparagine (free base)	--	50
L-Aspartic Acid	6.65	20

**Table 1. Compositions of Starting Medium**

L-Cystine·2HCl	31.29	65
L-Cysteine·HCl·H <sub>2</sub> O	17.56	—
L-Glutamic Acid	7.35	20
L-Glutamine	365	300
Glycine	18.75	10
L-Histidine·HCl·H <sub>2</sub> O	31.48	—
L-Histidine (free base)	—	5
L-Hydroxyproline	—	20
L-Isoleucine	54.47	50
L-Leucine	59.05	50
L-Lysine·HCl	91.25	40
L-Methionine	17.24	15
L-Phenylalanine	35.48	15
L-Proline	17.25	20
L-Serine	26.25	30
L-Threonine	53.45	20
L-Tryptophan	9.02	5
L-Tyrosine·2Na <sub>2</sub> H <sub>2</sub> O	55.79	29
L-Valine	52.85	20
<b>Vitamins:</b>		
Biotin	0.0035	0.2
D-Ca pantothenate	2.24	0.25
Choline Chloride	8.98	3
Folic Acid	2.65	1
I-Inositol	12.6	35
Niacinamide	2.02	1
Para-aminobenzoic Acid	—	1
Pyridoxine HCl	2.031	1
Pyridoxal HCl	—	—
Riboflavin	0.219	0.2
Thiamine HCl	2.17	1
Vitamin B <sub>12</sub>	0.68	0.005

**Table 2. Composition of DRF**

	1× Liquid, mg/L
<b>Inorganic Salts:</b>	
CaCl <sub>2</sub> (anhyd.)	77.7333
Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O	33.3333
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.0009
Fe(NO <sub>3</sub> ) <sub>2</sub> ·9H <sub>2</sub> O	0.0333
FeSO <sub>4</sub> ·7H <sub>2</sub> O	0.2780
KCl	341.2000
MgSO <sub>4</sub> (anhyd.)	48.8400
MgCl <sub>2</sub> (anhyd.)	19.0933
NaCl	6663.6667
NaHCO <sub>3</sub>	1466.6667

**Table 2. Composition of DRF (cont'd)**

NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	41.6667
Na <sub>2</sub> HPO <sub>4</sub> (anhyd.)	314.0133
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.2880
<b>Other Components:</b>	
D-Glucose	2767.3333
Glutathione (reduced)	0.3333
Hypoxanthine Na	1.5933
Linoleic Acid	0.2800
Lipoic Acid	0.0700
Phenol Red	7.0667
Putrescine 2HCl	1.7207
Sodium Pyruvate	36.6667
Thymidine	0.2433
HEPES	1766.6667
<b>Amino Acids:</b>	
L-Alanine	2.9667
L-Arginine	66.6667
L-Arginine·HCl	98.3333
L-Asparagine·H <sub>2</sub> O	5.0000
L-Asparagine (free base)	16.6667
L-Aspartic Acid	11.1000
L-Cystine·2HCl	42.5267
L-Cysteine·HCl·H <sub>2</sub> O	11.7067
L-Glutamic Acid	11.5667
L-Glutamine	343.3333
Glycine	15.8333
L-Histidine·HCl·H <sub>2</sub> O	20.9867
L-Histidine (free base)	1.6667
L-Hydroxyproline	6.6667
L-Isoleucine	52.9800
L-Leucine	56.0333
L-Lysine·HCl	74.1667
L-Methionine	16.4933
L-Phenylalanine	28.6533
L-Proline	18.1667
L-Serine	27.5000
L-Threonine	42.3000
L-Tryptophan	7.6800
L-Tyrosine·2Na <sub>2</sub> H <sub>2</sub> O	46.8600
L-Valine	41.9000
<b>Vitamins:</b>	
Biotin	0.0690
D-Ca pantothenate	1.5767
Choline Chloride	6.9867
Folic Acid	2.1000
I-Inositol	20.0667
Niacinamide	1.6800
Para-aminobenzoic Acid	0.3333
Pyridoxine HCl	1.6873
Pyridoxal HCl	—
Riboflavin	0.2127
Thiamine HCl	1.7800
Vitamin B <sub>12</sub>	0.4550

**Table 3. Composition of cDRF**

	1× Liquid
DRF (Table 2)	99%
ITS-X supplement (insulin, transferrin, selenium, ethanolamine)	1%
<b>Supplements:</b>	
Linoleic Acid	5 µg/ml
Gentamycin	50 µg/ml
Hydrocortisone	40 ng/ml
Fibronectin	1 µg/ml
Basic Fibroblast Growth Factor (bFGF)	10 ng/ml
Human Serum Albumin	1 mg/ml

**Table 4. Composition of cDRFm**

	1× Liquid
DRF (Table 2)	99%
ITS-X supplement (insulin, transferrin, selenium, ethanolamine)	1%
<b>Supplements:</b>	
Gentamycin	50 µg/ml
Hydrocortisone	160 ng/ml
Fibronectin	1 µg/ml
Basic Fibroblast Growth Factor (bFGF)	10 ng/ml
Human Serum Albumin	0.5 mg/ml

## II. Supplementation of Basal Medium

### A. Platelet-Derived Growth Factor (PDGF)

[0029] In some embodiments, the basal medium is supplemented with substantially pure PDGF.

[0030] PDGF is a major mitogenic factor present in serum but not in plasma. PDGF is a dimeric molecule consisting of two structurally related chains designated A and B. The dimeric isoforms PDGF-AA, AB and BB are differentially expressed in various cell types. In general, all PDGF isoforms are potent mitogens for connective tissue cells, including dermal fibroblasts, glial cells, arterial smooth muscle cells, and some epithelial and endothelial cell.

[0031] Recombinantly produced PDGF is commercially available from various sources. Human recombinant PDGF-BB (hrPDGF-BB) used in the Examples below was purchased from R&D Systems (Minneapolis, MN; catalog # 220-BB) and reconstituted and handled according to the manufacturer's instructions. The E. coli expression of hrPDGF-BB and the DNA sequence encoding the 109-amino-acid-residue mature human PDGF-B chain protein (C-terminally processed from that ends with threonine residue 190 in the precursor sequence) is described by Johnson et al. (EMBO J. 3:921 (1984)). The disulfide-linked homodimeric rhPDGF-BB consists of two 109-amino-acid-residue B chains and has molecular weight of about 25 kDa. The activity of PDGF is measured by its ability to stimulate <sup>3</sup>H-thymidine incorporation in quiescent NR6R-3T3 fibroblast as described by Raines et al. (Meth. Enzymol. 109:749-773 (1985)). The ED<sub>50</sub> for PDGF in this assay is typically 1-3 ng/ml.

[0032] The concentration of PDGF is chosen from 0.1-1 ng/ml, 1-5 ng/ml, 5-10 ng/ml, 10 ng/ml, 10-15 ng/ml, 15-50 ng/ml, and 50-100 ng/ml. In certain embodiments, cDRF is supplemented with 1-25 ng/ml, more preferably, 5 - 15 ng/ml and, most preferably, about 10 ng/ml of PDGF. In a particular embodiment, the PDGF is PDGF-BB. Alternatively, PDGF could be of another type, e.g., PDGF-AB, PDGF-BB, or a mix of any PDGF types. In related embodiments, the DM of the invention further or alternatively comprises additional supplements as described below.

## **B. Lipids**

[0033] In some embodiments, the basal medium is supplemented with CDLM (Table 5) or, alternately, one or more lipids from CDLM.

[0034] Lipids are important as structural components as well as potential energy sources in living cells. In vitro, most cells can synthesize lipids from glucose and amino acids present in the culture medium. However, if extracellular lipid is available, lipid biosynthesis is inhibited and the cells utilize free fatty acids, lipid esters, and cholesterol in the medium. Serum is rich in lipids and has been the major source of extracellular lipid for cultured cells. Chemically undefined lipid preparations based on marine oils have been found to be effective in promoting growth of cells in serum free-media in several systems. See, e.g., Weiss et al., *In Vitro* 26:30A (1990); Gorfien et al., *In Vitro* 26:37A (1990); Fike et al., *In Vitro* 26:54A (1990). Thus, supplementation of serum-free media with various lipids to replace those normally supplied by serum may be desirable.

[0035] Suitable lipids for use in the DM of this invention include stearic acid, myristic acid, oleic acid, linoleic acid, palmitic acid, palmitoleic acid, arachidonic acid, linolenic acid, cholesterol, and alpha-tocopherol acetate. In one embodiment, the basal medium is supplemented with the chemically defined lipid mixture (CDLM), shown in Table 5. CDLM is available from Invitrogen. As supplied by Invitrogen, in addition to the lipid components, CDLM contains ethanol (100 g/L) and emulsifiers Pluronic F68<sup>®</sup> (100 g/L) and Tween 80<sup>®</sup> (2.2 g/L).

[0036] In practicing the methods of the invention, the concentrations of individual lipid components of CDLM shown in Table 5 may be adjusted for specific cell culture conditions. Such adjustments can easily be made by a person skilled in the art using routine techniques. Furthermore, not all components of CDLM may be essential. A component or a subset of components is non-essential if, when its concentration is reduced, or the component is eliminated, the properties of the medium related to chondrocyte attachment, proliferation, and redifferentiation, remain substantially the same.

[0037] In certain embodiments, the DM of the invention comprises at least one, two, four, six, eight, or all lipid components of CDLM. In one

embodiment, the DM comprises PDGF and CDLM as defined in Table 5. In other nonlimiting embodiments, the DM comprises PDGF and lipid combinations as set forth in Table 6.

**Table 5. Composition of chemically defined lipid mixture (CDLM)**

Lipid components	mg/L
DL-alpha-tocopherol acetate	70
Stearic acid	10
Myristic acid	10
Oleic acid	10
Linoleic acid	10
Palmitic acid	10
Palmitoleic acid	10
Arachidonic acid	2
Linolenic acid	10
Cholesterol	220

**Table 6. Illustrative Lipid Combinations**

No.	Lipid(s)
1	cholesterol
2	cholesterol, arachidonic acid
3	cholesterol, arachidonic acid, linoleic acid
4	cholesterol, arachidonic acid, linoleic acid, linolenic acid
5	cholesterol, arachidonic acid, linoleic acid, linolenic acid, alpha-tocopherol acetate
6	cholesterol, arachidonic acid, linoleic acid, linolenic acid, alpha-tocopherol acetate, stearic acid
7	cholesterol, arachidonic acid, linoleic acid, linolenic acid, alpha-tocopherol acetate, stearic acid
8	cholesterol, arachidonic acid, linoleic acid, linolenic acid, alpha-tocopherol acetate, stearic acid, myristic acid
9	cholesterol, arachidonic acid, linoleic acid, linolenic acid, alpha-tocopherol acetate, stearic acid, myristic acid, oleic acid
10	cholesterol, arachidonic acid, linoleic acid, linolenic acid, alpha-tocopherol acetate, stearic acid, myristic acid, oleic acid, palmitic acid
11	cholesterol, arachidonic acid, linoleic acid, linolenic acid, alpha-tocopherol acetate, stearic acid, myristic acid, oleic acid, palmitic acid, palmitoleic acid
12	arachidonic acid, linoleic acid, linolenic acid, alpha-tocopherol acetate, stearic acid, myristic acid, oleic acid, palmitic acid, palmitoleic acid
13	arachidonic acid, linoleic acid, linolenic acid, stearic acid, myristic acid, oleic acid, palmitic acid, palmitoleic acid
14	arachidonic acid, linoleic acid, linolenic acid, stearic acid, myristic acid, oleic acid, palmitic acid
15	arachidonic acid, linoleic acid, linolenic acid, stearic acid, myristic acid, oleic acid
16	arachidonic acid, linoleic acid, linolenic acid, stearic acid, myristic acid

**Table 6. Illustrative Lipid Combinations (cont'd)**


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17	arachidonic acid, linoleic acid, linolenic acid, acetate, stearic acid
18	arachidonic acid, linoleic acid, linolenic acid, stearic acid
19	arachidonic acid, linoleic acid, linolenic acid
20	arachidonic acid, linoleic acid
21	arachidonic acid
22	cholesterol, linoleic acid
23	cholesterol, linoleic acid, linolenic acid
24	cholesterol, linoleic acid, linolenic acid, stearic acid
25	cholesterol, linoleic acid, linolenic acid, stearic acid, myristic acid
26	cholesterol, linoleic acid, linolenic acid, stearic acid, myristic acid, oleic acid
27	cholesterol, linoleic acid, linolenic acid, stearic acid, myristic acid, oleic acid, palmitic acid
28	cholesterol, linoleic acid, linolenic acid, stearic acid, myristic acid, oleic acid, palmitic acid, palmitoleic acid
29	cholesterol, linoleic acid, linolenic acid, alpha-tocopherol acetate, stearic acid, myristic acid, oleic acid, palmitic acid, palmitoleic acid
30	linoleic acid
31	cholesterol, linoleic acid
32	cholesterol, arachidonic acid, linoleic acid
33	cholesterol, arachidonic acid, linoleic acid, linolenic acid
34	cholesterol, arachidonic acid, linoleic acid, linolenic acid, alpha-tocopherol acetate
35	cholesterol, arachidonic acid, linoleic acid, linolenic acid, alpha-tocopherol acetate, stearic acid
36	cholesterol, arachidonic acid, linoleic acid, linolenic acid, alpha-tocopherol acetate, stearic acid, myristic acid
37	cholesterol, arachidonic acid, linoleic acid, linolenic acid, alpha-tocopherol acetate, stearic acid, myristic acid, oleic acid
38	cholesterol, arachidonic acid, linoleic acid, linolenic acid, alpha-tocopherol acetate, stearic acid, myristic acid, oleic acid
39	cholesterol, arachidonic acid, linoleic acid, linolenic acid, alpha-tocopherol acetate, stearic acid, myristic acid, oleic acid, palmitic acid, palmitoleic acid
40	linolenic acid
41	cholesterol, linolenic acid
42	cholesterol, alpha-tocopherol acetate, stearic acid, myristic acid, oleic acid, palmitic acid, palmitoleic acid
43	cholesterol, alpha-tocopherol acetate
44	cholesterol, stearic acid, myristic acid, oleic acid, palmitic acid, palmitoleic acid
45	stearic acid, myristic acid, oleic acid, palmitic acid, palmitoleic acid
46	cholesterol, myristic acid, oleic acid, palmitic acid, palmitoleic acid
47	cholesterol, oleic acid, palmitic acid, palmitoleic acid
48	cholesterol, stearic acid, myristic acid, oleic acid, palmitic acid, palmitoleic acid
49	cholesterol, myristic acid, oleic acid, palmitic acid
50	cholesterol, arachidonic acid, linoleic acid, linolenic acid, palmitic acid, palmitoleic acid

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[0038] In certain embodiments, the concentration (v/v) of lipids in the culture medium is chosen from 0.05-0.1%, 0.1-0.5%, 0.5%, 0.5-1%, 1-2%, and 2-5%. In certain other embodiments, the DM is additionally supplemented with 1

to 25 ng/ml, more preferably, 5 to 15 ng/ml, and, most preferably, about 10 ng/ml of PDGF. In a particular embodiment, the DM comprises approximately 0.5% (v/v) CDLM and 10 ng/ml PDGF.

### **C. IL-6-Family Cytokines**

[0039] Members of the IL-6 family of cytokines each can utilize a shared signal transducing receptor subunit, gp130, which is found in a wide range of cell types. See, e.g., Hirano et al. (2001) IL-6 Ligand and Receptor Family. In: Cytokine Reference, Academic Press, San Diego, 523-535. Examples of IL-6-family cytokines include, but are not limited to, oncostatin M (OSM), interleukin-6 (IL-6), leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), interleukin-11 (IL-11), cardiotrophin 1 (CT-1), and neurotrophin 1 / B cell-stimulating factor 3 (NNT-1/BSF-3). Cytokines of the IL-6 family have been found to regulate cell growth and differentiation in a wide variety of biological systems, including hematopoiesis, neurogenesis, and osteogenesis. Bruce et al., Prog. Growth Factor Res. 4:157-170 (1992).

#### **1. Oncostatin M (OSM)**

[0040] Human OSM is a secreted glycoprotein that is initially translated as a 252-amino-acid polypeptide with a 25-residue hydrophobic signal sequence at the N-terminus that is removed during the secretion process. An additional post-translational cleavage event removes 31 C-terminal residues, leaving a 192-amino-acid disulfide-linked mature protein. Rose et al., Proc. Nat. Acad. Sci. USA 88:8641-8645 (1991); Robinson et al., Cell 77:1101-1116 (1994). In humans, OSM binds and signals through two different receptor complexes - the LIF receptor (LIFR) / gp130 heterodimer and the OSM receptor (OSMR) / gp130 heterodimer. Binding to either receptor complex leads to activation of the Janus kinase / signal transducers and activators of transcription (JAK/STAT) and mitogen-activated protein kinase (MAPK) signaling pathways. Heinrich et al., Biochem. J. 374:1-20 (2003).

[0041] OSM has been reported to inhibit the growth of some, but not all, human tumor cell lines. In contrast, OSM has also been reported to stimulate the growth of some normal fibroblasts, such as human foreskin fibroblasts or WI-38 cells. Zarling et al., Proc. Nat. Acad. Sci. USA 83:9739-9743 (1986). Thus, OSM

may be useful for stimulating the growth of certain cells in vitro. A more detailed description of OSM can be found in U.S. Patent Nos. 5,202,116 and 5,814,307.

[0042] OSM is readily available from commercial sources. In the Examples below, a 196-amino-acid recombinant OSM produced in *E. coli* was obtained from R&D Systems (Minneapolis, MN) (catalog No.295-OM, see also Linsley et al., *Mol. Cell. Biol.* 10:1882-1890 (1990)). The biological activity of OSM may be assayed by testing in a human erythroleukemic cell line proliferation assay, as described, e.g., in Kitamura et al., *J. Cell. Physiol.* 140:323-334 (1989). In a preferred embodiment, human OSM, is used to produce the media of the invention. However, one skilled in the art would recognize that OSM from other species, naturally occurring mutants, and engineered mutants may also be effective.

## **2. Interleukin-6 (IL-6)**

[0043] IL-6 has many alternative names, including: interferon  $\beta$ 2; B-cell differentiation factor; B-cell stimulatory factor 2; hepatocyte stimulatory factor; hybridoma growth factor; and CTL differentiation factor. Human IL-6 is a 186-amino-acid secreted glycoprotein that is synthesized as a 212-amino-acid precursor protein. Matsuda et al., (2001) IL-6. In: *Cytokine Reference*, Academic Press, San Diego, 538-563. In humans, IL-6 binds and signals through a complex of the IL-6 receptor (IL-6R) and a gp130 homodimer. Binding of IL-6 to the IL-6R receptor leads to activation of the Janus kinase / signal transducers and activators of transcription (JAK/STAT) and mitogen-activated protein kinase (MAPK) signaling pathways. Heinrich et al., *Biochem. J.* 374:1-20 (2003).

[0044] IL-6 has been reported to induce differentiation of PC12 neuronal cells, to induce clonogenic maturation of bone marrow progenitor cells, and to induce the growth of T cells. In contrast, IL-6 has also been shown to inhibit the growth of myeloid leukemia cells and breast cancer cells. Thus, IL-6 may be useful for stimulating the growth of certain cells in vitro. A more detailed description of IL-6 biology can be found in U.S. Patent No. 5,188,828.

[0045] IL-6 is available from commercial sources. In the Examples below, a 184-amino-acid recombinant IL-6 produced in *E. coli* was obtained from R&D Systems (Minneapolis, MN) (catalog No.206-IL, see also Hirano et al.,

Nature 324:73-76 (1986)). The biological activity of IL-6 is assayed by testing in a plasmacytoma proliferation assay as described in, e.g., Nordan et al., J. Immunol. 139:813 (1987). In a preferred embodiment, human IL-6 is used to produce the media of the invention. However, one skilled in the art would recognize that IL-6 from other species, naturally occurring mutants, and engineered mutants may also be effective.

### **3. Leukemia Inhibitory Factor (LIF)**

[0046] LIF has several alternative names, including: cholinergic differentiation factor; human interleukin in DA cells; differentiation stimulating factor; MLPLI; and Emfilermin. Human LIF is a 180-amino-acid secreted glycoprotein. Kondera-Anasz et al., Am. J. Reprod. Immunol. 52:97-105 (2004). In humans, LIF binds and signals through the LIF receptor (LIFR) / gp130 heterodimer. Binding of LIF to the LIF receptor leads to activation of the Janus kinase / signal transducers and activators of transcription (JAK/STAT) and mitogen-activated protein kinase (MAPK) signaling pathways. Heinrich et al., Biochem. J. 374:1-20 (2003).

[0047] LIF has been reported to inhibit the proliferation of M1 myeloid leukemia cells. See, e.g., U.S. Patent No. 5,443,825. In contrast, LIF has also been reported to stimulate the growth of neurons as well as to promote the differentiation of neurons from an adrenal medullary phenotype to an acetylcholinergic phenotype. See, e.g., U.S. Patent No. 5,968,905. The addition of LIF to severed nerves can also enhance nerve regeneration. See, e.g., U.S. Patent No. 6,156,729. Thus, LIF may be useful for promoting the growth of certain cells in vitro.

[0048] LIF is available from commercial sources. In the Examples below, a 181-amino-acid recombinant human LIF produced in *E. coli* was obtained from Sigma-Aldrich (St. Louis, MO) (catalog No. L 5283, see also Gearing et al., EMBO J. 6:3995 (1987)). The biological activity of LIF is assayed by testing for its ability to stimulate the differentiation of M1 mouse myeloid leukemia cells as described, e.g., in Gearing et al., EMBO J. 6:3995 (1987). In a preferred embodiment, human LIF is used to produce the media of the invention. However,

one skilled in the art would recognize that LIF from other species, naturally occurring mutants, and engineered mutants may also be effective.

[0049] In certain embodiments, the DM of the invention is cDRF supplemented with PDGF, one or more lipids selected from the group consisting of stearic acid, myristic acid, oleic acid, linoleic acid, palmitic acid, palmitoleic acid, arachidonic acid, linolenic acid, cholesterol, and alpha-tocopherol acetate, and one or more cytokines. In particular embodiments, DM of the invention is cDRF supplemented with PDGF, one or more lipids selected from the group consisting of stearic acid, myristic acid, oleic acid, linoleic acid, palmitic acid, palmitoleic acid, arachidonic acid, linolenic acid, cholesterol, and alpha-tocopherol acetate, and one or more of the group consisting of OSM, IL-6, and LIF. The concentration of cytokine is chosen from 0.01-0.1 ng/ml, 0.1-1 ng/ml, 1-5 ng/ml, 5-10 ng/ml, 10-15 ng/ml, 15-50 ng/ml, and 50-100 ng/ml. In certain embodiments, cDRF is supplemented with 0.01-10 ng/ml, more preferably, 0.1-2 ng/ml and, most preferably, 0.5-1 ng/ml of OSM, IL-6, and/or LIF. In a preferred embodiment, cDRF is supplemented with approximately 10 ng/ml PDGF, 0.5% CDLM, 1 ng/ml IL-6, and 0.5 ng/ml OSM. In related embodiments, the DM of the invention further comprises additional supplements as described below.

[0050] In certain embodiments, the DM of the invention comprises at least one, two, or all three of OSM, IL-6, and LIF. In other nonlimiting embodiments, the DM comprises combinations of OSM, IL-6, and LIF as set forth in Table 7. In additional nonlimiting embodiments, the DM comprises any combination of OSM, IL-6, and LIF set forth in Table 7, PDGF, and CDLM as defined in Table 5. In additional nonlimiting embodiments, the DM comprises any combination of OSM, IL-6, LIF, PDGF, and lipids set forth in Table 7. In a preferred embodiment, the DM comprises OSM, IL-6, PDGF and CDLM as defined in Table 5. In a further preferred embodiment, the DM is cDRFm as defined in Table 4. For example, the medium may comprise cDRFm, OSM, IL-6, PDGF and CDLM.

**Table 7. Illustrative Combinations of OSM, IL-6, and LIF**

	Supplemented with IL-6-family Cytokine(s)	Supplemented with PDGF	Supplemented with CDLM or lipids of Table 6
1	OSM	no	no
2	OSM	yes	no
3	OSM	no	yes
4	OSM	yes	yes
5	IL-6	no	no
6	IL-6	yes	no
7	IL-6	no	yes
8	IL-6	yes	yes
9	LIF	no	no
10	LIF	yes	no
11	LIF	no	yes
12	LIF	yes	yes
13	OSM, IL-6	no	no
14	OSM, IL-6	yes	no
15	OSM, IL-6	no	yes
16	OSM, IL-6	yes	yes
17	OSM, LIF	no	no
18	OSM, LIF	yes	no
19	OSM, LIF	no	yes
20	OSM, LIF	yes	yes
21	IL-6, LIF	no	no
22	IL-6, LIF	yes	no
23	IL-6, LIF	no	yes
24	IL-6, LIF	yes	yes
25	OSM, IL-6, LIF	no	no
26	OSM, IL-6, LIF	yes	no
27	OSM, IL-6, LIF	no	yes
28	OSM, IL-6, LIF	yes	yes

**D. Additional Supplements**

[0051] The DM of the invention may optionally be supplemented with any number of additional supplements needed to promote the growth of cells in culture. Such supplements may include, but are not limited to, BMP family members, TGF- $\beta$  family members, IGF, and insulin.

[0052] The medium of the invention can be used to seed, grow, and maintain chondrocytes capable of redifferentiation in culture without the use of serum. The stated ranges of concentrations of PDGF, lipids, OSM, IL-6, and LIF may need to be adjusted for specific cell culture conditions. Such adjustments can easily be made by a person skilled in art using routine techniques.

[0053] In some embodiments, the culture medium of the invention is not supplemented with substantially pure jagged 1 (JAG1) and/or substantially pure interleukin-13 (IL-13).

[0054] In some embodiments, the culture medium of the invention is not supplemented with any of the specific combinations of supplements set forth in U.S. Patent Application Publication Nos. US 2005/0265980 A1 (e.g., at paragraphs 59 to 68) and US 2005/0090002 A1 (e.g., at paragraphs 10 to 14), although it may be supplemented with a subset of any combination disclosed therein as long as the medium excludes at least one or more of the supplements from that combination. For example, in some embodiments, the culture medium of the invention is not supplemented with any specific one, two, three, four or more supplements selected from the group consisting of substantially pure epidermal growth factor (EGF), substantially pure stem cell factor (SCF), substantially pure insulin-like growth factor 1 (IGF-1), substantially pure brain-derived neurotrophic factor (BDNF), substantially pure erythropoietin (EPO), substantially pure FMS-related tyrosine kinase-3 (Flt-3/Flk-2) ligand, and/or a substantially pure member of the wingless-type MMTV integration site (WNT) family. In some embodiments, the medium of the invention does not contain dexamethasone.

### **III. Chondrocytes and Other Suitable Cells**

[0055] The methods of the invention can be used with any suitable cells. The methods are particularly suitable for ex vivo propagation of cells capable of producing cartilaginous tissue, such as chondrocytes.

[0056] Chondrocytes are cells found in various types of cartilage, e.g., hyaline cartilage, elastic cartilage, and fibrocartilage. Chondrocytes are mesenchymal cells that have a characteristic phenotype based primarily on the

type of extracellular matrix they produce. Precursor cells produce type I collagen, but when they become committed to the chondrocyte lineage, they stop producing type I collagen and start synthesizing type II collagen, which constitutes a substantial portion of the extracellular matrix. In addition, committed chondrocytes produce proteoglycan aggregate, called aggrecan, which has glycosaminoglycans that are highly sulfated.

[0057] The term "chondrocyte", as used herein, refers to a differentiated cell obtained from the cartilage, including a de-differentiated chondrocyte as grown in culture which retains the capacity to differentiate into a chondrocyte. The term "chondrocyte" refers to a chondrocyte regardless of whether it is primary or passaged, autologous, heterologous, allogeneic, xenologous, etc.

[0058] Chondrocytes used in the present invention can be isolated by any suitable method. Various starting materials and methods for chondrocyte isolation are well known in the art. Freshney, *Culture of Animal Cells: A Manual of Basic Techniques*, 2d ed. A. R. Liss, Inc., New York, pp. 137-168 (1987); Klagsburn, *Methods Enzymol.* 58:560-564 (1979); R. Tubo and L. Brown, *Articular Cartilage*. In: *Human Cell Culture; Volume V*, Koller et al. (eds.) (2001); and Kandel et al., *Art. Cells, Blood Subs., and Immob. Biotech.* 25(5), 565-577 (1995). By way of example, articular cartilage can be harvested from femoral condyles of human donors, and chondrocytes can be released from the cartilage by overnight digestion in 0.1% collagenase/DMEM. The released cells are expanded as primary cells in a suitable medium such as the DM of this invention or DMEM containing 10% FBS.

[0059] It may be desirable in certain circumstances to grow chondrocyte progenitor stem cells such as mesenchymal stem cells rather than cells from cartilage biopsies that are already differentiated into chondrocytes. Chondrocytes can be obtained upon differentiation of such cells into chondrocytes. Examples of tissues from which such stem cells can be isolated include synovium, placenta, umbilical cord, bone marrow, adipose, skin, muscle, periosteum, or perichondrium.

[0060] Besides chondrocytes and chondrocyte progenitor stem cells, it may be desirable in certain circumstances to utilize other cells with chondrocytic

potential, such as cells of mesenchymal lineage that can be trans-differentiated into chondrocytes. Chondrocytes can be obtained by inducing differentiation of such cells into chondrocytes in vitro. Examples of such other cells with chondrocytic potential include osteoblasts, myocytes, adipocytes, fibroblasts, epithelial cells, keratinocytes, and neuronal cells.

[0061] Chondrocytes, chondrocyte progenitor cells, and other cells with chondrocytic potential may be cultured to a state that is suitable for treating a patient suffering from a cartilage defect. Such therapeutically useful chondrocytes should express articular cartilage-specific extracellular matrix components, including, but not limited to, type II collagen and proteoglycans characteristic of hyaline-like articular cartilage. Assays to determine the differentiation state of chondrocytes are known in the art and described in, e.g., R. Tubo and L. Brown, *Articular Cartilage*. In: *Human Cell Culture*; Volume V, Koller et al., eds. (2001) and the Examples.

[0062] Other cells for which the DM of the present invention may be used include any primary or passaged cells, or cells as part of cultured tissues, that are capable of growing in the DM. Examples of other cells include hepatocytes, beta cells, and islet cells.

[0063] Chondrocytes and other cells can be isolated from any mammal, including, without limitation, human, orangutan, monkey, chimpanzee, dog, cat, rat, rabbit, mouse, horse, cow, pig, elephant, etc. Cells for which the DM of the present invention may be used include any primary or passaged cells, or cells as part of cultured tissues, that are capable of growing in the DM.

#### **IV. Methods of Cell Culture**

[0064] The cell can be cultured using any suitable cell culture methods appropriate for a particular cell type and application. Methods cell culture are well known in the art and described in, e.g., J. M. Davis, *Basic Cell Culture*, 2d ed. Oxford U. Press, 2002.

[0065] For example, chondrocytes can be passaged at 80-90% confluence using 0.05% trypsin-EDTA, diluted for subculture, and reseeded for second and subsequent passages to allow for further expansion. Trypsin and

EDTA are both readily available from Invitrogen (Carlsbad, CA). Alternatively, cells may be passaged by incubation with a solution containing a chelating agent such as EDTA. The use of such chelating agents for the non-enzymatic detachment of cells is well known in the art. In a particular embodiment, cells grown in the DM of the invention are passaged using 0.1 mM to 1 mM EDTA. In a preferred embodiment, cells grown in the DM of the invention are passaged using less than 0.0025% (or 325 units/ml), preferably 0.00025% (or 32.5 units/ml), recombinant trypsin in 0.1 mM to 1 mM EDTA. At any time, cells can be collected and frozen in DMEM containing 10% DMSO and 40% HSA or in other compositions known in the art, e.g., as described in U.S. Patent No. 6,365,405.

[0066] In some embodiments, cells can be initially cultured at low density. The term "low density" refers to seeding densities less than 20,000 cells/cm<sup>2</sup>.

[0067] The methods of this invention are suitable for cells growing in cultures under various conditions including, but not limited to, monolayers, multilayers, on solid support, in suspension, and in 3D cultures.

## **V. Methods of Evaluating Media**

[0068] In some embodiments, a medium of the invention can be tested for the capacity to maintain cells in a differentiation-competent state, and in particular, for differentiation/redifferentiation into chondrocytes when the cells are placed in a permissive environment. Proteoglycan, aggrecan and collagen II are examples of components of the extracellular matrix normally secreted by chondrocytes in vivo and may serve as markers of chondrocyte function. The capacity of medium to maintain chondrocyte differentiation potential may be determined by agarose and/or alginate assays. The agarose assay identifies the formation of proteoglycan by cells grown in a three-dimensional agarose matrix and is described in, e.g., Benya et al., *Cell* 30:215-224 (1982). The alginate assay measures expression of aggrecan and collagen II genes in cells cultured in an alginate suspension and is described in, e.g., Yaeger et al., *Exp. Cell. Res.* 237(2):318-25 (1997); and Gagne et al., *J. Orthop Res.* 18(6):882-890 (2000).

## **VI. Methods of Using Cells**

[0069] The invention further provides cells cultured using the methods of the invention and methods of using such cells, e.g., in therapy, e.g., for treating a subject by administering to the subject such cells. For example, the methods include repair of cartilage defects (e.g., due to trauma or osteoarthritis) by administering chondrocytes (e.g., autologous chondrocytes) cultured in accordance with the methods of the invention.

### **EXAMPLES**

[0070] Various aspects of the invention are further described and illustrated in the Examples presented below.

#### **Example 1: IL-6 increases cell yield and proliferation of primary human chondrocytes**

[0071] Primary human chondrocytes were isolated from biopsies of articular cartilage by mincing of the sample followed by enzymatic digestion with 0.25% protease type XIV (*Streptomyces griseus*) for one hour and then 0.1% collagenase overnight at 37 °C. Cells were recovered by centrifugation for five minutes at 1,000 x g and resuspended in the appropriate test medium. Cells grown in DMEM + 10% FBS were plated at a density of 3,000 cells per cm<sup>2</sup> and cells grown in serum-free medium were plated at a density of 5,000 cells per cm<sup>2</sup>. T75 flasks were used for all experiments. The following media were tested:

- 1) DMEM + 10% FBS
- 2) cDRF/P/L as defined in Table 8
- 3) cDRF/P/L as defined in Table 8, supplemented with 0.2 ng/ml IL-6
- 4) cDRF/P/L as defined in Table 8, supplemented with 1.0 ng/ml IL-6

[0072] Cells were passaged upon reaching 50% to 80% confluence. Cells grown in DMEM + 10% FBS were rinsed with PBS, harvested by exposure to 325 units/ml trypsin in EDTA, counted, and reseeded. Cells grown in serum-free media were rinsed with PBS, harvested by exposure to 0.00025% Trypzean<sup>TM</sup> (0.1x recombinant trypsin; Sigma-Aldrich, St. Louis, MO) in 0.5 mM EDTA, counted and reseeded. Cell yield was determined and population

doublings calculated at the end of each passage. Cell yield was greatest for cells grown in cDRF/P/L + IL-6 at all passages examined (Table 9). The growth index for cells grown in cDRF/P/L + IL-6 was roughly equal to the growth index for cells grown in DMEM + 10% FBS and exceeded that of cells grown in cDRF/P/L alone (Table 10). These results indicate that cDRF/P/L supplemented with IL-6 is an effective replacement for serum-containing media.

**Table 8. Composition of cDRF/P/L**

<b>1x Liquid</b>	
DRF (Table 2)	99%
ITS-X supplement (insulin, transferrin, selenium, ethanolamine)	1%
<b>Supplements:</b>	
Linoleic Acid	5 µg/ml
Gentamycin	50 µg/ml
Hydrocortisone	40 ng/ml
Fibronectin	1 µg/ml
Basic Fibroblast Growth Factor (bFGF)	10 ng/ml
Human Serum Albumin	1 mg/ml
Chemically defined lipid mixture (CDLM)	5 µl/ml
Platelet derived growth factor (PDGF)	10 ng/ml

**Table 9.**

Medium	Cell yield per T75, x 10 <sup>5</sup>		
	Passage #1	Passage #2	Passage #3
DMEM + 10% FBS	7.1	22.1	35.7
cDRF/P/L	13.7	29.1	26.0
cDRF/P/L + 0.2 ng/ml IL-6	20.6	29.1	119.0
cDRF/P/L + 1 ng/ml IL-6	20.3	50.1	118.0

**Table 10.**

Medium	Growth Index (population doubling/day)		
	Passage #1	Passage #2	Passage #3
DMEM + 10% FBS	0.23	0.41	0.67
cDRF/P/L	0.19	0.27	0.28
cDRF/P/L + 0.2 ng/ml IL-6	0.22	0.42	0.71
cDRF/P/L + 1 ng/ml IL-6	0.22	0.54	0.71

**Example 2: OSM increases cell yield and proliferation of primary human chondrocytes**

[0073] Primary human chondrocytes were isolated from biopsies of articular cartilage by mincing of the sample followed by enzymatic digestion with 0.25% protease type XIV (*Streptomyces griseus*) for one hour and then 0.1% collagenase overnight at 37 °C. Cells were recovered by centrifugation for five minutes at 1,000 x g and resuspended in the appropriate test medium. Cells grown in DMEM + 10% FBS were plated at a density of 3,000 cells per cm<sup>2</sup> and cells grown in serum-free medium were plated at a density of 5,000 cells per cm<sup>2</sup>. T75 flasks were used for all experiments. The following media were tested:

- 1) DMEM + 10% FBS
- 2) cDRF/P/L as defined in Table 8
- 3) cDRF/P/L as defined in Table 8, supplemented with 0.1 ng/ml OSM
- 4) cDRF/P/L as defined in Table 8, supplemented with 0.5 ng/ml OSM
- 5) cDRF/P/L as defined in Table 8, supplemented with 1.0 ng/ml OSM

[0074] Cells were passaged upon reaching 50% to 80% confluence. Cells grown in DMEM + 10% FBS were rinsed with PBS, harvested by exposure to 325 units/ml trypsin in EDTA, counted, and reseeded. Cells grown in serum-free media were rinsed with PBS, harvested by exposure to 0.00025% Trypzean™ (0.1x recombinant trypsin; Sigma-Aldrich, St. Louis, MO) in 0.5 mM EDTA, counted and reseeded. Cell yield was determined and population doublings calculated at the end of each passage. The data in Table 11 indicates that cell yield was greatest for cells grown in cDRF/P/L + OSM at all passages

examined. The growth index for cells in cDRF/P/L + OSM was roughly equal to the growth index for cells in DMEM + 10% FBS and exceeded that of cells in cDRF/P/L alone (Table 12). These results indicate that cDRF/P/L supplemented with OSM is an effective replacement for serum-containing media.

**Table 11.**

Medium	Cell yield per T75, x 10 <sup>5</sup>		
	Passage #1	Passage #2	Passage #3
DMEM + 10% FBS	12.7	25.6	23.4
cDRF/P/L	17.7	22.4	13.8
cDRF/P/L + 0.1 ng/ml OSM	37.5	26.4	48.6
cDRF/P/L + 0.5 ng/ml OSM	25.6	62.2	41.6
cDRF/P/L + 1 ng/ml OSM	22.0	45.8	41.2

**Table 12.**

Medium	Growth Index (population doubling/day)		
	Passage #1	Passage #2	Passage #3
DMEM + 10% FBS	0.37	0.5	0.42
cDRF/P/L	0.24	0.32	0.19
cDRF/P/L + 0.1 ng/ml OSM	0.31	0.47	0.37
cDRF/P/L + 0.5 ng/ml OSM	0.33	0.58	0.50
cDRF/P/L + 1 ng/ml OSM	0.37	0.52	0.43

### **Example 3: LIF increases cell yield and proliferation of primary human chondrocytes**

[0075] Primary human chondrocytes were isolated from biopsies of articular cartilage by mincing of the sample followed by enzymatic digestion with 0.25% protease type XIV (*Streptomyces griseus*) for one hour and then 0.1% collagenase overnight at 37 °C. Cells were recovered by centrifugation for five minutes at 1,000 x g and resuspended in the appropriate test medium. Cells grown in DMEM + 10% FBS were plated at a density of 3,000 cells per cm<sup>2</sup> and cells grown in serum-free medium were plated at a density of 5,000 cells per cm<sup>2</sup>. T75 flasks were used for all experiments. The following media were tested:

- 1) DMEM + 10% FBS

- 2) cDRF/P/L as defined in Table 8
- 3) cDRF/P/L as defined in Table 8, supplemented with 0.1 ng/ml LIF
- 4) cDRF/P/L as defined in Table 8, supplemented with 0.5 ng/ml LIF
- 5) cDRF/P/L as defined in Table 8, supplemented with 2.0 ng/ml LIF

[0076] Cells were passaged upon reaching 50% to 80% confluence. Cells grown in DMEM + 10% FBS were rinsed with PBS, harvested by exposure to 325 units/ml trypsin in EDTA, counted, and reseeded. Cells grown in serum-free media were rinsed with PBS, harvested by exposure to 0.00025% Trypzean™ (0.1x recombinant trypsin; Sigma-Aldrich, St. Louis, MO) in 0.5 mM EDTA, counted and reseeded. Cell yield was determined and population doublings calculated at the end of each passage. The data in Table 13 indicates that after the first passage, cell yield was greatest for cells grown in cDRF/P/L + LIF. The growth index for cells in cDRF/P/L + LIF was greater than the growth index for cells in cDRF/P/L alone after the second passage (Table 14). These results indicate that cDRF/P/L supplemented with LIF is an effective replacement for serum-containing media.

**Table 13.**

Medium	Cell yield per T75, x 10 <sup>5</sup>		
	Passage #1	Passage #2	Passage #3
DMEM + 10% FBS	16.1	30.2	20.8
cDRF/P/L	18.5	34.4	7.8
cDRF/P/L + 0.1 ng/ml LIF	18.5	45.4	49.6
cDRF/P/L + 0.5 ng/ml LIF	18.7	46.6	44.6
cDRF/P/L + 2 ng/ml LIF	15.0	51.6	43.6

**Table 14.**

Medium	Growth Index (population doubling/day)		
	Passage #1	Passage #2	Passage #3
DMEM + 10% FBS	0.44	0.54	0.40
cDRF/P/L	0.20	0.20	0.15
cDRF/P/L + 0.1 ng/ml LIF	0.20	0.40	0.27
cDRF/P/L + 0.5 ng/ml LIF	0.20	0.40	0.30
cDRF/P/L + 2 ng/ml LIF	0.19	0.54	0.25

**Example 4: IL-6 and OSM together increase cell yield of primary human chondrocytes**

[0077] Primary human chondrocytes were isolated from biopsies of articular cartilage by mincing of the sample followed by enzymatic digestion with 0.25% protease type XIV (*Streptomyces griseus*) for one hour and then 0.1% collagenase overnight at 37 °C. Cells were recovered by centrifugation for five minutes at 1,000 x g and resuspended in the appropriate test medium. Cells grown in DMEM + 10% FBS were plated at a density of 3,000 cells per cm<sup>2</sup> and cells grown in serum-free medium were plated at a density of 5,000 cells per cm<sup>2</sup>. T75 flasks were used for all experiments. The following media were tested:

- 1) DMEM + 10% FBS
- 2) cDRF/P/L as defined in Table 8
- 3) cDRF/P/L as defined in Table 8, supplemented with 1.0 ng/ml IL-6
- 4) cDRF/P/L as defined in Table 8, supplemented with 0.5 ng/ml OSM
- 5) cDRF/P/L as defined in Table 8, supplemented with 1.0 ng/ml IL-6 + 0.5 ng/ml OSM

[0078] Cells were passaged upon reaching 50% to 80% confluence. Cells grown in DMEM + 10% FBS were rinsed with PBS, harvested by exposure to 325 units/ml trypsin in EDTA, counted, and reseeded. Cells grown in serum-free media were rinsed with PBS, harvested by exposure to 0.00025% Trypzean™ (0.1x recombinant trypsin; Sigma-Aldrich, St. Louis, MO) in 0.5 mM EDTA, counted and reseeded. Cell yield was determined and population doublings calculated at the end of each passage. The data in Table 15 indicates that cell yield was greatest for cells grown in cDRF/P/L + IL-6, cDRF/P/L + OSM, or cDRF/P/L + IL-6 + OSM. These results indicate that cDRF/P/L supplemented with IL-6 and OSM is an effective replacement for serum-containing media.

**Table 15.**

Medium	Cell yield per T75, x 10 <sup>5</sup>		
	Passage #1	Passage #2	Passage #3
DMEM + 10% FBS	9.8	14.0	9.0
cDRF/P/L	14.0	30.3	32.4
cDRF/P/L + 1.0 ng/ml IL-6	24.1	29.6	46.5
cDRF/P/L + 0.5 ng/ml OSM	19.4	68.0	30.5
cDRF/P/L + 1 ng/ml IL-6 + 0.5 ng/ml OSM	16.9	77.3	91.7

**Example 5: JAG-1 inhibits growth of chondrocytes in serum-free medium**

[0079] Primary human chondrocytes were isolated from biopsies of articular cartilage by mincing of the sample followed by enzymatic digestion with 0.25% protease type XIV (*Streptomyces griseus*) for one hour and then 0.1% collagenase overnight at 37 °C. Cells were recovered by centrifugation for five minutes at 1,000 x g and resuspended in the appropriate test medium. Cells grown in DMEM + 10% FBS were plated at a density of 3,000 cells per cm<sup>2</sup> and cells grown in serum-free medium were plated at a density of 5,000 cells per cm<sup>2</sup>. T75 flasks were used for all experiments. The following media were tested:

- 1) DMEM + 10% FBS
- 2) cDRF/P/L as defined in Table 8
- 3) cDRF/P/L as defined in Table 8, supplemented with 2.0 µg/ml JAG-1

[0080] Cells were passaged upon reaching 50% to 80% confluence. Cells grown in DMEM + 10% FBS were rinsed with PBS, harvested by exposure to 325 units/ml trypsin in EDTA, counted, and reseeded. Cells grown in serum-free media were rinsed with PBS, harvested by exposure to 0.00025% Trypzean™ (0.1x recombinant trypsin; Sigma-Aldrich, St. Louis, MO) in 0.5 mM EDTA, counted and reseeded. Cell yield was determined and population doublings calculated at the end of each passage. The data in Table 16 indicates that the cell yield in cDRF/P/L was roughly 2-fold greater than the yield in DMEM + 10% FBS. However, when JAG-1 was added to cDRF/P/L, the cell yield

decreased approximately 16-fold compared to cDRF/P/L alone. Likewise, the growth index for cells grown in the presence of JAG-1 was only 0.004, compared to 0.24 for cells in cDRF/P/L without JAG-1. These results indicate that, under the conditions tested, cDRF/P/L supplemented with JAG-1 is not an effective replacement for serum-containing media.

**Table 16.**

Medium	Cell Yield (per T75, x 10 <sup>5</sup> )	Growth Index (Pop. Doubling/Day)
DMEM + 10% FBS	10.90	0.23
cDRF/P/L	21.70	0.24
cDRF/P/L + 2 µg/ml JAG-1	1.32	0.004

**Example 6: IL-13 inhibits growth of chondrocytes in serum-free medium**

[0081] Primary human chondrocytes were isolated from biopsies of articular cartilage by mincing of the sample followed by enzymatic digestion with 0.25% protease type XIV (*Streptomyces griseus*) for one hour and then 0.1% collagenase overnight at 37 °C. Cells were recovered by centrifugation for five minutes at 1,000 x g and resuspended in the appropriate test medium. Cells grown in DMEM + 10% FBS were plated at a density of 3,000 cells per cm<sup>2</sup> and cells grown in serum-free medium were plated at a density of 5,000 cells per cm<sup>2</sup>. T75 flasks were used for all experiments. The following media were tested:

- 1) DMEM + 10% FBS
- 2) cDRF/P/L as defined in Table 8
- 3) cDRF/P/L as defined in Table 8, supplemented with 3.0 ng/ml IL-13
- 4) cDRF/P/L as defined in Table 8, supplemented with 10.0 ng/ml IL-13

[0082] Cells were passaged upon reaching 50% to 80% confluence. Cells grown in DMEM + 10% FBS were rinsed with PBS, harvested by exposure to 325 units/ml trypsin in EDTA, counted, and reseeded. Cells grown in serum-free media were rinsed with PBS, harvested by exposure to 0.00025% Trypzean™ (0.1x recombinant trypsin; Sigma-Aldrich, St. Louis, MO) in 0.5 mM

EDTA, counted and reseeded. Cell yield was determined and population doublings calculated at the end of each passage. The data in Table 17 indicates that the cell yield in cDRF/P/L was roughly 2-fold greater than the yield in DMEM + 10% FBS. However, when IL-13 was added to cDRF/P/L, the cell yield decreased in a dose-dependent manner. IL-13 concentrations of 3 ng/ml and 10 ng/ml decreased the cell yield by 32% and 46%, respectively, compared to cDRF/P/L alone. In addition, the time in culture to required to achieve 50% to 80% confluence was increased in the presence of IL-13, resulting in a growth index of only 0.07 or 0.06. These results indicate that, under the conditions tested, cDRF/P/L supplemented with IL-13 is not an effective replacement for serum-containing media.

**Table 17.**

Medium	Cell Yield (per T75, x 10 <sup>5</sup> )	Growth Index (Pop. Doubling/Day)
DMEM + 10% FBS	6.84	0.40
cDRF/P/L	14.90	0.21
cDRF/P/L + 3 ng/ml IL-13	10.20	0.07
cDRF/P/L + 10 ng/ml IL-13	7.98	0.06

**Example 7: The E93 medium increases cell yield and proliferation of chondrocytes**

[0083] Primary human chondrocytes were isolated from biopsies of articular cartilage by mincing of the sample followed by enzymatic digestion with 0.25% protease type XIV (*Streptomyces griseus*) for one hour and then 0.1% collagenase overnight at 37 °C. Cells were recovered by centrifugation for five minutes at 1,000 x g and resuspended in the appropriate test medium. Cells grown in DMEM + 10% FBS were plated at a density of 3,000 cells per cm<sup>2</sup> and cells grown in serum-free medium were plated at a density of 5,000 cells per cm<sup>2</sup>. T75 flasks were used for all experiments. The following media were tested:

- 1) DMEM + 10% FBS
- 2) cDRFm as defined in Table 4, supplemented with 5 µl/ml CDLM as defined in Table 5, 10 ng/ml PDGF, 1 ng/ml IL-6 and 0.5 ng/ml OSM (referred herein as "E93").

[0084] Cells were passaged upon reaching 50% to 80% confluence. Cells grown in DMEM + 10% FBS were rinsed with PBS, harvested by exposure to 325 units/ml trypsin in EDTA, counted, and reseeded. Cells grown in the E93 medium were rinsed with PBS, harvested by exposure to 0.00025% Trypzean™ in 0.5 mM EDTA, counted and reseeded. Cell yield was determined and population doublings calculated at the end of each passage. The growth index for cells in E93 was equal to or greater than the growth index for cells in DMEM + 10% FBS (Table 18, Figure 1). The cell yield for cells grown in E93 was greatly increased compared to cells grown in DMEM + 10% FBS (Table 19, Figure 2). These results indicate that cDRFm supplemented with CDLM, PDGF, IL-6 and OSM is an effective replacement for serum-containing media.

**Table 18.**

Medium	Growth Index (population doubling/day)		
	Passage #1	Passage #2	Passage #3
DMEM + 10% FBS	0.41	0.55	0.57
E93	0.50	1.01	0.81

**Table 19.**

Medium	Cell yield per T75, x 10 <sup>5</sup>		
	Passage #1	Passage #2	Passage #3
DMEM + 10% FBS	35.9	30.8	33.5
E93	116	143	186

**Example 8: Medium supplemented with IL-6 and OSM maintains re differentiation capacity of chondrocytes in three-dimensional culture**

[0085] Primary human chondrocytes were isolated from biopsies of articular cartilage by mincing of the sample followed by enzymatic digestion with 0.25% protease type XIV (*Streptomyces griseus*) for one hour and then 0.1% collagenase overnight at 37 °C. Cells were recovered by centrifugation for five minutes at 1,000 x g and resuspended in the appropriate test medium. Cells grown in DMEM + 10% FBS were plated at a density of 3,000 cells per cm<sup>2</sup> and

cells grown in serum-free medium were plated at a density of 5,000 cells per cm<sup>2</sup>. T75 flasks were used for all experiments. The following media were used:

- 1) DMEM + 10% FBS
- 2) Serum-free E93 medium as described in Example 7

[0086] Cells were passaged upon reaching 50% to 80% confluence. Cells grown in DMEM + 10% FBS were rinsed with PBS, harvested by exposure to 325 units/ml trypsin in EDTA, counted, and reseeded. Cells grown in serum-free medium were rinsed with PBS, harvested by exposure to 0.00025% Trypzean™ in 0.5 mM EDTA, counted and reseeded.

[0087] After the third passage, cells were tested for the ability to redifferentiate as measured by colony formation and the production of proteoglycan in agarose (Benya et al., Cell 30:215-224 (1982)). Fifty thousand cells were resuspended in 2% agarose and plated in 60 mm dishes. Cells in agarose were cultured in DMEM + 10% FBS at 37 °C, and refed 24 hours after plating, and every 2 to 3 days thereafter. After 21 days in culture, the plates were fixed with 10% formalin, rinsed, stained with 0.2% safranin, and rinsed extensively to remove background stain. The number of colonies that stained positive for proteoglycan, and were equal to or greater than 50 microns in size, was determined. Plates on which more than 6.8% of the cells formed proteoglycan-positive colonies and met the minimum size criteria were scored as "pass". All strains were tested in triplicate. Cell strains from six biopsies were examined. As shown in Table 17, all six strains passed the agarose assay whether they were grown in serum-containing or serum-free media. These results further indicate that cDRFm supplemented with CDLM, PDGF, IL-6 and OSM is an effective replacement for serum-containing media.

**Table 20.**

Strain No.	DMEM + 10% FBS	E93
1	21.5%	14.8%
2	13.5%	12.3%
3	20.8%	11.8%
4	36.2%	31.3%
5	16.6%	17.3%
6	15.0%	28.4%

**Example 9: Mean cell yield for ten strains of chondrocytes is greater in medium supplemented with IL6 and OSM than in DMEM supplemented with serum**

[0088] Primary human chondrocytes were isolated from biopsies of articular cartilage by mincing of the sample followed by enzymatic digestion with 0.25% protease type XIV (*Streptomyces griseus*) for one hour and then 0.1% collagenase overnight at 37 °C. Cells were recovered by centrifugation for five minutes at 1,000 x g and resuspended in the appropriate test medium. Cells grown in DMEM + 10% FBS were plated at a density of 3,000 cells per cm<sup>2</sup>. Cells grown in serum-free medium were plated at either 5,000 cells per cm<sup>2</sup> (E93 high) or 3,000 cells per cm<sup>2</sup> (E93 low). T75 flasks were used for all experiments. The following media were tested:

- 1) DMEM + 10% FBS;
- 2) E93 media, as described in Example 7.

[0089] Cells were passaged upon reaching 50% to 80% confluence. Cells grown in DMEM supplemented with 10% FBS were rinsed with PBS, harvested by exposure to 325 units/ml trypsin in EDTA, counted, and reseeded. Cells grown in E93 media were rinsed with PBS, harvested by exposure to 0.00025% Trypzean™ (0.1x recombinant trypsin; Sigma-Aldrich, St. Louis, MO) in 0.5 mM EDTA, counted and reseeded. A total of ten biopsies were processed to generate ten different strains. Cell yield per flask was determined at the end of each passage and normalized to the P1 yield of cells in DMEM supplemented with 10% FBS. The mean cell yield for cells grown in E93 at the high or low plating density was greatly increased compared to cells grown in DMEM supplemented with 10% FBS (Table 21). These results indicate that E93 is an effective replacement for serum-containing media.

**Table 21.**

Medium	Normalized yield per T-75 flask (mean of 10 strains)
	P1 in DMEM + 10% FBS = 100

	Passage #1	Passage #2	Passage #3
DMEM + 10% FBS	100	68	86
E93 low	350	202	180
E93 high	334	314	195

**Example 10: Medium supplemented with IL6 and OSM maintains capacity of chondrocytes to re-express type 2 collagen and aggrecan in alginate suspension culture**

[0090] Primary human chondrocytes were prepared as describe in Example 9. Cells grown in DMEM + 10% FBS or E93 were harvested in third passage for alginate culture. Alginate cultures were set up by seeding  $1 \times 10^6$  cells into a 1.2% alginate solution. Alginate cultures were fed every 3-5 days with EGHIC (DMEM, 20 ng/mL rhIGF-1, 25  $\mu$ g/mL ascorbic acid, and 1 mM sodium pyruvate). After 21 days of culture, the chondrocytes were extracted from the alginate beads and mRNA for type I collagen, type II collagen and aggrecan were detected using a ribonuclease protection assay (RPA). In this assay, type II collagen is detected as a 310 base pair (bp) band on a gel, type I collagen is a 260 bp band, and aggrecan is a 210 bp band. Figure 3 shows that increasing amounts of cell lysate from cells grown in E93 (lanes 2, 3 and 4) or DMEM supplemented with 10% serum (lanes 5, 6 and 7) contain mRNA for type II collagen and aggrecan. This indicates that human chondrocytes grown in E93 media are capable of re-expression these important cartilage markers.

**Example 11: Karyotype and senescence of chondrocytes grown in medium supplemented with IL6 and OSM**

[0091] It may be important that cells maintain a normal karyotype and enter senescence during culture, for example, if cells are used for human therapy. Chondrocytes grown in E93 displayed a normal karyotype through at least ten passage and senesced after approximately thirty population doublings.

**Example 12: Low levels of cytokines stimulate growth of chondrocytes**

[0092] Primary human chondrocytes were isolated from biopsies of articular cartilage by mincing of the sample followed by enzymatic digestion with 0.25% protease type XIV (*Streptomyces griseus*) for one hour and then 0.1% collagenase overnight at 37 °C. Cells were recovered by centrifugation for five minutes at 1,000 x g and resuspended in the appropriate test medium. Cells grown in DMEM + 10% FBS were plated at a density of 3,000 cells per cm<sup>2</sup>. Cells grown in serum-free medium were plated at either 5,000 cells per cm<sup>2</sup>. T75 flasks were used for all experiments. The following media were tested:

- 1) DMEM + 10% FBS
- 2) E93 media, as described in example 7
- 3) E93 media with 0.5 ng/ml IL-6 and 0.25 ng/ml OSM
- 4) E93 media with 0.1 ng/ml IL6 and 0.05 ng/ml OSM

[0093] Cells were passaged upon reaching 50% to 80% confluence. Cells grown in DMEM + 10% FBS were rinsed with PBS, harvested by exposure to 325 units/ml trypsin in EDTA, counted, and reseeded. Cells grown in E93 media were rinsed with PBS, harvested by exposure to 0.00025% Trypzean™ (0.1x recombinant trypsin; Sigma-Aldrich, St. Louis, MO) in 0.5 mM EDTA, counted and reseeded. The growth rate, expressed as population doublings per day, was calculated at the end of each passage (Table 22). These results indicate that low levels of IL-6 and OSM in E93 support growth of primary human chondrocytes.

**Table 22.**

Medium	Population Doublings per Day		
	Passage #1	Passage #2	Passage #3
E93	0.40	0.72	0.63
E93 w/ 0.5 ng/ml IL-6, 0.25 ng/ml OSM	0.40	0.82	0.56
E93 w/ 0.1 ng/ml IL-6, 0.05 ng/ml OSM	0.34	0.72	0.42

[0094] All references cited within the specification are incorporated by reference in their entirety.

[0095] The term 'comprise' and variants of the term such as 'comprises' or 'comprising' are used herein to denote the inclusion of a stated integer or stated integers but not to exclude any other integer or any other integers, unless in the context or usage an exclusive interpretation of the term is required.

[0096] Any reference to publications cited in this specification is not an admission that the disclosures constitute common general knowledge in Australia.

**CLAIMS**

1. A method for propagating chondrocytes, wherein the method comprises a step of incubating the chondrocytes with a culture medium comprising a basal medium supplemented with oncostatin M (OSM).
2. The method of claim 1, wherein the basal medium is additionally supplemented with interleukin-6 (IL-6).
3. The method of claim 1, wherein the basal medium is additionally supplemented with leukemia inhibitory factor (LIF).
4. The method of claim 1, wherein the basal medium is additionally supplemented with IL-6 and LIF.
5. The method of any one of claims 1-4, wherein the basal medium is additionally supplemented with platelet-derived growth factor (PDGF).
6. The method of any one of claims 1-5, wherein the basal medium is additionally supplemented with one or more lipids selected from the group consisting of stearic acid, myristic acid, oleic acid, linoleic acid, palmitic acid, palmitoleic acid, arachidonic acid, linolenic acid, cholesterol, and alpha-tocopherol acetate.
7. The method of any one of claims 1-6, wherein the basal medium is cDRF.
8. The method of claim 1, wherein the basal medium is cDRF, and wherein the basal medium is additionally supplemented with PDGF and a chemically defined lipid mixture (CDLM).
9. The method of claim 1, wherein the basal medium is cDRFm, and wherein the basal medium is additionally supplemented with PDGF and CDLM.
10. The method of claim 1, wherein the basal medium is cDRFm, and wherein the basal medium is additionally supplemented with IL-6, PDGF and CDLM.

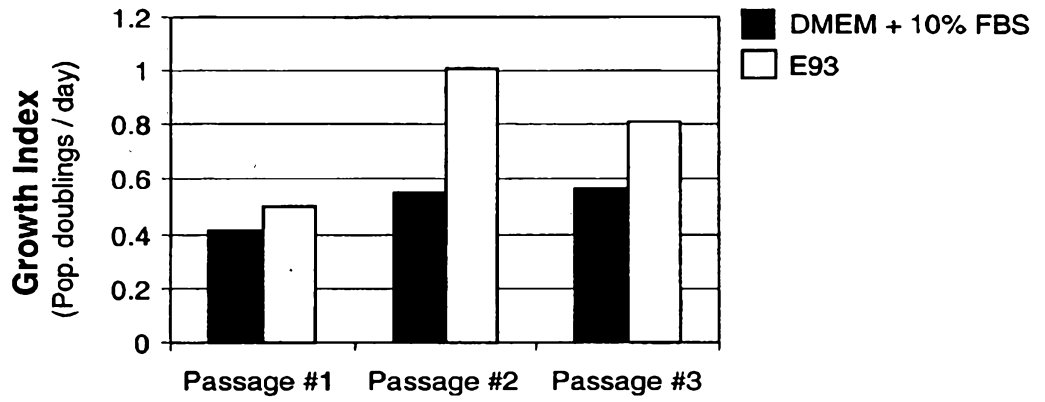
11. The method of any one of claims 1-10, wherein the basal medium is not supplemented with substantially pure jagged 1 (JAG1) and/or substantially pure interleukin-13 (IL-13).
12. The method of any one of claims 1-11, wherein OSM is present in the culture medium at a concentration of from 0.01 ng/ml to 10 ng/ml.
13. The method of claim 12, wherein OSM is present in the culture medium at a concentration of from 0.1 ng/ml to 2 ng/ml, and preferably from 0.5ng/ml to 1 ng/ml.
14. The method of claim 2, wherein OSM and IL-6 are each present in the culture medium at a concentration of from 0.01 ng/ml to 10 ng/ml.
15. The method of claim 3, wherein OSM and LIF are each present in the culture medium at a concentration of from 0.01 ng/ml to 10 ng/ml.
16. The method of claim 4, wherein OSM, IL-6 and LIF are each present in the culture medium at a concentration of from 0.01 ng/ml to 10 ng/ml.
17. The method of any one of claims 1-16, wherein the culture medium is serum-free.
18. The method of any one of claims 1-16, wherein the culture medium further comprises serum.
19. The method of claim 1, wherein the culture medium comprises cDRFm; 0.1-100 ng/ml PDGF; 0.05-5% CDLM; 0.01-10 ng/ml OSM; and 0.01-10 ng/ml IL-6.
20. The method of any one of claims 1-19, wherein the chondrocytes are de-differentiated.
21. The method of any one of claims 1-19, wherein the chondrocytes are derived from mesenchymal stem cells.
22. The method of claim 20 or claim 21, wherein the chondrocytes are human chondrocytes.

23. The method of claim 22, wherein the chondrocytes are human articular chondrocytes.
24. The method of any one of claims 20-23, wherein the chondrocytes are primary chondrocytes.
25. The method of any one of claims 1-24, wherein the method further comprises a step of passaging the chondrocytes.
26. The method of claim 25, wherein the chondrocytes are passaged by incubating the chondrocytes with a solution comprising a chelating agent.
27. The method of claim 26, wherein the chelating agent is EDTA.
28. The method of claim 27, wherein the EDTA is present in the solution at a concentration of from 0.1 mM to 1 mM.
29. The method of claim 25, wherein the chondrocytes are passaged by incubating the chondrocytes with a solution containing trypsin at a concentration of less than 325 units/ml.
30. The method of claim 29, wherein the solution further comprises from 0.1 mM to 1 mM EDTA.
31. The method of any one of claims 1-30, wherein at the start of incubation, the chondrocytes are seeded at a density less than 20,000 cells/cm<sup>2</sup>.
32. The method of any one of claims 1-31, wherein the culture medium comprises cDRFm; 0.1 – 100 ng/ml PDGF; 0.05 – 5% CDLM; 0.01 – 10 ng/ml OSM; and 0.01 – 10 ng/ml IL-6.
33. A method of treating a cartilage defect in a subject, comprising propagating the chondrocytes according to the method of any one of claims 1 to 32.
34. Use of chondrocytes in the manufacture of a medicament for the treatment of a cartilage defect in a subject, wherein the manufacture comprises

- propagating the chondrocytes according to the method of any one of claims 1-32.
35. A method for manufacturing a medicament intended for the treatment of a cartilage defect in a subject, wherein the method comprises propagating chondrocytes according to the method of any one of claims 1-32 and the use of said chondrocytes for the manufacture of said medicament.
  36. A culture medium when used for propagating chondrocytes, wherein the culture medium comprises a basal medium that is supplemented with substantially pure oncostatin M (OSM), and wherein the OSM is present at a concentration of from 0.01 ng/ml to 5 ng/ml.
  37. The culture medium of claim 36, wherein the OSM is present at a concentration of from 0.1 ng/ml to 2 ng/ml, and preferably from 0.5 ng/ml to 1 ng/ml.
  38. The method of any one of claims 1-33 and 35 or the use of claim 34, wherein the culture medium has the features that are recited in claim 36 or claim 37.

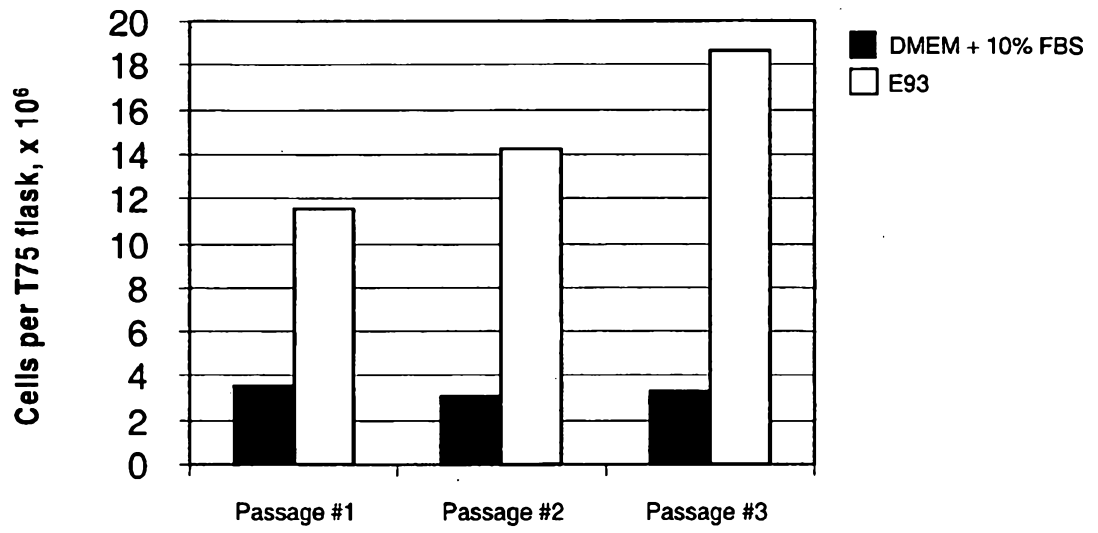
Dated: 14 January 2014

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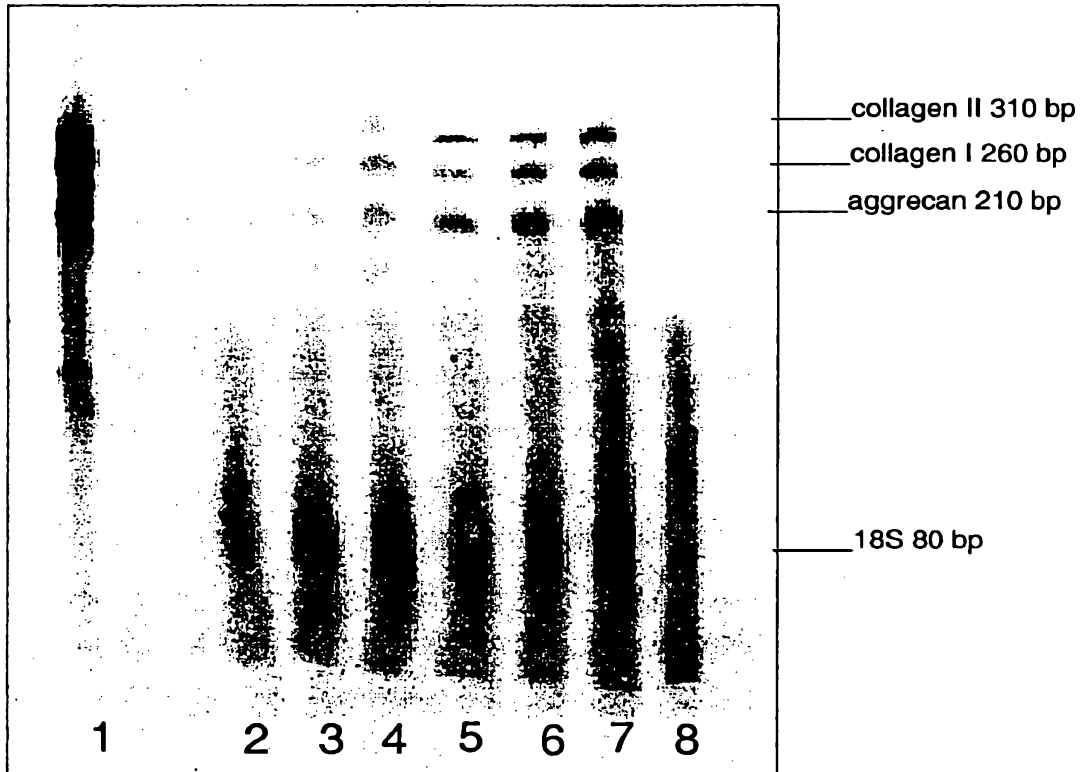
**FIG. 1**

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**FIG. 2**

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**FIG. 3**