

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
19 January 2012 (19.01.2012)

PCT

(10) International Publication Number
WO 2012/009538 A2

(51) International Patent Classification:
A61K 35/36 (2006.01)

(21) International Application Number:
PCT/US2011/044010

(22) International Filing Date:
14 July 2011 (14.07.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/364,459 15 July 2010 (15.07.2010) US

(71) Applicant (for all designated States except US): **RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY** [US/US]; Old Queens, Somerset Street, New Brunswick, NJ 08903 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **YARMUSH, Martin, L.** [US/US]; 164 Ward Street, Newton, MA 02459 (US). **SHARMA, Nripen** [IN/US]; 4055 Woodbridge Avenue, Edison, NJ 08837 (US). **MAGUIRE, Tim** [US/US]; 37 East Burgess Drive, Piscataway, NJ 08854 (US). **NAGRATH, Deepak** [IN/US]; 3477 Ashburnam Road, Ann Arbor, MI 48105 (US).

(74) Agents: **NORTON, Gerard, P.** et al.; Fox Rothschild LLP, 997 Lenox Drive, Building 3, Lawrenceville, NJ 08648-2311 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report (Rule 48.2(g))

(54) Title: ADIPOCYTE-DERIVED MEMBRANE EXTRACT WITH BIOLOGICAL ACTIVITY

(57) Abstract: The present invention relates, generally, to a cell media extract that supports cell growth and provides base components for derived basement membrane complexes. Such extract may be incorporated within other cell media to support cell growth *in vitro* or otherwise provide a scaffold to support and accelerate growth *in vivo*. The invention provides isolation and purification of the cell media extract and broad applications of the isolated extract, for example, in hepatocyte culture systems, wound healing, treatment of aging skins, and stem cell differentiation systems.



WO 2012/009538 A2

ADIPOCYTE-DERIVED MEMBRANE EXTRACT WITH BIOLOGICAL ACTIVITY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. §119(e) to U.S. Provisional Patent Application Serial No. 61/364,459, filed on July 15, 2010, which is hereby incorporated
5 by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

The invention described herein was supported in whole or in part by grants from the National Institutes of Health (Grant Nos. R01 DK 059766 and R01 AI 063795) and National Science Foundation (Grant No. 828244). The U.S. Government has certain rights
10 in this invention.

FIELD OF THE INVENTION

The present invention relates, generally, to a cell media extract that supports cell growth and provides components for derived basement membrane complexes. The invention also concerns isolation methods of the cell media extract and wide applications
15 of the cell media extract, for example, in hepatocyte culture systems, wound healing, treatment of aging skins, stem cell differentiation systems, tissue engineering, and regenerative medicine applications such as ECM arrays, human embryonic stem cell cultures, obesity research and cancer cell invasion.

BACKGROUND OF THE INVENTION

20 Synthetic biomaterials and derived basement membrane (BM) complexes represent state-of-the-art tools for drug delivery, cellular engineering, and 3-dimensional scaffold generation. While these materials often attempt to mimic *in vivo* tissue architecture, there are many challenges to developing the ideal biomaterial. One such challenge is to define an extracellular matrix and non-matrix composition that adequately supports native cell
25 growth and functionality in both an *in vitro* and *in vivo* context. Existing compositions fail to account for both of these fronts because they either do not adequately mimic native *in vivo* cell responses or, immunogenically, are not suitable for transplant. Accordingly, they are limited in use and a more naturally-derived biomaterial is desirable.

Extracellular matrix components are a complex mixture of matrix molecules, including glycoproteins, fibronectin, collagens, laminins, and proteoglycans, as well as nonmatrix proteins. Non-matrix components include a wide array of signal molecules, e.g. growth factors, chemokines, cytokines, ligands, and the like. The entirety of this composition, rather than simply the presence of an extracellular scaffold, is critical for regulating cell phenotype. This is particularly true in the basement membrane (BM) complexes, which are extremely diverse, tissue specific, and dynamic. To mimic *in vivo* environmental conditions, the BM protein array should possess, at minimum, binding sites for cell adhesion molecules and cell signaling molecules that serve as ligands for triggering cell surface receptors. Such sites and molecules assist in guiding cellular differentiation and inhibit or promote cell proliferation, functionality, and migration.

The main challenge with previously developed synthetic matrix proteins is the effective control of dynamics and spatial organization of multiple signal presentation. Naturally derived compositions possess the inherent properties of biological recognition, including presentation of receptor-binding ligands, susceptibility to cell triggered proteolytic degradation, and remodeling implicated in tissue morphogenesis. Most synthetic analogs, however, represent oversimplified mimics of the natural model in that they lack the spatial, temporal, and protein complexity.

Cell matrix biologists and bioengineers, recently, have attempted to overcome these limitations by using more naturally-derived biomaterials. Numerous techniques have been developed to isolate natural proteins from a variety of mammalian sources, such as decellularized submucosal intestine, urinary bladder, liver, and skeletal muscle for tissue engineering and regenerative medical applications. Such an approach supersedes both previously established synthetic analogs and tissue-based methods because it has the potential to (1) obviate chemical and enzymatic procedures used to isolate basement membrane extract and (2) prevent disruption of protein-protein interactions. Thus, it potentially presents a less cumbersome procedure with minimal batch-to-batch variability, reduction of pathogen transmission and potentially affords the ability to modulate supramolecular composition of the extract utilizing various *in vitro* biochemical perturbations.

One early generation example of such a biomaterial is set forth in U.S. Patent Nos. 4,829,000 and 5,158,874. The membrane, or “matrigel,” set forth in these patents is rich in the extracellular matrix proteins laminin, collagen IV, heparan sulfate proteoglycans, entactin, and nidogen. It is formed by first extracting these components from Engelbreth
5 Holm-Swarm (EHS) mouse sarcomas, then heating and polymerized the extract to form a three dimensional matrix. Such a matrix is embodied within the biological membrane and cell culture reagent BD Matrigel™, available from BD Biosciences. This product, while widely used by researchers for studying cell interactions, differentiation and identifying putative therapeutic agents, is still limited in its ability to predict accurately predict *in vivo*
10 behavior of cells. Moreover, due in-part to its immunological effects, this material is also not available to provide *in vivo* scaffold formation for cell growth, i.e. tissue engineering. Accordingly, new and advanced biomaterials are still desirable.

To develop a naturally cross-linked basement membrane that meets the above criteria, there is a pressing need to develop a novel *in vitro* cell culture system that has the
15 ability to generate substantial amounts of a naturally-derived extract with defined and optimized matrix and non-matrix protein compositions. Such a design would be able to accurately predict *in vivo* effects because its composition more accurately mimics those observed. Moreover, such a composition, ideally, would be directly transplantable into a host and would be optimized to support tissue engineering.

20 SUMMARY OF THE INVENTION

The instant invention addresses such a need. The invention has a number of applications, including, *inter alia*, hepatocyte culture systems, quantitative modeling of effect of Adipogel on hepatic metabolism, wound healing of partial and full thickness *in vitro* and *in vivo* skin models, cell culture optimization ECM arrays, and stem cell
25 differentiation systems.

In one embodiment, the instant invention relates to an isolated cellular extract of extracellular matrix and non-matrix proteins purified from a cell-exposed differentiation media. The proteins are > 10kDa or >100 kDa in size and are provided at a total concentration of about 100 mg/ml. Matrix components may include one or more proteins
30 selected from the group collagen I, collagen IV, collagen V, collagen VI, fibronectin,

laminin, hyaluronan, and combinations thereof, and non-matrix proteins may be selected from chemokines, cytokines, lipoproteins, glycoproteins, receptor proteins, ligands, signal transduction proteins, enzymes, hormones, growth factors and combinations thereof. In one embodiment, the non-matrix proteins include one or more growth factors, which may be selected from the group Coagulation factor III (CFIII), Dickkopf-1 (DKK-1), Dickkopf-3 (DKK-3), Developmental receptor tyrosine kinase (Dtk), Gremlin, Hepatocyte growth factor (HGF), Interleukin-1 receptor II (IL-1RII), Interleukin-4 (IL-4), Interleukin-5 receptor α (IL-5 α), Interleukin-20 (IL-20), Leptin receptor (Leptin R), Leukemia inhibitor factor (LIF), Tumor necrosis factor SuperFamily 14 (TNFSF14), Lymphotoxin, Macrophage inflammatory protein-1 α (MIP α), Osteopontin, Osteoprotegerin, Progranulin, P-Selectin, Resistin-like molecule β (RELM β), Secretory leukocyte peptidase inhibitor (SLPI), Tissue inhibitor of metalloproteinases 1 (TIMP-1), Toll-like receptor 4 (TLR4), Tomoregulin-1, Tumor necrosis factor SuperFamily 10 (TNFSF10), Ubiquitin, Vascular endothelial growth factor (VEGF), and combinations thereof.

In certain embodiments of the instant invention, the extract includes the extracellular matrix proteins collagen IV, fibronectin, and laminin and the non-matrix proteins include VEGF, HGF, and LIF. Collagen IV may be provided in a concentration range between about 20 $\mu\text{g/ml}$ and about 60 $\mu\text{g/ml}$, fibronectin at a concentration range between about 2 $\mu\text{g/ml}$ and about 5 $\mu\text{g/ml}$, laminin at a concentration range between about 10 $\mu\text{g/ml}$ and about 15 $\mu\text{g/ml}$. These concentrations are not necessarily limiting to the instant invention, however, and may be adapted as provided herein.

The cell differentiation media used to derive the foregoing extract includes at least one soluble factor, at least one steroid, and isobutylmethylxanthine. Soluble factors may include, but are not limited to, an agonist and/or agent that induces natural cell growth pathways. In one embodiment, such an agonist or agent may be selected from the group rosiglitazone, troglitazone, thiazolidinedione, insulin or combinations thereof. The soluble factor may be provided in an effective amount, which may be between about 0.1 μM and 1.0 μM .

Steroid(s) also may be provided as a cell growth modulator and/or differentiation enhancer. Such agent may be selected from the group dexamethasone, cortisol, aldosterone, hydrocortisone, triiodothyronine, corticosterone, or combinations thereof.

The steroid(s) may be provided in an effective amount, as defined herein, which may be between about 0.1 μ M and 1.0 μ M. Similarly, the remaining component of the differentiation media, isobutylmethylxanthine, may also be provided in an effective amount, which may be between about 0.1 μ M and 1.0 μ M.

5 Any cell line may be used to generate the foregoing extract, as discussed in greater detail below. In certain embodiments, however, the isolated cellular extract is prepared from a preadipocyte cell line exposed to the foregoing differentiation media.

In further aspects, the instant invention also relates to a method for isolating the foregoing cellular extract by (1) optionally culturing a cell line on a first cell media until
10 confluent; (2) differentiating the cell line on a second cell media of at least one soluble factor, at least one steroid, and isobutylmethylxanthine; and (3) isolating and purifying a second cell media extract. The second cell media extract may be the differentiation media, as defined herein, and may include one or more of the extracellular matrix and non-matrix proteins. The first cell media may include DMEM supplemented with 10% FBS, 2%
15 Penicillin and Streptomycin. Each of the cell line, soluble factor, and steroid may be as defined above or otherwise herein.

In further embodiments, the instant invention also relates to a method for proliferating and/or maintaining cell functionality a cell line by culturing the cells on the extract of the instant invention. In one aspect, the cell line includes hepatocytes or
20 hepatocyte-like cells, which are incubated on the extract either directly or after it is incorporated into additional cell media. This method results in improved hepatocytic cell albumin secretion rates and cell morphology that approximates those observed in a natural environment.

In another embodiment, the cell line includes endothelial or endothelial-like cells
25 which are incubated on the extract either directly or after it is incorporated into additional cell media. This method stimulates angiogenesis and results in native functionality of the endothelial cell, i.e. formation of HUVEC tubes.

In a further embodiment, the cell line includes human embryonic stem cells which are incubated on the extract either directly or after it is incorporated into additional cell

media. This method results in the proliferation of the cells, while maintaining the original phenotype.

5 In alternative embodiments, the instant invention also relates to a derived basement membrane formed, at least in part, from the extract of the instant invention. Such basement membrane is used to facilitate *in vitro* or *in vivo* cell growth and proliferation of a cell line. In one aspect, the basement membrane is used for healing a wound in a subject by administering to the wound site basement membrane complex containing the cell media derived extract. As discussed herein, such a method results in at least a 1.5 fold increase in normalized cell numbers, as compared to cell proliferation without the extract.

10 In further embodiments, the invention relates to a method for treating aging skin of a subject also by administering to a dermal layer of a patient the extract containing basement membrane of the instant invention. The administration of this extract results in enhanced expression of epidermal keratinocyte function and viability, as compared to untreated keratinocyte cells of approximately the same age.

15 In additional embodiments, the instant invention relates to a kit for proliferating or maintaining cell functionality having an extract of extracellular matrix and non-matrix proteins isolated and purified from a cell-exposed differentiation media comprising at least one soluble factor, at least one steroid and isobutylmethylxanthine; and cellular growth media or a basement membrane composition.

20 Synthetic biomaterials and natural extracellular matrix derived basement membrane complexes represent state-of-the art tools for drug delivery, cellular engineering and 3D scaffold generation to mimic *in vivo* tissue architecture for biomedical applications. Synthetic ECM analogs represent oversimplified mimics of natural ECMs lacking the spatial and temporal complexity.

25 Challenges of developing an ideal biomaterial include: a) host biocompatibility, b) batch to batch variability, c) ease of availability, d) ability to form scaffolds, powders and gels, e) biodegradability, and f) defined extracellular matrix and growth factor composition. ECM derivative approaches include synthetic and semi-synthetic ECMs (Extracel), shape controlled hydrogels, puramatrix, tissue derived ECM (perfusion-based

UBM, DCLM; Cartrigel, Matrigel, Myogel), cell culture based ECM secretions, and cell-cell co-culture based ECM secretions and applications.

Bioengineered natural and synthetic biomaterials necessitate a dynamic interplay of complex macromolecular compositions of hydrated extracellular matrices (ECMs) and soluble growth factors. The challenges in utilizing synthetic ECMs is the effective control of temporal and spatial complexity of multiple signal presentation as compared to natural ECMs that possess the inherent properties of biological recognition including presentation of receptor-binding ligands, susceptibility to cell-triggered proteolytic degradation and remodeling. The present invention provides a murine preadipocyte differentiation system for generating a natural basement membrane extract (Adipogel) comprising ECM proteins (collagen IV, laminin, hyaluronan, and fibronectin) and relevant growth factors (hepatocyte growth factor, vascular endothelial growth factor, and leukemia inhibitory factor). The demonstrated synthesis, isolation, characterization and application of Adipogel provide immense potential for applications such as hepatocyte culture systems, wound healing and stem cell differentiation systems as described below.

The invention has demonstrated the effective utilization of Adipogel for enhanced albumin synthesis rate of primary hepatocyte cultures for a period of 10 days as compared to collagen sandwich cultures and comparable or higher function as compared to Matrigel cultures. The invention also demonstrated comparable cytochrome P450 1A1 activity for the collagen-Adipogel condition to the collagen sandwich and Matrigel culture conditions. The invention provides effective utilization of Adipogel for accelerated healing of partial and full thickness wounds using *in vitro* human skin equivalent cultures and *in vivo* mouse models. In addition, the invention also shows comparable urea secretion rates of human embryonic stem cell derived endodermal cells in presence of Adipogel as compared to collagen cultures. Thus, Adipogel has the potential to be effectively utilized for tissue engineering and regenerative medicine applications including ECM arrays, human embryonic stem cell cultures, obesity research and cancer cell invasion.

Adipogel system has a number of advantages, including but not limited to: a) Adipogel involves generation of normal ECM, whereas Matrigel involves tumor derived ECM; b) Adipogel yield is significantly higher than normal cell lines independent of chemical extraction procedures to generate ECM as compared to tissue derived natural

ECMs; c) a complex mixture of multiple ECM proteins resembles basement membranes closely as compared to synthetic polymers or in vitro reconstituted ECMs; d) a growth factor-enriched ECM is easy to obtain with low variability in composition; e) a mixture of ECM is naturally complexed via protein-protein interactions rather than in vitro mixing of individual proteins utilized in ECM arrays; f) the utilization of a fibroblast-like phenotype as an originating cell type to generate Adipogel provides reason to harness the basement membrane biogenesis phenomena for ubiquitous applications; g) Adipogel is comprised of collagen IV, fibronectin, laminin and hyaluronic acid that is present in basement membranes from numerous tissues; and h) a reductionist approach of utilizing individual growth factors/ECMs cannot be used to predict accurate cellular response, but instead, utilization of a naturally complexed basement membrane like Adipogel can potentiate identification of individual and/or multiple growth factors/ECMs implicated in cellular applications using a top-bottom approach.

Thus, in other aspects the present invention provides use of an extract comprising extracellular matrix and non-matrix proteins prepared according to any embodiments described herein for (1) proliferating and/or maintaining cell functionality of hepatocytes, (2) proliferating and/or maintaining cell functionality of endothelial or endothelial-like cells, (3) manufacture of a medicament for treatment of a wound in a subject, (4) manufacture of a medicament for treatment aging skin in a subject, or for any other similar purposes, wherein the extract is isolated from a cell-exposed differentiation media comprising at least one soluble factor, at least one steroid, and isobutylmethylxanthine.

Additional embodiments and advantages will be readily apparent to one of skill in the art, based on the teaching provided herein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGs. 1A and 1B illustrate hepatocyte culture technique and a schematic to culture primary hepatocytes using Adipogel.

FIG. 2 provides a functional analysis of rat hepatocytes *in vitro* using Adipogel: urea (left panel) and albumin (right panel). Dotted line [1] is the Collagen Single Gel condition to which the remaining experimental values are normalized.

FIG. 3 illustrates hepatocyte morphology in Adipogel supplemented medium on collagen single gel coated 12 well plates.

FIG. 4 illustrates HUVEC tube formation in presence of Adipogel and no gel conditions. In Gel type 1, Adipogel was generated by insulin stimulation of cells. In Gel Type 2, Adipogel generated by rosiglitazone stimulation of cells.

FIG. 5 illustrates dermal fibroblast migration/ keratinocyte proliferation in a wound assay. (A) In vitro scratch assay for fibroblasts. (B) Normal human dermal fibroblasts (NHDF) cell number and C) Human keratinocyte attachment on different culture substrates. NHDFs/Human keratinocytes were plated on tissue culture plastic (TCP), thin layer of d2 and d4 Adipogel (150 mg/ml) for 4 days at same initial cell seeding density. NHDFs were subjected to *in vitro* scratch assay with images captured at initial time point and after 24 hrs using phase-contrast microscope. The region between the two dark lines in figure (A) corresponds to the open wound area. As shown (Inset), the number of cells and hence the rate of migration is higher in the wound area 24 hrs post-scratch wound for the d2 and d4 Adipogel coated wells as compared to the control populations. Note that the Early stage ECM corresponds to day 2 Adipogel, whereas Late stage ECM corresponds to day 4 Adipogel.

FIG. 6 illustrates MTT staining of cell viability in presence of Adipose derived gels, controls and no gel conditions. In Gel type 1, Adipogel was generated by insulin stimulation of cells. In Gel Type 2, Adipogel generated by rosiglitazone stimulation of cells. Purple staining is indicative of cell proliferation.

FIG. 7 illustrates keratinocyte proliferation/migration in small puncture wounds for different experimental conditions. As shown, keratinocyte proliferation/ migration is higher in presence of Gel 1 and 2 as compared to neosporin and no gel conditions on day 2 and 4 of culture.

FIG. 8 illustrates morphology and characterization of Day 7 undifferentiated human embryonic stem cells *in vitro*. Human embryonic stem cells (hESCs) were plated in Adipogel coated 12 well plate for 7 days with Mouse Embryonic Feeder Layer Conditioned Media. (A) Morphology of undifferentiated ES cells. (B) SSEA-4 staining of cells implying the presence of undifferentiated phenotype. (C) Urea secretion of Day 20

differentiated endodermal cells on Adipogel and Collagen coated dishes. The cells were exposed to DMEM supplemented with 100 ng/ml OSM, 10^{-7} M Dex, 5 ug/ml ITS.

FIG. 9 represents a schematic diagram of Adipogel generation using a preadipocyte differentiation system. Murine 3T3-L1 preadipocytes were cultured to confluence in basal medium. After 2 days post-confluency, cells were differentiated in the presence of IBMX, dexamethasone and insulin agonist. After 2 days post differentiation, media was switched to differentiation medium supplemented with insulin agonist only. On days 2 and 4, media was collected and protein extract > 10 or 100 kDa MW cutoffs were extracted using Amicon centrifugal filters. The protein solution was plated on culture dishes for substrate formation. Preadipocyte differentiation secretes collagen I-VI, laminin, fibronectin, proteoglycans.

FIG. 10 illustrates extracellular matrix (ECM) protein arrays and their applications.

FIG. 11 illustrates effect of natural ECM on breast cancer cell adhesion and proliferation.

FIG. 12 illustrates metabolic engineering processes of identification, characterization and modification/introduction of biochemical pathways to improve cellular function.

FIG. 13 illustrates the disease treatment approach of the present invention.

FIG. 14 illustrates metabolic network model for hepatocyte cultures, which provides rationale for mathematical programming techniques to optimize urea and albumin secretion in adipogel cultures. Arrows indicate direction of reaction assumed in the model. Numbers refer to reaction numbers listed in Table 4 and Table 5. Albumin reaction is not shown for purposes of clarity.

FIG. 15 illustrates metabolic flux analysis of rat hepatocytes in vitro using Adipogel. [A] Recovery stage; [B] Pre-stable stage; and [C] Stable stage of culture. Hepatocytes cultured in five different configurations at a density of 500,000 cells/well in a 12 well plate; CSG corresponds to culture on single collagen gel; CDG corresponds to culture in collagen double gel sandwich configuration; CSG+solASG corresponds to hepatocytes cultured on collagen single gel with soluble Adipogel in the media; CSG+ASG corresponds to culture on collagen single gel with Adipogel overlaid on

top; Adipogel was utilized at a 1:5 ratio with culture media and media was changed on days 0, 1, 2, 5, 7 and 9. Metabolic Flux Analysis was performed on [A] recovery stage [B] pre-stable stage and [C] stable stage of culture. MFA results for CDG vs. CSG+solASG conditions are represented in the figures. Note that the fluxes in red correspond to significantly upregulated fluxes [with statistical significance of $p, 0.05$] for the CSG+solASG condition as compared to the CDG condition. Fluxes in blue correspond to significantly downregulated metabolite fluxes.

FIG. 16 illustrates experimental design of hepatocyte cultures. Four different conditions were utilized for the metabolite measurements. Collagen single gel [CSG], collagen double gel [CDG] collagen-soluble Adipogel sandwich [CSG+solASG] and collagen-Adipogel sandwich cultures [CSGASG]. Secreted products were measured at the recovery stage, pre-stable stage and stable stage of culture. Urea and albumin synthesis was determined from day 3 to day 10 of culture.

FIG. 17 contains images of polystyrene + Adipogel.

FIG. 18 illustrates a wound-healing model (*in vitro* system I): decellularized partial thickness wound with intact dermis. EpiSkin consists of a 'type I bovine collagen matrix, representing the dermis, surfaced with a film of type IV human collagen, upon which is laid, after 13 days in culture, stratified differentiated epidermis derived from second passage human keratinocytes.

FIG. 19 illustrates another wound healing model (*in vitro* system II): partial thickness wound with thin layer of intact dermal collagen.

FIGs. 20A and 20B illustrate the results from the *in vitro* system II, which demonstrate that Adipogel in the presence of collagen induces accelerated and uniform wound closure in slow healing skin wounds as compared to collagen or collagen + Hemagel conditions.

FIG. 21 illustrates an *in vivo* assessment.

FIGs. 22A and 22B demonstrate the results of the *in vivo* assessment. The *in vivo* skin wounds treated with Adipogel heal in presence of Alloderm shows a high promise in wound dressing applications. In particular, combination of Adipogel with Alloderm leads to the largest improvement after 2 weeks.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to an isolated cell media extract that supports cell growth and native functionality. The extract includes both extra-cellular matrix and non-matrix proteins, which are implicated in facilitating cell proliferation, maintenance of differentiated cell function, migration, and/or angiogenesis. It may also be incorporated into other cell culture media or within a basement membrane complex to support cell growth *in vitro* or otherwise provide a scaffold that supports and/or accelerates growth *in vivo*. Additional embodiments and advantages of the instant invention will be apparent to one of ordinary skill in the art based on the disclosure provided herein.

In one aspect, the instant invention relates to an isolated cell media extract of extracellular matrix and non-matrix proteins. Extracellular matrix proteins may include one or any combination of at least collagen I, collagen IV, collagen V, collagen VI, fibronectin, laminin, and hyaluronan. The non-matrix proteins may include, but are not limited to, chemokines, cytokines, lipoproteins, glycoproteins, receptor proteins, ligands, signal transduction proteins, enzymes, hormones, growth factors, and combinations thereof

In one embodiment, the matrix components of the extract are comprised of at least collagen IV, fibronectin, and laminin. Collagen IV may be the most abundant protein and may account for up to and including 50% of the proteins extracted. While not intending to be bound by theory, it provides both the integral component of BM structures and ligands used for cell adhesion and motility. The isolated collagen IV, forms a sheet-like network made by a mesh of filaments rather than by linear fibrils. Its mammalian peptide form is comprised of at least six different genes, e.g. COL4A1, COL4A2, COL4A3, COL4A4, COL4A5, COL4A6. To this end, and as used herein, the structure of the isolated Collagen IV may mimic the size and amino acid composition of such naturally occurring sequences within mammalian organisms. Its structure is not so limited, however, and may also include any other naturally occurring or synthetic sequences, homologues and structural variants thereof. Ideally, though not exclusively, the Collagen IV structure (or any component of the extract for that matter) would not be immunogenic when transplanted into a recipient organism.

Fibronectin refers to a high-molecular weight (~440kDa) extracellular matrix glycoprotein that binds to membrane-spanning receptor proteins called integrins. While not intending to be bound by theory, it influences cell growth and differentiation through its effects on gene-encoding cell cycle components and also facilitates self-assembly of extracellular matrix (ECM) molecules such as collagen I fibril formation. Fibronectin typically exists as a dimer of two nearly identical monomers linked by a pair of disulfide bonds. While it is produced by a single gene, alternative splicing of its pre-mRNA leads to the creation of numerous isoforms. The structure of fibronectin contemplated herein may include any such isoforms or any other naturally occurring or synthetic sequences, homologues and structural variants thereof.

Laminin refers to large trimeric proteins that contain an α -chain, a β -chain and a γ -chain, which form a cross that is able to bind to other cell membranes and extracellular matrix molecules. While not intending to be bound by theory, it is a biologically active portion of the basal lamina, influencing cell differentiation and proliferation, migration, adhesion as well as phenotype and survival. Species of laminins are presented as combinations of different alpha-, beta-, and gamma-chains. There are five forms of alpha-chains (LAMA1, LAMA2, LAMA3, LAMA4, LAMA5), four of beta-chains (LAMB1, LAMB2, LAMB3, LAMB4), and three of gamma-chains (LAMC1, LAMC2, LAMC3). Laminins of the instant invention may be provided in any combination of such chains, or any other naturally occurring or synthetic sequences, homologues and structural variants thereof.

As noted above, one or more non-matrix proteins are also included in the extract. While any of the aforementioned proteins may be included, in one embodiment the non-matrix proteins include one or more growth factors. Examples of such agents include, but are not limited to, Coagulation factor III (CFIII), Dickkopf-1 (DKK-1), Dickkopf-3 (DKK-3), Developmental receptor tyrosine kinase (Dtk), Gremlin, Hepatocyte growth factor (HGF), Interleukin-1 receptor II (IL-1RII), Interleukin-4 (IL-4), Interleukin-5 receptor α (IL-5 α), Interleukin-20 (IL-20), Leptin receptor (Leptin R), Leukemia inhibitor factor (LIF), Tumor necrosis factor SuperFamily 14 (TNFSF14), Lymphotoxin, Macrophage inflammatory protein-1 α (MIP α), Osteopontin, Osteoprotegerin, Progranulin, P-Selectin, Resistin-like molecule β (RELM β), Secretory leukocyte

peptidase inhibitor (SLPI), Tissue inhibitor of metalloproteinases 1 (TIMP-1), Toll-like receptor 4 (TLR4), Tomoregulin-1, Tumor necrosis factor SuperFamily 10 (TNFSF10), Ubiquitin, Vascular endothelial growth factor (VEGF), and combinations thereof.

In certain embodiments, the growth factors include a combination of at least
5 VEGF, HGF, and LIF. While not intending to be bound by theory, VEGF is a proangiogenic factor that promotes formation of new blood vessels at sites of injury and is also active in vasculogenesis and endothelial cell growth. It also induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis, and induces permeabilization of blood vessels and binds to the VEGFR1/Flt-1 and VEGFR2/Kdr receptors, heparan
10 sulfate, and heparin.

While not intending to be bound by theory, HGF is a paracrine cellular growth, motility, and morphogenic factor. It is typically secreted by mesenchymal cells and targets and acts primarily on epithelial cells and endothelial cells but also on hemopoietic progenitor cells. It is shown to have a major role in embryonic organ development, adult
15 organ regeneration, and wound healing. HGF also regulates cell growth, cell motility, and morphogenesis by activating a tyrosine kinase signaling cascade after binding to the proto-oncogenic c-Met receptor. Its ability to stimulate mitogenesis, cell motility, and matrix invasion gives it a central role in angiogenesis, tumorigenesis, and tissue regeneration. It is secreted as a single inactive polypeptide and is cleaved as serine proteases into a 69-kDa α
20 chain and 34-kDa β chain.

While not intending to be bound by theory, LIF derives its name from its ability to induce the terminal differentiation of myeloid leukemic cells. Other properties attributed to the cytokine include the growth, promotion, and cell differentiation of different types of target cells, influence on bone metabolism, cachexia, neural development, embryogenesis,
25 and inflammation.

Based on the foregoing, the isolated cell extract may be comprised of at least the matrix proteins collagen IV, fibronectin, and laminin and at least the non-matrix proteins VEGF, HGF, and LIF. The total concentration of matrix and non-matrix proteins may be about 100 mg/ml. To this end, collagen IV may be provided in a concentration range
30 between about 20 μ g/ml and about 60 μ g/ml. The fibronectin also may be provided in a concentration range between about 2 μ g/ml and about 5 μ g/ml, and the laminin in a

concentration range between about 10 µg/ml and about 15 µg/ml. In certain embodiments of the instant invention, the extract contains at least approximately 60 µg/ml collagen IV, approximately 2 µg/ml of fibronectin, approximately 15 µg/ml of laminin. Such a composition may be incorporated into a cell culture media or basement membrane substrate, as discussed in greater detail below. The term “about” or “approximately,” or the like, as used herein, refers to a range of values within ten percent (10%) of a baseline value. Thus, for example, the phrase “about 100” refers to a range of values between 90 and 110. When the term “about” or “approximately,” or the like, is applied to a range, it indicates that both the upper limit and lower limit can vary up to ten percent (10%) of the base line value.

The term “a,” “an,” or “the,” as used herein, represents both singular and plural forms. In general, when either a singular or a plural form of a noun is used, it denotes both singular and plural forms of the noun.

The instant invention also relates to novel methods for generating the foregoing cellular extract. In one embodiment, this method includes an *in vitro* cell culture system that generates substantial amounts of natural extracellular matrix and non-matrix proteins. Cells, specifically, are grown on differentiation media adapted to optimize the production and excretion of such proteins, which are then isolated and purified using methods discussed below or otherwise known in the art. While any cell line is suitable for this method, in certain embodiments, stem cells and preadipocytes cells are used because of their pluripotent capabilities, ability to produce natural BM extracts, and/or ability to produce a sufficient level of ECM proteins and non-matrix proteins. Preadipocytes are particularly preferred, although not limiting, because of their ability to produce such extracts, which is advantageous over current systems and protocols. Such cells may be derived from any species, but in certain embodiments are derived from mammalian sources including, but not limited to, those of a murine or human host. While preadipocytes are discussed below in the context of the instant methodology, this is not considered limiting to the invention, as any of the foregoing or otherwise known cell types may be readily substituted.

As an example, Adipogel is compared with Matrigel in Table 1.

Table 1. Comparison between Adipogel with Matrigel.

Feature	Matrigel	Adipogel
Method of synthesis	Purified from extract of murine EHS tumor	Purified from mammalian preadipocyte secretions
Composition	Collagen IV, Laminin, perlecan, nidogen, FGF, IGF, EGF (16)	Collagen IV, laminin, fibronectin, hyaluronan, HGF, VEGF, LIF
Applications	Angiogenesis, transplantation, tissue engineering	Primary cells and cell line culture, functional maintenance
Disadvantages	Batch-to-batch variability, animal derived, chemical digestion, not completely characterized, complex mixture	Not completely characterized, complex mixture, animal cell line derived
Advantages	Tested in multiple applications, basement membrane-like complex, gelation procedure easy	No chemical enzymatic procedures, less cumbersome, cheap basement membrane-like complex

In a first embodiment, the cells are optionally incubated to confluency using any standard growth media. By way of example, such media may include, but is not limited to, Dulbecco's Modified Eagle Medium (DMEM), which may be supplemented with bovine serum and one or more antibiotics. In certain embodiments, the media is supplemented with 10% FBS and 2% penicillin and streptomycin. The cells are incubated until confluent or until forming a single cell mass across the media, and are then transferred to the differentiation media. While cells may be transferred at any time post-confluency, in certain instances they are transferred approximately 48 hours after confluency.

The differentiation media used to develop the extract includes at least one soluble factor, at least one steroid, and isobutylxanthine. Soluble factors, ideally, though not exclusively, include one or more agonists or agents that induce natural cell growth pathways. In one embodiment, for example, the soluble factor is an agonist that induces the lipogenic pathway. Such agonists may include, but are not limited to, rosiglitazone, troglitazone, thiazolidinediones, and combinations thereof. In certain embodiments, the agonist is rosiglitazone. In alternative embodiments, the solubilizing factor that stimulates cell growth is insulin. The soluble factor may be provided, either individually or collectively, at any effective concentration able to initiate, facilitate, or assist with the induction of a natural cell growth pathway. In certain embodiments, such an effective amount is between about 0.1 μ M and 1.0 μ M. In further non-limiting embodiments, for example, the soluble factor(s) is provided at a concentration of about 1.0 μ M.

The steroids used within the differentiation media also may include any steroid that acts as an receptor agonist and an inducer of cellular differentiation and/or proliferation.

In one embodiment, such steroids may include, but are not limited to, dexamethasone, cortisol, aldosterone, hydrocortisone, triiodothyronine, corticosterone, and combinations thereof. In certain embodiments, the steroid is dexamethasone. The steroids may be provided, either individually or collectively, at any effective concentration able to act as a
5 receptor agonist and/or inducer of cell differentiation and/or proliferation. In certain embodiments, such an effective amount is a concentration between about 0.1 μ M and 1.0 μ M. In further non-limiting embodiments, the steroid(s) is provided at a concentration of about 1.0 μ M.

While not intending to be bound by theory, isobutylxanthine is provided as a cyclic
10 AMP activator and decreases cell proliferation. It may be provided at any effective concentration able to achieve such effects. In one embodiment, an effective concentration is between about 0.1 μ M and 1.0 μ M. In further embodiments, isobutylxanthine is provided at a concentration of about 0.1 μ M.

Based on the foregoing, in one embodiment of the instant invention, the
15 differentiation media is comprised of approximately 1 μ M dexamethasone, 0.1 μ M isobutyl-methylxanthine, and 1 μ M rosiglitazone and/or 1 μ M insulin.

The foregoing concentrations are not considered limiting to the invention, however, may be adjusted in accordance with the foregoing teachings or ranges to any alternative amount that would achieve the foregoing effects and would optimize
20 extracellular matrix and non-matrix protein production and excretion. Because cell types have unique requirements for proliferation and maintenance of cell functionality, optimization may be dependent upon the intended use of the extract, i.e. type of cells to be grown on an extract-containing media or basement membrane complex. As illustrated below, such concentrations may be specifically optimized for specific cell types such as
25 hepatocytes, stem cells, endothelial cells, keratinocytes, fibroblasts, tumor cells, or similar cells types which would be desirable to grow on a basement membrane matrix. Optimization techniques for these, or other cells types, would be readily apparent to one of skill in the art based on the Experiments and disclosure provided herein.

Preadipocytes, or other cell types, are incubated on the differentiation media for
30 any effective amount of time to elicit the desired amounts biomaterial extract provided herein. In one embodiment, cells are incubated from 0-10 days with medium changes

approximately every 2 d. In another embodiment, cells are incubated from 0-5 days with medium changes approximately every 2 d. In even further embodiments, cells are incubated from 2-4 days, preferably 4 days, with medium changes approximately every 2 d, as this period results in a highly viscoelastic material resembling ECM components secreted maintain adipose tissue cell-cell contact, cell morphological induction, and functional and gene expression indicative of mature cell lineage. In alternative embodiments, however, the cells may be incubated for 2 days. On the second day post-differentiation, cells may be optionally exposed to culture medium supplemented only with one or more solubilizing factors provided herein. Again, the instant invention is not limited to the foregoing and incubation time periods may be optimized based upon the intended use of or cells to be grow upon the extract. Optimization techniques would be readily apparent to one of skill in the art based on the Experiments and disclosure provided herein.

Cell-exposed differentiation media is then collected and purified using standard techniques. In one example, the media is centrifuged at a sufficient speed and using an appropriate sized filter to removed unwanted elements, while retaining the proteinaceous portion of the isolate. Centrifuge speeds and times and filter sizes may be in any amount to isolate all proteins greater than 10 kDa in size; greater than 100 kDa in size; or any size that is desirable. For example, the extract may be centrifuged at 4000 g for 1.5 h with filter sizes include 100- or 10-kDa centrifugal filter.

The isolated extract may then be stored or otherwise incorporated into a cell media or basement membrane substrate for use in either *in vitro* or *in vivo* culture systems. With respect to the former, the extract may be used directly or otherwise may be combined with additional cell media in any combination or ratio effective to facilitate cell growth, proliferation, migration, functionality or the like. Such an effective ratio of extract to cell media, where necessary, may be between 1:1 and 1:10. In certain embodiments, the ratio of extract is cell media is approximately 1:5. The ratio, however, is not limited to this range and may be adjusted depending upon the type of cells to be grown and media requirements of such cells.

The additional cell culture media may include any culturing media that is known in the art, particularly for mammalian cell systems, such as, but are not limited to, DMEM

media, Williams Media E, CSG media, stem cell culture media, basal media, RPMI, alpha-MEM, IMDM, or the like. While one of skill in the art will readily appreciate the applicability of the techniques below to a wide range of cell culture systems, exemplified, but non-limiting, cell lines useful for such methods include hepatocyte, stem cells,
5 keratinocytes, fibroblasts, tumor cells and endothelial cells.

In hepatocyte cell systems, the cell media extract was generated using a differentiation media of dexamethasone, isobutylxanthine, and rosiglitazone in accordance with the foregoing and the Examples below. The isolated extract was then laden on top of CSG hepatocyte cultures and was shown to induce increased albumin-differentiated
10 function, when compared to otherwise known culture media and basement membranes. In culture period of at least 10 days, the cells exhibited enhanced albumin secretion, comparable cytochrome P450 1A1 activity, improved differentiated function, and improved cell morphology, as compared to other synthetic culture types (FIGs. 2-3). While not intending to be bound by theory, it is surmised that such effects are due, at least
15 in part, the natural biological material in the extract, particularly the ECM proteins laminin and collagen IV.

In endothelial cells, a cell media extract was generated using a differentiation media of dexamethasone, isobutylxanthine, and either insulin or rosiglitazone. The isolated extract was then laden on top of endothelial cells plated on basal media and
20 incubated with the cell extract being added to the cells every two days. After day four, and as illustrated in FIG. 4, the endothelial cells exhibited Human umbilical vein endothelial cell (HUVEC) tube formation. This, again, suggests that the more natural biological material of the instant invention maintained natural cell functionality.

Over three million patients suffer from chronic wounds in the U.S. Numerous
25 types of wound healing processes, for example, partial thickness wound healing, full thickness wound healing, incisional wound healing, diabetic foot ulcers, venous leg ulcers, pressure sores, and burns. Issues associated with wound healing include expensive regimens, difficulty to achieve complete wound closure, wound scarring, and no growth factor, cytokine enriched hydrated matrix available for wound healing. Wound contraction
30 process includes inflammation, proliferation, and remodeling. One objective of the present invention is to use Adipogel to cause increased host integration for a faster and

better wound healing. Adipogel, as an extracellular matrix enriched with natural growth factor can cause a complete wound closure in 2-4 days.

Single and multi-component extracellular matrix (ECM) protein arrays have been developed in academia and the industry for probing cell adhesion, growth, proliferation and differentiation. The drawbacks in the commercial success of these technologies include: a) expensive systems utilizing commercially available ECM molecules, b) lack of complete set of basement membrane proteins in sub-arrays due to difficulty in availability of rare components, c) reconstituted ECMs that have been chemically or enzymatically derived that do not mimic the natural protein-protein interactions present in basement membranes *in vivo*, d) single ECM per sub-array not mimicking *in vivo* basement membrane-like architecture and e) utilization of ECMs in arrays without manufacture of matrix components in a controlled environment (FIG. 10).

A preliminary *in vitro* wound assay was developed to illustrate the effect of Adipogel on fibroblast migration and keratinocyte cell attachment in the wound closure phenomena. As shown in the figure, following a scratch-wound, there is an increase in cell migration rate and number of cells migrating into the wound 24 hours post scratch wound for human dermal fibroblasts on the matrix as compared to tissue culture plastic. In addition, there is increased fibroblast cell proliferation in the presence of extracellular matrix with keratinocyte attachment on the ECM [FIG. 5]. In addition, the effect of natural ECM on breast cancer cell adhesion and proliferation is also illustrated in FIG. 11, which compares Adipogel, Matrigel, Adipogel + Matrigel with untreated plates using MDA-MB231 cells. In another aspect, the present invention provides a method for proliferating and maintaining the phenotype of human embryonic stem cells comprising: culturing human embryonic stem cells on a media extract comprising cellular matrix and non-matrix proteins isolated and purified from a cell-exposed differentiation media comprising at least one soluble factor, at least one steroid, and isobutylmethylxanthine. The human embryonic stem cells are incubated on the extract either directly or after it is incorporated into additional cell media. This method results in the proliferation of the cells, while maintaining the original phenotype.

The complex developmental process leading to generation of adult tissues from embryonic stem cells include early embryonic development, commitment, and late

embryonic development stages, specifically from zygote, through morula, blastocyst, three germ layers (ectoderm, mesoderm, endoderm), and progenitors, finally to adult tissues. The challenge is to understand the mechanisms controlling differentiation.

In one embodiment the present invention provides introduction of metabolic engineering and biochemical pathways (i.e., identification, characterization, and modification) to improve cellular function. [FIG. 12]. The disease treatment approach of the present invention in this aspect is illustrated in FIG. 13. In this aspect the present invention provides cellular metabolism-novel biological substrate integrated crosstalk to elucidate Cell-ECM-Metabolism interactions in various applications, such as stem cell differentiation, primary cell culture, tumor invasions, spinal cord injury, obesity research, and high-throughput screening systems.

In embryonic stem cells, the cell media extract was also generated using a differentiation media dexamethasone, isobutylxanthine, and either insulin or rosiglitazone. Isolated extract was then laden on top of embryonic stem cells plated on mouse embryonic feeder layer conditioned media, which resembled human Embryonic Stem Cell media. As illustrated in FIG. 8, after day 7 of the incubation cell growth was observed and the cell exhibited an undifferentiated phenotype.

In addition to *in vitro* techniques, the extract of the instant invention may also be used as a platform for assisted *in vivo* cell differentiation, functionality maintenance, recruitment, angiogenesis, migration, and the like. Generally speaking, the extract may be applied directly or otherwise combined with one or more other natural or synthetic compositions to form a basement membrane that is suitable for the development of a desired cell type. This basement membrane is then applied directly applied to a target site on a patient or used in an *in vitro* assay to mimic *in vivo* cell behavior. Optionally, additional cell types (e.g. embryonic stem cells, endothelial cells, etc.) also may be applied to the matrix *in situ*, or otherwise *in vitro* prior to application of the biomaterial.

While one of skill in the art will readily appreciate the applicability of the techniques below to a wide range of uses, the composition of the additional basement membrane components and ratio of such components to the extract are provided below for wound healing and anti-aging. These uses are not considered limiting, however, and the basement membrane may be optimized depending upon alternative uses and alternative

target cell system. As discussed previously, optimization techniques would be readily apparent to one of skill in the art based on the Experiments and disclosure provided herein.

In a first embodiment, the extract of the instant invention is used in an *in vitro* wound assay to illustrate its effect on fibroblast migration and keratinocyte cell attachment in the wound closure phenomena. As shown in FIG. 5, following a scratch-wound, there was an increase in cell migration rate and number of cells migrating into the wound 24 hours post scratch wound for human dermal fibroblasts on the matrix as compared to tissue culture plastic. In addition, there was increased fibroblast cell proliferation in the presence of extracellular matrix with keratinocyte attachment on the ECM.

In another embodiment, the extract of the instant invention is applied directly or combined with additional basement membrane components for deriving a scaffold to facilitate wound healing. Such additional basement membrane materials, where applicable, are preferably, though not exclusively, naturally derived and of mammalian or human origin. Materials may be specifically manufactured to avoid immunogenicity upon application. In one embodiment, additional basement membrane components may be derived from mammalian cells and/or primary cells, in general. Such components may be derived using one or more of the purification methods discussed herein, or otherwise using known or standard methods in the art.

The extract containing basement membrane may be applied to the wound in any amount or mechanism effective to achieve the desired wound healing. While not limited thereto, in one embodiment, it is provided at a ratio of about 2.5 – 20.0 μ l of biomembrane per about 1 mm of wound and may be reapplied as necessary, e.g. every 6 hours, 12 hours, 24 hours, 48 hours, etc. One of skill in the art will readily appreciate, however, that this ratio may be varied based on the ratio of extract to basement membrane components and, otherwise, upon the desired healing rate. The basement membrane containing the extract may be applied directly to the wound site or otherwise incorporated into known wound dressings, bandages, or the like.

As illustrated in FIGs. 6 and 7, when the basement membrane containing the extract of the instant invention is incorporated within the wound, it results in keratinocyte cell proliferation, migration and accelerated wound healing. As further illustrated in FIG. 7, administration of the extract resulted in a 1.5 fold increase in normalized cell increase,

as compared to cell generation rates in the absence of the instant extract or basement membrane. Accordingly, the extract containing biomaterial increases the rate of cell differentiation and the healing process.

5 In addition to wound healing, the instant invention also relates to a method of treating aging skin using an extract containing basement membrane. More specifically, the extract of the instant invention is applied to a site of the aging skin alone or in combination with additional basement membrane components using one or more of the components/methods/ratios discussed above.

10 The extract containing basement membrane may be applied to the site of aged skin in any amount or mechanism effective to achieve the anti-aging effect. While no limited thereto, in one embodiment, it is provided at a ratio of about 2.5 – 20.0 μ l of biomembrane per about 1 mm of the site. One of skill in the art will readily appreciate, however, that this ratio may be varied based on the ratio of extract to basement membrane components and, otherwise, upon the desired healing rate. The basement membrane containing the extract
15 may be applied topically to aged or aging skin or otherwise incorporated into known dressings, bandages, or the like. Such application, in accordance with the methods and ranges above, results in enhanced expression and functionality of epidermal cells, particularly keratinocytes, and improves cell viability. Thus, it reduces the appearance of aging in the patient.

20 In further embodiments, the instant invention also relates to a kit for supporting cell growth and providing base components for scaffold formation and tissue engineering. The kit may include an extract prepared in accordance with the foregoing methods and specifically optimized for proliferating a cell type. The kit may also include one or more additional cell culture media or basement membrane scaffolds, also in accordance with the
25 foregoing, which may be admixed with the extract and used for *in vitro* or *in vivo* cell proliferation.

Further, the adipocyte derived basement membrane extract (adipogel) of the present invention may find wide applications in addition to hepatocyte functional maintenance, wound healing, cell adhesion ECM arrays, and stem cell differentiation as
30 described above, for example, but not limited to, 3-D tissue microenvironments for toxicogenomics; angiogenesis and anti-angiogenic compound identification; drug delivery;

in vitro cell culture, growth, proliferation, functional maintenance, preservation and differentiation (ECM Arrays); in vitro burn models; diabetic foot ulcers; integrated signaling and metabolic network for matrix-metabolism interactions; multiplex transfected/non-transfected cell line ECM array factories for HCS; cell-biomaterial
5 interactions; biomimetic materials; tumor therapy; bone disease; nerve repair; medical devices; and skin care.

In other aspects the invention provides use of an extract comprising extracellular matrix and non-matrix proteins prepared according to any embodiments described herein for (1) proliferating and/or maintaining cell functionality of hepatocytes, (2) proliferating
10 and/or maintaining cell functionality of endothelial or endothelial-like cells, (3) manufacture of a medicament for treatment of a wound in a subject, (4) manufacture of a medicament for treatment aging skin in a subject, or for any other similar purposes, wherein the extract is isolated from a cell-exposed differentiation media comprising at least one soluble factor, at least one steroid, and isobutylmethylxanthine.

15 In the foregoing description, numerous specific details are set forth in order to provide a thorough understanding of the invention. It should be understood by one of skill in the art, however, that the present invention is not limited to these details and that adaptations may be made to achieve similar effects. To this end, additional embodiments and advantages of the instant invention will be readily apparent to one of skill in the art,
20 based on the foregoing. The invention will be further described in the following non-limiting examples.

EXAMPLES

Materials

Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/L glucose, fetal
25 bovine serum (FBS), penicillin, streptomycin, and trypsin-EDTA were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Dexamethasone, isobutylmethylxanthine, epidermal growth factor, insulin, glucagon, rosiglitazone, and hydrocortisone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Protein centrifugal filters (10- and 100-kDa cutoff) were purchased from Millipore Technologies
30 (Billerica, MA, USA). Matrigel at high concentration was purchased from BD Biosciences

(Franklin Lakes, NJ, USA). (See Sharma N, et al., *PLOS One*, 6(5): e20137 (2011), which is hereby incorporated by reference in its entirety.)

Example 1 - Adipogel generation and characterization using an agonist

A. Generation using preadipocyte differentiation

5 Mammalian preadipocytes were cultured in T-175 cm² flasks in DMEM supplemented with 10% FBS and 2% penicillin and streptomycin until the cells attained confluency. Then, at 48 h postconfluency, the cells were differentiated in culture medium supplemented with 1 μ M dexamethasone, 0.1 μ M isobutyl-methylxanthine, and 1 μ M rosiglitazone for 2 d with medium changes every 2 d. On the second day post-
10 differentiation, cells were exposed to culture medium supplemented with 1 μ M rosiglitazone for an additional 2 d. Medium supernatant was collected on d 2 and 4 of differentiation and stored at 4°C prior to further processing.

During the differentiation process, cell-exposed medium was collected and processed further for generation of cell-derived ECMs. A highly viscoelastic material was
15 identified on d 2 and 4 of adipocyte differentiation resembling ECM components secreted by preadipocytes to maintain adipose tissue cell-cell contact, morphological induction of adipocytes, and functional and gene expression indicative of mature adipocyte lineage.

To purify the ECM-rich material, the differentiated preadipocyte-conditioned medium was centrifuged at 4000 g for 1.5 h using an Amicon 100- or 10-kDa centrifugal
20 filter. The concentrate, primarily composed of medium constituents with molecular mass cutoff of 100 and 10 kDa, comprised the cell culture supernatant-derived protein concentrate, including ECM. About 250 μ l of protein concentrate was obtained at the end of the purification step per 15 ml of conditioned medium, with a yield of ~60 fold. Because the concentrate was derived from an adipocyte-related cell type and had a gel-like
25 configuration, it was termed “Adipogel.”

B. Adipogel composition determination using protein arrays

In addition to characterization of ECM proteins, the protein composition of the d 4 Adipogel was identified. The protein composition was determined using Biotin-Labeled Antibody Arrays (Ray Biotech, Norcross, GA, USA) for simultaneous detection of 308
30 mouse proteins in d 4 Adipogel. Through a simple process, the diluted Adipogel samples were biotinylated and dialyzed overnight in preparation for incubation with the array. The

biotinylated sample was added onto the glass slide antibody arrays and incubated at room temperature with gentle shaking. After incubation with fluorescent dye streptavidin, the signals were visualized by either chemiluminescence or fluorescence. Protein concentrate purified from FBS-supplemented basal medium was utilized as negative control. A
 5 normalization of up-regulated proteins in Adipogel vs. controls was performed to detect fold changes. Fold changes >1.5 were identified and categorized into different protein subtypes.

The protein content of the gel consists of 27 up-regulated proteins as compared to controls (basal medium protein concentrate). Table 2, below, illustrates the protein
 10 characterization of Adipogel: upregulated protein composition of Adipogel compared to DMEM basal medium at d 4; 10-kDa cutoff.

Table 2. Protein characterization of Adipogel.

Symbol	Fold Change	Description	Protein Category	Tissue Specificity/Biological Activity
Epiregulin	2.78	Epiregulin	Mitogen	NIH3T3 condition medium and human colonic subepithelial myofibroblasts;/ Potential mitogen for renal epithelial cells Autocrine growth factor for human keratinocyte proliferation; Cancer development; Wound closure
HGF	12.54	Hepatocyte Growth Factor	Growth Factor	Paracrine cellular growth, motility and morphogenic factor, embryonic organ development, adult organ regeneration, wound healing
LIF	1.91	Leukemia Inhibitory Factor	Cytokine	Interleukin 6 cytokine; terminal differentiation of myeloid leukaemic cells. Growth promotion and cell differentiation of different types of target cells. Influence on bone metabolism, cachexia, neural development, embryogenesis and inflammation
Osteopontin	1.66	Osteopontin	Cytokine	Preosteoblasts, osteoblasts, osteocytes, extraosseous cells in the inner ear, brain, kidney, deciduum, placenta, odontoblasts, some bone marrow cells, hypertrophic chondrocytes, macrophages, smooth muscle, and endothelial cells/Bone remodeling and anchoring osteoclasts to the mineral matrix of bones
Progranulin	6.09	Progranulin	Growth Factor	Neurotrophic factor regulates neurite outgrowth and enhance neurite survival; Nervous system development and neurodegeneration
P-Selectin	3.74	P-selectin	Signal Transduction Protein	Recruits leukocytes (white blood cells) to the site of injury during inflammation
VEGF	16.14	Vascular Endothelial Growth Factor	Growth Factor	Angiogenesis, vasculogenesis and endothelial cell growth, proliferation; promotes cell migration, inhibits apoptosis and induces permeabilization of blood vessels

The applications of Adipogel of the present invention in hepatocyte functional augmentation *in vitro* is compared with products from other sources favorably. See Table

15 3.

Table 3. Comparison of Adipogel with other products.

	In Vitrogen Celsis Lonza	ATCC	Gerone Yecuris	Rutgers Technology
Product	•Fresh human hepatocytes •Cryopreserved human hepatocytes	•Cell lines	•Stem cell products	•Adipogel sandwich with primary hepatocytes •Multiple species
Difference	•Suboptimal donors •Lot to lot variation •Poor in vivo – in vitro correlation	•Poor in vivo – in vitro correlation •Limited in cellular function	•Mixed cell populations •Recovery of specific cells is difficult •Limited to lab scale	•Ability to maintain cellular function for weeks •Cheap production process

Example 2 – Hepatocyte cell function on Adipose gelA. Primary rat hepatocyte isolation

5 Female Lewis rats (Charles River Laboratories, Wilmington, MA, USA) weighing 180 –200 g (2–3 mo old) were used as a hepatocyte source and were maintained in accordance with National Research Council guidelines. Experimental protocols were approved by the Subcommittee on Animal Care, Committee on Research, Massachusetts General Hospital. Using a modification of the 2-step collagenase perfusion method, which
 10 involves purification of the cell suspension by means of centrifugation over Percoll, $\sim 1\text{--}2 \times 10^6$ cells were routinely isolated from one rat with viability between 85 and 98%, as judged by Trypan blue exclusion.

B. Hepatocyte culture in collagen sandwich, Adipogel, and Matrigel

15 Type 1 collagen was prepared by extracting acid-soluble collagen from Lewis rat-tail tendons. To create a thin layer of collagen gel in 12-well tissue culture plates, 400 μl of an ice-cold mixture of 1 part of 10X concentrated DMEM and 9 parts of 1.25 mg/ml rat tail tendon type I collagen were evenly distributed over the bottom of each well. The plates were incubated at 37°C for 60 min to induce collagen gelation before cell seeding.
 20 Each well of the 12-well culture plates received 5×10^5 primary hepatocytes in

suspension in 0.5 ml standard hepatocyte culture medium, which consisted of DMEM supplemented with 14 ng/ml glucagon, 7.5 µg/ml hydrocortisone, 0.5 U/ml insulin, 20 ng/ml EGF, 200 U/ml penicillin, 200 µg/ml streptomycin, and 10% FBS. Cultures were incubated in 90% air/10% CO₂ at 37°C. Cells were rinsed with 1X PBS to remove nonadherent cells 4–6 h after seeding. For the double-collagen-gel culture configuration, a second layer of 250 µl collagen was laden on top of the cells 48 h postseeding. Medium was changed every 24 h and collected from d 3 onwards until d 10. Three additional culture conditions were utilized as described below.

For the Adipogel conditions, the BME prepared in accordance with Example 1 and isolated on d 4 was utilized for the entire set of hepatocyte experiments. For the soluble Adipogel condition, 100 µl of Adipogel was solubilized in 400 µl of culture medium by continuous pipeting. The supplemented medium was added to cell cultures on d 0, 1, 2, 5, 7, and 9 of culture [FIG. 1B]. For the second condition, to form the adipocyte-derived gel, 100 µl of Adipogel was uniformly spread over each well by slow dripping along the wall. To promote gelation, the plates were incubated at 37°C for 60 min, followed by addition of culture medium.

For Matrigel cultures, a single layer of 200 µl Matrigel was added to each well of a 12-well plate, as described previously. The coated plates were incubated for 30 min at 37°C for gelation. Following this, hepatocytes were seeded on top of the single-gel Matrigel cultures.

C. Hepatocyte functional assessment

Albumin concentration in the collected medium samples was analyzed using a competitive ELISA. Albumin protein and the antibody were purchased from MP Biomedicals (Solon, OH, USA). Urea concentration was determined *via* its specific reaction with diacetyl monoxime with a commercially available assay kit (Fisher Scientific, Pittsburgh, PA, USA). The absorbance was measured with a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

D. Biochemical Assays

The biochemical assays were performed at the recovery, pre-stable and stable stages of culture with media samples. Amino acids were fluorescently labeled using the AccQ-Tag system [Waters Co., Milford, MA], separated by high performance liquid

chromatography [HPLC Model 2690, Waters Co.], and quantitated by a fluorescence detector [Model 474, Waters Co.]. Glucose and lactate levels were measured with commercially available kits [Sigma], the former based on the reaction of glucose catalyzed by glucose oxidase and the latter based on the conversion of lactate to pyruvate catalyzed by lactate oxidase. Acetoacetate and bhydroxybutyrate were measured using a commercially available kit [Bioassay Systems].

E. Metabolic Flux Analysis

Metabolic flux analysis (MFA) is a useful methodology to characterize the differential activation of metabolic pathways in hepatocyte cultures. Thus, based on a stoichiometric model for the metabolic reaction network prevalent in hepatocytes, intracellular reaction fluxes are estimated by mass balances around each intracellular metabolite and extracellular flux measurements. This gives the possibility to calculate intracellular metabolite fluxes, which are difficult to measure from relatively few measurements and to corroborate the metabolic network. The model used in this work was originally developed for perfused liver and modified subsequently for cultured hepatocytes with incorporation of lipid metabolism reactions. FIG. 14 illustrates the metabolic network considered. Table 4 and Table 5 show the list of reactions and metabolites included. The main assumptions for the application of MFA to the hepatic metabolic network are as follows: 1. The metabolic network is based on known stoichiometry of hepatic intermediary metabolism with consideration of carbon and nitrogen balances. 2. Albumin is a major protein product of hepatocytes and hence only this protein is considered. 3. The cellular uptake/secretion rates of metabolites are distinct from the intracellular fluxes of the corresponding metabolites. Thus, the intracellular and extracellular pools of substrates have been distinguished. Also, the mechanisms of active and passive transport have not been incorporated. 4. The metabolite pools are at pseudo steady-state with a single pool in the cell. The influx and efflux of metabolites into/from hepatocytes are calculated from the amount of metabolites remaining in the extracellular media after 24 h.

Following the above assumptions, the mathematical model consists of mass balances around 45 intracellular metabolites considering 72 reactions [Table 4, Table 5 and FIG. 14]. The common pathways in Table 4 and Table 5 include pentose phosphate pathway; lipid, glycerol and fatty acid metabolism; lactate metabolism and tricarboxylic acid (TCA) cycle; urea cycle; amino acid metabolism; oxygen uptake and electron

transport and albumin protein metabolism. The sum of fluxes to and from the metabolite pools is assumed to be zero [pseudo steady-state assumption]:

$$S \cdot v = 0 \quad (1)$$

where the matrix S contains the stoichiometric coefficients of the incorporated reactions.

- 5 Each element S_{ij} of S is the coefficient of metabolite i in reaction j , and each v_j of vector v is the net flux or conversion rate of reaction j . Equation (1) is separated into measured and unknown fluxes, v_m and v_u , respectively, as follows:

$$S_u \cdot v_u = S_m \cdot v_m \quad (2)$$

- 10 The measured fluxes represent measured rates of uptake or release of extracellular metabolites and thus, solving Equation (2) gives estimates of intracellular fluxes.

The intracellular metabolites used for mass balance include MetabolitePools, Glucose-6Phosphate, Ribulose-5Phosphate, Ribose-5Phosphate, Xylulose-5Phosphate, Erythrose-4Phosphate, Glyceraldehyde-3Phosphate, Fructose-6Phosphate, Fructose-1,6BisPhosphate, PhosphoEnolPyruvate, Pyruvate, NADH, FADH₂, Acetyl-CoA, 15 Oxaloacetate, Citrate, 2-oxo-gluterate, Succinyl-CoA, Fumarate, Malate, Ammonia, Ornithine, Citrulline, Acetoacetyl-CoA, Acetoacetate, Alanine, Cysteine, Aspartate, Glutamate, Phenylalanine, Glycine, Histidine, Isoleucine, Lysine, Leucine, Methionine, Asparagine, Proline, Glutamine, Arginine, Serine, Threonine, Valine, Tyrosine, CO₂, Oxygen (45 metabolites).

20 **Table 4: List of reactions in metabolic network utilizing Gluconeogenic fluxes.**

Flux #	Reaction	Pathway
1	Glucose 6-phosphate → Glucose	Gluconeogenesis
2	Glucose 6-phosphate → 2 NADPH + CO ₂ + Ribulose 5-P	PPP
3	Ribulose 5-P ↔ Ribose 5-P	PPP
4	Ribulose 5-P ↔ Xylulose 5-P	PPP
5	Ribose 5-P + Xylulose 5-P ↔ Fructose6-P Erythrose 4-P	PPP
6	Erythrose 4-P + Xylulose 5-P ↔ Glyceraldehyde 3-P + Fructose 6-P	PPP
7	Fructose 6-phosphate ↔ Glucose 6-phosphate	Gluconeogenesis
8	Fructose 1,6-Bisphosphate → Fructose6-phosphate	Gluconeogenesis
9	2 Glyceraldehyde 3-P ↔ Fructose 1,6-Bisphosphate	Gluconeogenesis
10	Glycerol + ATP → Glyceraldehyde 3-P + NADH	Lipid metabolism

Flux #	Reaction	Pathway
11	Phosphoenolpyruvate + ATP + NADH $\leftrightarrow$ Glyceraldehyde 3-P	Gluconeogenesis
12	Oxaloacetate + GTP $\leftrightarrow$ CO ₂ + Phosphoenolpyruvate	Gluconeogenesis
13	Pyruvate + CO ₂ + ATP $\leftrightarrow$ Oxaloacetate	Gluconeogenesis
14	Lactate $\leftrightarrow$ Pyruvate + NADH	Lactate/TCA cycle
15	Acetyl-CoA + Oxaloacetate $\rightarrow$ Citrate	Lactate/TCA cycle
16	Citrate $\leftrightarrow$ 2-oxo-Glutarate + NADH + CO ₂	Lactate/TCA cycle
17	2-oxo-Glutarate $\rightarrow$ Succinyl-CoA + NADH + CO ₂	Lactate/TCA cycle
18	Succinyl-CoA $\leftrightarrow$ GTP + FADH ₂ + Fumarate	Lactate/TCA cycle
19	Fumarate $\leftrightarrow$ Malate	Lactate/TCA cycle
20	Malate $\leftrightarrow$ Oxaloacetate + NADH	Lactate/TCA cycle
21	Ornithine + CO ₂ + NH ₄ + 2 ATP $\rightarrow$ Citrulline	Urea cycle
22	Citrulline + Aspartate + ATP $\rightarrow$ Arginine + Fumarate	Urea cycle
23	Arginine $\rightarrow$ Ornithine + Urea	Urea cycle
24	Alanine + 2-oxo-Glutarate $\leftrightarrow$ Pyruvate + Glutamate	AA metabolism
25	Serine $\rightarrow$ Pyruvate + NH ₄	AA metabolism
26	Cysteine + 2-oxo-Glutarate + HCN $\leftrightarrow$ Glutamate + HSCN + Pyruvate	AA metabolism
27	Threonine + ATP $\rightarrow$ NADH + Glycine + Acetyl-CoA	AA metabolism
28	2 Glycine $\leftrightarrow$ Serine + CO ₂ + NH ₄ + NADH	AA metabolism
29	Valine + 2-oxo-Glutarate + ATP $\rightarrow$ Glutamate + Succinyl-CoA + 2 NADH + FADH ₂ + CO ₂	AA metabolism
30	Isoleucine + 2-oxo-Glutarate + ATP $\rightarrow$ Glutamate + Succinyl-CoA + Acetyl-CoA + 2 NADH + FADH ₂	AA metabolism
31	Leucine + 2-oxo-Glutarate $\rightarrow$ Glutamate + NADH + FADH ₂ + ATP + Acetoacetate + Acetyl-CoA	AA metabolism
32	Lysine + 2 2-oxo-Glutarate $\rightarrow$ 2 Glutamate + 3 NADH + FADH ₂ + 2 CO ₂ + Acetoacetyl-CoA	AA metabolism
33	Phenylalanine + O ₂ $\rightarrow$ Tyrosine	AA metabolism
34	Tyrosine + 2-oxo-Glutarate + 2 O ₂ $\rightarrow$ Glutamate + CO ₂ + Fumarate + Acetoacetate	AA metabolism
35	Glutamate $\leftrightarrow$ 2-oxo-Glutarate + NADPH + NH ₄	AA metabolism
36	Glutamine $\rightarrow$ Glutamate + NH ₄	AA metabolism
37	Proline $\rightarrow$ Glutamate + NADH	AA metabolism
38	Histidine $\rightarrow$ NH ₄ + Glutamate	AA metabolism
39	Methionine + 3 ATP + Serine $\rightarrow$ Cysteine + NADH + Succinyl-CoA + NH ₄	AA metabolism
40	Oxaloacetate + NH ₄ + NADH $\leftrightarrow$ Aspartate	AA metabolism

Flux #	Reaction	Pathway
41	Asparagine $\leftrightarrow$ Aspartate + NH ₄	AA metabolism
42	Triacylglycerol + 4 ATP $\rightarrow$ Glyceraldehyde 3-P + 24 Acetyl-CoA + 21 FADH ₂ + 22 NADH	Lipid metabolism
43	2 Acetyl-CoA $\leftrightarrow$ Acetoacetyl-CoA	Lipid metabolism
44	Acetoacetyl-CoA $\rightarrow$ Acetoacetate	Lipid metabolism
45	Acetoacetate + NADH $\leftrightarrow$ Hydroxybutyrate	Lipid metabolism
46	NADH + 0.5 O ₂ $\rightarrow$ NAD	Electron transport
47	FADH ₂ + 0.5 O ₂ $\rightarrow$ FAD	Electron transport
48	Albumin Synthesis	Protein metabolism
49	O ₂ Input	Electron transport
50	CO ₂ Output	Electron transport
51	Acetoacetate Output	Lipid metabolism
52	Ornithine Output	Urea cycle
53	Ammonia Output	Urea cycle
54	Alanine Output	AA metabolism
55	Cysteine Output	AA metabolism
56	Aspartate Output	AA metabolism
57	Glutamate Output	AA metabolism
58	Phenylalanine Output	AA metabolism
59	Glycine Output	AA metabolism
60	Histidine Output	AA metabolism
61	Isoleucine Output	AA metabolism
62	Lysine Output	AA metabolism
63	Leucine Output	AA metabolism
64	Methionine Output	AA metabolism
65	Asparagine Output	AA metabolism
66	Proline Output	AA metabolism
67	Glutamine Output	AA metabolism
68	Arginine Output	Urea cycle
69	Serine Output	AA metabolism
70	Threonine Output	AA metabolism
71	Valine Output	AA metabolism
72	Tyrosine Output	AA metabolism

Table 5: List of reactions in metabolic network utilizing Glycolytic fluxes.

Flux #	Reaction	Pathway
1	Glucose → Glucose-6-phosphate	Glycolysis
2	Glucose 6-phosphate → 2 NADPH + CO ₂ + Ribulose 5-P	PPP
3	Ribulose 5-P ↔ Ribose 5-P	PPP
4	Ribulose 5-P ↔ Xylulose 5-P	PPP
5	Ribose 5-P + Xylulose 5-P ↔ Fructose 6-P + Erythrose 4-P	PPP
6	Erythrose 4-P + Xylulose 5-P ↔ Glyceraldehyde 3-P + Fructose 6-P	PPP
7	Glucose 6-phosphate ↔ Fructose 6-phosphate	Glycolysis
8	Fructose 6-phosphate → Fructose 1,6-Bisphosphate	Glycolysis
9	Fructose 1,6-Bisphosphate ↔ 2 Glyceraldehyde 3-P	Glycolysis
10	Glycerol + ATP → Glyceraldehyde 3-P + NADH	Lipid metabolism
11	Glyceraldehyde 3-P ↔ Phosphoenolpyruvate + ATP + NADH	Glycolysis
12	Phosphoenolpyruvate + ADP ↔ Pyruvate + ATP	Glycolysis
13	Pyruvate + CoA + NAD ↔ Acetyl-CoA + CO ₂ + NADH	Glycolysis
14	Pyruvate + NADH ↔ Lactate	Lactate/TCA cycle
15	Acetyl-CoA + Oxaloacetate → Citrate	Lactate/TCA cycle
16	Citrate ↔ 2-oxo-Glutarate + NADH + CO ₂	Lactate/TCA cycle
17	2-oxo-Glutarate → Succinyl-CoA + NADH + CO ₂	Lactate/TCA cycle
18	Succinyl-CoA ↔ GTP + FADH ₂ + Fumarate	Lactate/TCA cycle
19	Fumarate ↔ Malate	Lactate/TCA cycle
20	Malate ↔ Oxaloacetate + NADH	Lactate/TCA cycle
21	Ornithine + CO ₂ + NH ₄ + 2 ATP → Citrulline	Urea cycle
22	Citrulline + Aspartate + ATP → Arginine + Fumarate	Urea cycle
23	Arginine → Ornithine + Urea	Urea cycle
24	Alanine + 2-oxo-Glutarate ↔ Pyruvate + Glutamate	AA metabolism
25	Serine → Pyruvate + NH ₄	AA metabolism
26	Cysteine + 2-oxo-Glutarate + HCN ↔ Glutamate + HSCN + Pyruvate	AA metabolism
27	Threonine + ATP → NADH + Glycine + Acetyl-CoA	AA metabolism
28	2 Glycine ↔ Serine + CO ₂ + NH ₄ + NADH	AA metabolism
29	Valine + 2-oxo-Glutarate + ATP → Glutamate + Succinyl-CoA + 2 NADH + FADH ₂ + CO ₂	AA metabolism
30	Isoleucine + 2-oxo-Glutarate + ATP → Glutamate + Succinyl-CoA + Acetyl-CoA + 2 NADH + FADH ₂	AA metabolism
31	Leucine + 2-oxo-Glutarate → Glutamate + NADH + FADH ₂ + ATP + Acetoacetate + Acetyl-CoA	AA metabolism
32	Lysine + 2 2-oxo-Glutarate → 2 Glutamate + 3 NADH + FADH ₂ + 2 CO ₂ + Acetoacetyl-CoA	AA metabolism
33	Phenylalanine + O ₂ → Tyrosine	AA metabolism
34	Tyrosine + 2-oxo-Glutarate + 2 O ₂ → Glutamate + CO ₂ + Fumarate + Acetoacetate	AA metabolism

Flux #	Reaction	Pathway
35	Glutamate $\leftrightarrow$ 2-oxo-Glutarate + NADPH +NH ₄	AA metabolism
36	Glutamine -> Glutamate + NH ₄	AA metabolism
37	Proline -> Glutamate + NADH	AA metabolism
38	Histidine -> NH ₄ +Glutamate	AA metabolism
39	Methionine +3 ATP + Serine -> Cysteine + NADH + Succinyl-CoA + NH ₄	AA metabolism
40	Oxaloacetate + NH ₄ +NADH $\leftrightarrow$ Aspartate	AA metabolism
41	Asparagine $\leftrightarrow$ Aspartate + NH ₄	AA metabolism
42	Triacylglycerol + 4 ATP -> Glyceraldehyde 3-P + 24 Acetyl-CoA +21 FADH ₂ + 22 NADH	Lipid metabolism
43	2 Acetyl-CoA $\leftrightarrow$ Acetoacetyl-CoA	Lipid metabolism
44	Acetoacetyl-CoA -> Acetoacetate	Lipid metabolism
45	Acetoacetate + NADH $\leftrightarrow$ Hydroxybutyrate	Lipid metabolism
46	NADH + 0.5 O ₂ -> NAD	Electron transport
47	FADH ₂ + 0.5 O ₂ -> FAD	Electron transport
48	Albumin Synthesis	Protein metabolism
49	O ₂ Input	Electron transport
50	CO ₂ Output	Electron transport
51	Acetoacetate Output	Lipid metabolism
52	Ornithine Output	Urea cycle
53	Ammonia Output	Urea cycle
54	Alanine Output	AA metabolism
55	Cysteine Output	AA metabolism
56	Aspartate Output	AA metabolism
57	Glutamate Output	AA metabolism
58	Phenylalanine Output	AA metabolism
59	glycine Output	AA metabolism
60	Histidine Output	AA metabolism
61	Isoleucine Output	AA metabolism
62	Lysine Output	AA metabolism
63	Leucine Output	AA metabolism
64	Methionine Output	AA metabolism
65	Asparagine Output	AA metabolism
66	Proline Output	AA metabolism
67	Glutamine Output	AA metabolism
68	Arginine Output	Urea cycle
69	Serine Output	AA metabolism
70	Threonine Output	AA metabolism
71	Valine Output	AA metabolism
72	Tyrosine Output	AA metabolism

F. Statistical analysis

Each data point represents the mean of 2 or 3 experiments (each with 3 biological replicates), and the error bars represent the sem. Statistical significance was determined using Student's *t* test for unpaired data. Data was normalized to number of cells initially seeded and expressed as mmol/million cells/day. For extracellular metabolite measurements, the data-sets for each metabolite flux for each experimental condition were averaged from the sum of all replicates per experimental condition. The standard error of the mean was calculated from replicate data-set for each experimental condition. The mean and standard error of the mean of the extracellular metabolite measurements is quantitatively represented in the Table 6, Table 7 and Table 8 and qualitatively represented in FIGs. 15A, 15B, and 15C.

Table 6. Effect of Adipogel substrate on amino acid and glucose metabolism of recovery stage hepatocyte cultures.

FluxNo.	Metabolites	CSG	CDG	CSG+solASG	CSG+ASG
1	Glucose	-0.41±0.016	-0.388±0.362	-0.873±0.495	-0.373±0.419
14	Lactate	0.582±0.092*	0.743±0.061	1.274±0.555	0.971±0.759
23	Urea	1.952±0.051*	1.479±0.123	1.828±0.442	1.533±0.312
45	βhydroxybutyrate	-0.003±0.016	-0.012±0.002	0.041±0.011	0.019±0.003
48	Albumin	0.0001±0*	0.0002±0	0.0008±0.0004*	0.0003±0.0002
49	O ₂	0.916±0.128	1.169±0.163	2.005±0.28	1.528±0.213
50	CO ₂	0.102±0.018	0.13±0.023	0.223±0.039	0.17±0.029
51	Acetoacetate	0.124±0.005	0.257±0.149	0.094±0.059	0.133±0.097
52	Ornithine	-0.04±0.014*	-0.067±0.015	-0.031±0.027*	-0.04±0.018*
53	Ammonia	-0.562±0.045	-0.599±0.015	-0.646±0.028*	-0.601±0.006
55	Cysteine	0.016±0.067	-0.04±0.02	0.059±0.056*	0.053±0.052*
56	Aspartate	0.017±0.021	0.005±0.005	0.006±0.012	-0.002±0.01
57	Glutamate	0.027±0.075	0.02±0.014	0.042±0.027	0.032±0.011
58	Phenylalanine	-0.049±0.035	-0.052±0.015	-0.126±0.041*	-0.109±0.015*
59	Glycine	-0.163±0.075	-0.167±0.01	-0.209±0.026*	-0.206±0.017*
60	Histidine	-0.256±0.121	-0.176±0.04	-0.511±0.179*	-0.298±0.038*
61	Isoleucine	-0.025±0.025	-0.03±0.016	-0.092±0.042*	-0.073±0.023*
62	Lysine	-0.116±0.083	-0.159±0.059	-0.285±0.097*	-0.244±0.051*
63	Leucine	-0.03±0.031	-0.036±0.019	-0.135±0.087*	-0.085±0.025*
65	Asparagine	-0.008±0	-0.005±0	0±0	-0.004±0
66	Proline	-0.03±0.02	-0.027±0.003	-0.012±0.027	-0.002±0.021*
67	Glutamine	8.017±22.261	0.887±0.627	0.183±0.118	0.87±0.287
68	Arginine	-0.021±0.071	0.139±0.227	0.183±0.118*	-0.006±0.03
69	Serine	-0.104±0.066	-0.08±0.018	-0.052±0.052*	-0.13±0.004*
70	Threonine	-0.277±0.105	-0.316±0.035	-0.165±0.046*	-0.375±0.023*

FluxNo.	Metabolites	CSG	CDG	CSG+solASG	CSG+ASG
71	Valine	-0.018±0.031	-0.025±0.027	-0.373±0.018*	-0.078±0.027*
72	Tyrosine	0.169±0.007*	-0.146±0.01	-0.135±0.085*	-0.185±0.009*

Hepatocytes were cultured in four different configurations at a density of 500,000 cells/well in a 12 well plates for 10 days. Values are in $\mu\text{mol}/\text{million cells}/\text{day}$. * indicates $p < 0.05$ for each experimental condition vs. CDG. Sign conventions are in accordance with Table 4. -ve value for glucose corresponds to glucose uptake; -ve value for amino acids corresponds to amino acid uptake; +ve value for acetoacetate corresponds to acetoacetate release.

Table 7. Effect of Adipogel substrate on amino acid and glucose metabolism of pre-stable stage hepatocyte cultures.

FluxNo.	Metabolites	CSG	CDG	CSG+solASG	CSG+ASG
1	Glucose	0.37±0.046	0.222±0.274	-0.03±0.047*	0.297±2.35
14	Lactate	0.393±0.359	0.001±0	0.883±0.196	0.637±0.546*
23	Urea	0.97±0.06*	1.852±0.13	1.869±0.135	1.555±0.486
45	β hydroxybutyrate	-0.021±0.01	-0.012±0.016	0.026±0.003	-0.01±0.003
48	Albumin	0.0001±0*	0±0	0.001±0.001*	0.001±0*
49	O ₂	0.619±0.086	0.002±0.001	1.39±0.194	1.003±0.14
50	CO ₂	0.062±0.009	0.0002±0.0001	0.139±0.02	0.1±0.014
51	Acetoacetate	-0.054±0.014	-0.011±0.025	0.054±0.013*	-0.051±0.023
52	Ornithine	-0.048±0.016	-0.082±0.003*	-0.023±0.013	-0.066±0.01*
53	Ammonia	-0.394±0.135	-0.638±0.008*	-0.645±0.047	-0.615±0.03
55	Cysteine	-0.007±0.059	-0.042±0.005	0.083±0.02*	0.008±0.024*
56	Aspartate	0.002±0.011	0.004±0.006	0±0.009	0.006±0.016
57	Glutamate	0.021±0.049	0.025±0.008	0.031±0.043	0.044±0.034
58	Phenylalanine	-0.058±0.049	-0.143±0.015*	0.164±0.012*	-0.127±0.037
59	Glycine	-0.192±0.03	-0.22±0.018	-0.205±0.035	-0.179±0.046
60	Histidine	-0.374±0.172	-0.468±0.03	-0.537±0.081	-0.44±0.098
61	Isoleucine	-0.07±0.058	-0.13±0.116	-0.118±0.064	-0.073±0.053
62	Lysine	-0.156±0.16	-0.285±0.019	-0.312±0.058	-0.25±0.07
63	Leucine	-0.074±0.065	-0.087±0.018	-0.138±0.065	-0.086±0.058
65	Asparagine	-0.007±0.002	-0.002±0.003*	-0.001±0.002	-0.004±0*
66	Proline	-0.002±0.01	-0.005±0.003	-0.025±0.022	-0.016±0.017
67	Glutamine	6.395±14.477	1.121±0.377	0.136±0.188*	1.198±0.917
68	Arginine	-0.021±0.039	-0.007±0.021	-0.062±0.052	0.017±0.078
69	Serine	-0.073±0.101	-0.152±0.01	-0.181±0.026	-0.142±0.032
70	Threonine	-0.269±0.228	-0.378±0.008	-0.378±0.011	-0.363±0.016
71	Valine	-0.072±0.073	-0.081±0.02	-0.129±0.074	-0.07±0.073
72	Tyrosine	-0.095±0.068	-0.122±0.024	-0.166±0.041	-0.107±0.068

- Hepatocytes were cultured in four different configurations at a density of 500,000 cells/well in a 12 well plates for 10 days. *Values are in $\mu\text{mol}/\text{million cells}/\text{day}$. * indicates $p < 0.05$ for each experimental condition vs. CDG. Sign conventions are in accordance with Table 4. -ve value for glucose corresponds to glucose uptake; -ve value for amino acids corresponds to amino acid uptake; +ve value for acetoacetate corresponds to acetoacetate release.*

Table 8. Effect of Adipogel substrate on amino acid and glucose metabolism of stable stage hepatocyte cultures.

FluxNo.	Metabolites	CSG	CDG	CSG+solASG	CSG+ASG
1	Glucose	0.072±0.016*	-0.001±0.04	0.073±0.043*	0.072±0.0358*
14	Lactate	0.071±0.022	0.068±0.015	0.095±0.013*	0.103±0.039
23	Urea	1.074±0.77*	1.946±0.155	1.847±0.253	1.703±0.299
45	β hydroxybutyrate	-0.021±0.005	-0.021±0.005	0.073±0.022	0.073±0.022
48	Albumin	0±0*	0.0007±0.0001	0.0017±0.0002*	0.0008±0.0002
49	O ₂	0.112±0.016	0.107±0.015	0.149±0.021	0.162±0.023
50	CO ₂	0.013±0.003	0.012±0.002	0.017±0.003	0.018±0.003
51	Acetoacetate	0.071±0.009	0.071±0.009	-0.024±0.014	-0.024±0.014
52	Ornithine	0.052±0.016*	-0.082±0.003	-0.025±0.01*	-0.066±0.011*
53	Ammonia	0.346±0.545	-0.005±0.006	-0.045±0.007	0.005±0.011
55	Cysteine	0.017±0.052	0.042±0.005	-0.088±0.018	-0.008±0.027*
56	Aspartate	0.005±0.002	0.003±0.001	0.001±0.001	0.003±0.001
57	Glutamate	0.048±0.022*	0.142±0.006	0.085±0.01	0.092±0.021*
58	Phenylalanine	0.001±0.072*	-0.198±0.011	-0.252±0.01	-0.167±0.012*
59	Glycine	0.032±0.083	-0.035±0.014	-0.092±0.015*	-0.006±0.037
60	Histidine	0.034±0.019*	-0.091±0.001	-0.092±0.001	-0.086±0*
61	Isoleucine	0.037±0.109*	0.172±0.017	-0.133±0.036*	-0.019±0.057*
62	Lysine	0.038±0.073	-0.003±0.015	-0.096±0.013*	-0.041±0.017*
63	Leucine	0.021±0.098*	0.134±0.017	-0.164±0.032*	-0.045±0.051*
65	Asparagine	0.006±0.003*	0±0	-0.003±0.002*	0.004±0*
66	Proline	0.325±0.343	0.021±0.006	0.069±0.014*	0.063±0.006*
67	Glutamine	13.714±3.82*	6.444±0.746	0.125±0.518*	2.496±1.908*
68	Arginine	0.001±0.002*	0.008±0.001	-0.003±0.002*	-0.001±0.002*
69	Serine	0.027±0.035*	-0.038±0.004	-0.061±0.005*	-0.03±0.003*
70	Threonine	0.046±0.047*	-0.188±0.002	-0.411±0.617	-0.73±0.94*
71	Valine	0.051±0.108*	0.203±0.014	-0.222±0.35*	0.007±0.057*
72	Tyrosine	0.002±0.072*	0.104±0.02	-0.116±0.026*	0.03±0.047*

10

Hepatocytes were cultured in four different configurations at a density of 500,000 cells/well in a 12 well plates for 10 days. *Values are in $\mu\text{mol}/\text{million cells}/\text{day}$. * indicates $p < 0.05$ for each experimental condition vs. CDG. Sign conventions are in accordance*

with Table 4. -ve value for glucose corresponds to glucose uptake; -ve value for amino acids corresponds to amino acid uptake; +ve value for acetoacetate corresponds to acetoacetate release.

5 The MFA framework was applied to each biological experimental data-set of extracellular metabolite measurements in mmol/million cells/day input units. The output of the MFA for each biological experimental data-set was averaged using the sum over all computationally derived replicates for the unknown [non-measured] fluxes. The standard error of the mean was calculated from the computationally derived replicates for the
10 unknown [nonmeasured] fluxes.

 The summary statistics were calculated using t-test by comparing data from all experiments from one experimental condition for (e.g.: CSG+solASG) vs. data from all experiments from another experimental condition (for e.g.: CDG). This statistical method was used for extracellular metabolite measurements and computed intracellular fluxes
15 while comparing a specific metabolite flux between different experimental conditions. Statistical significance was determined using the Student's t-test for unpaired data. Differences were considered significant when the value of probability was less than or equal to 0.05 ($P \leq 0.05$).

G. Effect of Adipogel on hepatic differentiated function

20 Routine culture of primary hepatocytes is difficult and cumbersome because of their ability to develop compromised function. The instant invention includes a primary hepatocyte culture system that supersedes the traditional methodology of maintaining hepatocyte function and polarity in collagen double-gel sandwich systems. As shown in FIG. 2, hepatocytes cultured on single collagen gel with a soluble matrix of Adipogel in
25 the culture medium showed comparable urea secretion rates, but significantly higher albumin secretion rates, from d 4 to 10 of culture, as compared to collagen double-gel (CDG) cultures. The albumin secretion rate for collagen single gel (CSG) + soluble Adipogel (solASG) was higher as compared to Matrigel cultures.

 A comparison of the morphology of the hepatocytes in culture showed that
30 collagen sandwich and collagen-Adipogel sandwich (CSG+ASG) cultures result in uniform polygonal morphology (FIG. 3).

H. Effect of Adipogel on Hepatic Metabolism

A metabolic analysis was conducted on the different culture configurations viz. the collagen single gel [CSG], collagen double gel [CDG], collagen-Adipogel sandwich [CSG+ASG] and collagen single gel+soluble Adipogel [CSG+solASG]. To elucidate the effect of Adipogel on hepatocyte metabolism, the CDG was compared to the CSG+solASG conditions as described below. Day 4 [recovery stage], day 7 [pre-stable stage] and day 10 [stable stage] of analysis were chosen to investigate the delayed effect [day4, day7] as well as the immediate effect [day10] of Adipogel supplementation on hepatic metabolism. Primary rat hepatocytes isolated from livers of female Lewis rats recover within 4 days of culture from isolation induced injury while function is stabilized at 7 days post-isolation [52]. Thus, these stages were defined based on stabilization of function for the culture conditions from day 4 [recovery] to day 7 [pre-stable] to day 10 [stable] stage of cultures.

G. Glucose and Lactate Measurements

As shown in Table 3 and Table 5, for the CDG condition, hepatocytes are glycolytic [v_1] at the recovery stage and stable stage of culture respectively with glucose production at the prestable stage [Table 4]. On the other hand, CSG+solASG condition reveals significantly higher glucose consumption at the prestable stage and glucose production at the stable stage as compared to CDG condition. The lactate consumption rate [v_{14}] is significantly higher for the CSG+solASG condition as compared to the CDG condition only at the stable stage.

I. Amino Acid Fluxes

At the recovery stage and pre-stable stage, cysteine synthesis [v_{55}] is significantly higher for CSG+solASG vs. CDG condition. At the recovery stage, glutamine [v_{67}] and arginine synthesis rate [v_{68}] is also significantly higher for CSG+solASG vs. CDG condition. At the stable stage, serine [v_{69}], glycine [v_{59}], isoleucine [v_{61}], lysine [v_{62}], leucine [v_{63}], methionine [v_{64}], arginine [v_{68}], valine [v_{71}] and tyrosine [v_{72}] uptake rates are significantly higher for CSG+solASG condition as compared to CDG condition. Thus, we can correlate increased amino acid uptake rates and changes in glucose metabolism to hepatocellular function at the stable stage of culture.

J. Metabolic Flux Analysis of Hepatocyte Cultures

Using a previously developed hepatic metabolic network comprising of 72 reactions and 27 extracellular metabolite measurements, we performed a MFA for the different experimental conditions at 3 time points viz. the recovery stage, pre-stable stage and the stable stage of hepatocytes cultured in collagen single gel [CSG], cultured in collagen double gel sandwich configuration [CDG] and hepatocytes on collagen single gel with Adipogel overlay [CSG+solASG]. As shown in FIG. 15, the estimated intracellular fluxes were compared by representation of differentially regulated fluxes for the CSG+solASG condition vs. CDG condition. MFA revealed a rich intermediary metabolic data for the different conditions with an increase certain reactions in the TriCarboxylic Acid [TCA] cycle [v₁₇–v₂₀] for the recovery stage comparison [FIG. 15A]. At the pre-stable stage [FIG. 15B], there was an increase in PPP [v₂–v₉] and reduction in gluconeogenic pathway [v₁₁–v₁₃], glutamine metabolism [v₃₆–v₃₈] and acetyl-CoA synthesis [v₃₀, v₄₃] and clearance [v₂₇, v₄₂] with increased citrate synthesis rate [v₁₅] for the CSG+solASG condition as compared to the CDG condition. At the stable stage, increased amino acid uptake rates, decreased acetyl-CoA synthesis [v₃₀, v₃₁] with increased TCA cycle fluxes [v₁₇–v₂₀] were observed between the two conditions. Overall, from a functional standpoint the urea synthesis fluxes increased [v₂₁, v₂₂] and there were numerous differences in various pathway fluxes between the two conditions including an increase in albumin secretion for the CSG+solASG vs. CDG condition on all days of analysis.

A comparison of the CSG+solASG condition at different time points of the differentiation process was also conducted. A comparison of the recovery stage and pre-stable stage cultures revealed significant increase in PPP and decrease in TCA cycle fluxes for the CSG+solASG condition with no significant difference in albumin synthesis rate [Table 9]. A significant decrease in glucose uptake, lipid metabolism and increase in pyruvate synthesis rates, amino acid uptake rates and albumin synthesis with increase in majority of the transamination reactions were observed for the stable stage vs. the recovery stage of cultures.

Table 9. Summary of results for the CSG+solASG condition on different experimental days.

Pathways	FluxNo.	RecoveryStage	Pre-stableStage	StableStage
		CSG+solASG	CSG+solASG	CSG+solASG

Pathways	FluxNo.	RecoveryStage	Pre-stableStage	StableStage
Glucose Uptake	1	Base	Decrease	Increase significantly
PPP	2,3,5	Base	Decrease	Same
PPP	4,6	Base	Decrease	Decrease
Gluconeogenesis	7,8	Base	Increase significantly	Same
Gluconeogenesis	9	Base	Same	Increase significantly
Gluconeogenesis	10	Base	Decrease	Decrease
Glycerol Uptake	11-14	Base	Same	Decrease
TCA	15-18	Base	Decrease	Same
Pyruvate	24,26	Base	Same	Increase significantly
Amino Acid	25,27,28	Base	Same	Decrease
Amino Acid	30,31,33	Base	Same	Decrease
Amino Acid	36,37	Base	Same	Increase significantly
Amino Acid	38,40	Base	Same	Decrease
Lipid	42-44	Base	Same	Increase significantly
Amino acid	53-55	Base	Same	Increase significantly
Amino acid	56	Base	Same	Decrease
Amino acid	57	Base	Same	Decrease
Amino acid	58	Base	Decrease	Increase significantly
Amino acid	59-64	Base	Same	Increase significantly
Amino acid	65	Base	Same	Decrease
Amino acid	66	Base	Same	Decrease
Amino acid	68-72	Base	Same	Increase significantly

Hepatocytes were cultured on collagen single gel at a density of 500,000 cells/well in a 12 well plate. Adipogel was utilized at a 1:5 ratio with culture media and media was changed on days 0, 1,2,5,7 and 9 of culture. Metabolic Flux Analysis was performed on [A] recovery stage, [B] pre-stable stage and [C] stable stage of culture.

Example 3 – Angiogenesis Stimulation of Endothelial Cells

Cell extract and Adipogel was prepared in accordance with the forgoing using culture medium supplemented with 1 μ M dexamethasone, 0.1 μ M isobutylmethylxanthine, and 1 μ M of rosiglitazone or insulin. The purified ECM was then included within a basal media formulation for *in vivo* endothelial cell proliferation and to access tube formation, e.g. cell functionality.

Human umbilical vein endothelial cells (HUVECs) were cultured in medium, per the manufacturer's instructions (Lonza). They were then washed with PBS, trypsinized and plated at a density of 15,000 cells/well in 96 well plates on Adipogels. Media contain Adipogel (1:5 ratio) with endothelial cell medium was added to cells every 2 days and tube formation was assessed on day 4 of culture.

In FIG. 4, HUVEC tube formation is illustrated in the presence and absence of the Adipogel. In both Gel type 1 (manufactured with insulin) and Gel type 2 (manufactured with rosiglitazone) tube formation was exhibited. Images of Polystyrene + Adipogel are illustrated in FIG. 17.

5 Example 4 – Dermal fibroblast migration/ keratinocyte attachment

Normal human dermal fibroblasts/ human keratinocytes were plated on tissue culture plastic, thin layer of d2 and d4 Adipogel for 4 days at same initial cell seeding density. Fibroblasts were subjected to *in vitro* scratch assay with images captured at the initial time point and after 24 hours using phase-contrast microscope. The region between
10 the two dark lines in FIG. 5A corresponds to the open wound area. As shown (inset), the number of cells and hence the rate of migration is higher in the wound area 24 hrs post-scratch wound for the d2 and d4 Adipogel as compared to the control populations. FIG. 5B shows the cell number for the proliferating fibroblasts and FIG. 5C shows the attachment of keratinocytes to different substrates.

15 Example 5 – Cell proliferation of human embryonic stem cells

Cell extract and Adipogel was prepared in accordance with the forgoing using culture medium supplemented with 1 μ M dexamethasone, 0.1 μ M isobutylmethylxanthine, and 1 μ M of rosiglitazone or insulin. The purified ECM was then included in media formulation that embodies a hESC formulation, e.g. mouse embryonic
20 feeder layer conditioned media.

After a 7 day incubation period cell morphology and phenotype were measured. FIG. 8A illustrates normal cell morphology and the SSEA-4 staining of cells in FIG. 8B indicate cell proliferation and imply the presence of an undifferentiated phenotype.

Example 6 – Accelerated Wound Healing

25 Cell extract and Adipogel was prepared in accordance with the forgoing using culture medium supplemented with 1 μ M dexamethasone, 0.1 μ M isobutylmethylxanthine, and 1 μ M of rosiglitazone or insulin.

A. Episkin model culture

As a model for a patient's skin an Episkin® culture was used. Episkin® is a three
30 dimensional human skin equivalent construct and was comprised of human keratinocytes

cultured on bovine collagen I in a transwell system. Episkin® is cultured routinely in 12 well culture plates in Episkin® Medium [SkinEthic Laboratories] with media changes every 2 days. The skin tissue samples are stable for one week.

An 8 mm diameter hole was made in the skin using a biopsy punch. The collagen and keratinocytes were removed from the Episkin® using the biopsy punch. 20 ul of Adipogel was applied to the open wound area. In some cases, Wound-be-gone or Neosporin was also used as a control. The wound area was analyzed 1, 2 or 4 days post-puncture for closure.

B. Keratinocyte viability and proliferation in Episkin model

Viability and cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) conversion. After treating skin cells with 0.3 mg/ml MTT for 3 hours followed by acidic isopropanol extraction, the absorbance readings of formazan formation was obtained using a Biorad (Hercules, CA) Model 680 plate reader with a 585 nm emission filter. FIG 6 provides a comparison of the MTT stain for cell viability in the presence of Gel type 1 (manufactured with insulin), Gel type 2 (manufactured with rosiglitazone), no gel, Neosporin®, and a positive control. FIG. 7 illustrates the keratinocyte proliferation/migration in the puncture wound for each of these groups as well. As illustrated both the Gel 1 and Gel 2 exhibit keratinocyte proliferation and migration and a significantly higher rate than no gel or the use of Neosporin.

A wound-healing model (in vitro system I) is illustrated in FIG. 18. The results in the in vitro system I are illustrated in FIG. 7, which demonstrates that wound closure is the highest for Gels as compared to Neosporin and controls.

Another wound healing model (in vitro system II) is illustrated in FIG. 19. The results from the in vitro system II are illustrated in FIGs. 20A and 20B, which demonstrate that Adipogel in the presence of collagen induces accelerated and uniform wound closure in slow healing skin wounds as compared to collagen or collagen + Hemagel conditions.

In vivo assessment is illustrated in FIG. 21, and the results are shown in FIG. 22. The in vivo skin wounds treated with Adipogel heal in presence of Alloderm shows a high promise in wound dressing applications. In particular, combination of Adipogel with Alloderm leads to the largest improvement after 2 weeks.

These experiments indicate that: (1) Slow healing in vitro aged skin exhibit faster wound closure in 2 days with Adipogel as compared to Hema-gel and untreated controls; (2) Fast healing in vitro fresh skin exhibit faster wound closure in 4 days with Adipogel as compared to Hema-gel and untreated controls; (3) In vivo skin wounds treated with Adipogel heal in presence of Alloderm revealing promise in wound dressing applications; and (4) For full thickness wounds, Adipogel plus Alloderm led to the best results.

Example 7 – Anti-Aging Therapy

Cell extract and Adipogel is prepared in accordance with the foregoing using culture medium supplemented with 1 μ M dexamethasone, 0.1 μ M isobutyl-methylxanthine, and 1 μ M of rosiglitazone or insulin.

The purified extract is then included in a natural material composition, in accordance with the foregoing, that is topically applied to aged skin for anti-aging therapies. The distinguishing effect of the purified growth factor enriched ECM is enhanced expression of epidermal keratinocyte function and viability as opposed to untreated aged skin.

Although the invention herein has been described with reference to particular embodiments or examples, it is to be understood that these embodiments or examples are merely illustrative of the principles and applications of the present invention. It is therefore to be understood that numerous modifications may be made to the illustrative embodiments and that other arrangements may be devised without departing from the spirit and scope of the present invention as defined by the appended claims.

CLAIMS

WHAT IS CLAIMED IS:

1. An isolated cellular extract comprising extracellular matrix and non-matrix proteins isolated from a cell-exposed differentiation media.
- 5 2. The isolated cellular extract of claim 1 wherein the differentiation media is exposed to at least one preadipocyte cell line.
3. The isolated cellular extract of claim 1 wherein the extracellular matrix and non-matrix proteins are greater than 10 kDa in size.
4. The isolated cellular extract of claim 1 wherein the extracellular matrix and
10 non-matrix proteins are greater than 100 kDa in size.
5. The isolated cellular extract of claim 1 wherein the extracellular matrix and non-matrix proteins have a protein concentration of about 100 mg/ml.
6. The isolated cellular extract of claim 1 wherein the extracellular matrix proteins are selected from the group consisting of collagen I, collagen IV, collagen V,
15 collagen VI, fibronectin, laminin, hyaluronan, and combinations thereof.
7. The isolated cellular extract of claim 1 wherein the non-matrix proteins are selected from the group consisting of chemokines, cytokines, lipoproteins, glycoproteins, receptor proteins, ligands, signal transduction proteins, enzymes, hormones, growth factors, and combinations thereof.
- 20 8. The isolated cellular extract of claim 1 wherein the non-matrix proteins comprise one or more proteins selected from the group consisting of Coagulation factor III (CFIII), Dickkopf-1 (DKK-1), Dickkopf-3 (DKK-3), Developmental receptor tyrosine kinase (Dtk), Gremlin, Hepatocyte growth factor (HGF), Interleukin-1 receptor II (IL-1RII), Interleukin-4 (IL-4), Interleukin-5 receptor α (IL-5 α), Interleukin-20 (IL-20),
25 Leptin receptor (Leptin R), Leukemia inhibitor factor (LIF), Tumor necrosis factor SuperFamily 14 (TNFSF14), Lymphotoxin, Macrophage inflammatory protein-1 α (MIP α), Osteopontin, Osteoprotegerin, Progranulin, P-Selectin, Resistin-like molecule β

(RELM beta), Secretory leukocyte peptidase inhibitor (SLPI), Tissue inhibitor of mettalloproteinases 1 (TIMP-1), Toll-like receptor 4 (TLR4), Tomoregulin-1, Tumor necrosis factor SuperFamily 10 (TNFSF10), Ubiquitin, and Vascular endothelial growth factor (VEGF).

5 9. The isolated cellular extract of claim 1 wherein the extracellular matrix proteins are comprised of collagen IV, fibronectin, and laminin and the non-matrix proteins are comprised of VEGF, HGF, and LIF.

10 10. The isolated cellular extract of claim 9 wherein collagen IV is provided in a concentration range between about 20 µg/ml and about 60 µg/ml, fibronectin is provided in a concentration range between about 2 µg/ml and about 5 µg/ml, and laminin is provided in a concentration range between about 10 µg/ml and about 15 µg/ml.

11. The isolated cellular extract of claim 9 comprising approximately 60 µg/ml collagen IV, approximately 2 µg/ml of fibronectin, and approximately 15 µg/ml of laminin.

15 12. The isolated cellular extract of claim 1 wherein the differentiation media comprises at least one soluble factor, at least one steroid, and isobutylmethyloxanthine.

13. The isolated cellular extract of claim 12 wherein the soluble factor is an agonist.

20 14. The isolated cellular extract of claim 12 wherein the soluble factor is selected from the group consisting of rosiglitazone, troglitazone, thiazolidinedione, insulin and combinations thereof.

15. The isolated cellular extract of claim 12 wherein the steroid is selected from the group consisting of dexamethasone, cortisol, aldosterone, hydrocortisone, triiodothyronine, corticosterone, and combinations thereof.

25 16. A method for isolating a cellular extract comprising:
optionally culturing a cell line on a first cell media until confluent;

differentiating the cell line on a second cell media comprising at least one soluble factor, at least one steroid, and isobutylmethylxanthine; and

isolating a second cell media extract comprising extracellular matrix and non-matrix proteins.

5

17. The method of claim 16 wherein the cell line is comprised of one or more preadipocytes.

18. The method of claim 16 wherein the first cell media is comprised of DMEM
10 supplemented with about 10% FBS, about 2% Penicillin, and Streptomycin.

19. The method of claim 16 wherein the soluble factor is selected from the group consisting of rosiglitazone, troglitazone, thiazolidinedione, insulin and combinations thereof.

15

20. The method of claim 16 wherein the steroid is selected from the group consisting of dexamethasone, cortisol, aldosterone, hydrocortisone, triiodothyronine, corticosterone, and combinations thereof.

20 21. The method of claim 16 wherein the second cell media extract further comprises proteins of greater than 10 kDa in size.

22. The method of claim 16 wherein the second cell media extract further comprises proteins of great than 100 kDa in size.

25

23. The method of claim 16 wherein the extracellular matrix proteins are selected from the group consisting of collagen I, collagen IV, collagen V, collagen VI, fibronectin, laminin, hyaluronan, and combinations thereof.

30 24. The method of claim 16 wherein the non-matrix proteins are selected from the group consisting of chemokines, cytokines, lipoproteins, glycoproteins, receptor proteins, ligands, signal transduction proteins, enzymes, hormones, and combinations thereof.

25. The method of claim 16 wherein the non-matrix proteins are selected from the group consisting of Coagulation factor III (CFIII), Dickkopf-1 (DKK-1), Dickkopf-3 (DKK-3), Developmental receptor tyrosine kinase (Dtk), Gremlin, Hepatocyte growth factor (HGF), Interleukin-1 receptor II (IL-1RII), Interleukin-4 (IL-4), Interleukin-5
5 receptor α (IL-5 α), Interleukin-20 (IL-20), Leptin receptor (Leptin R), Leukemia inhibitor factor (LIF), Tumor necrosis factor SuperFamily 14 (TNFSF14), Lymphotoxin, Macrophage inflammatory protein-1 α (MIP α), Osteopontin, Osteoprotegerin, Progranulin, P-Selectin, Resistin-like molecule β (RELM β), Secretory leukocyte
10 peptidase inhibitor (SLPI), Tissue inhibitor of metalloproteinases 1 (TIMP-1), Toll-like receptor 4 (TLR4), Tomoregulin-1, Tumor necrosis factor SuperFamily 10 (TNFSF10), Ubiquitin, Vascular endothelial growth factor (VEGF), and combinations thereof.

26. The method of claim 16 wherein the extracellular matrix proteins comprise
15 collagen IV, fibronectin, and laminin and the non-matrix proteins comprise VEGF, HGF, and LIF.

27. The method of claim 26 wherein collagen IV is provided in a concentration range between about 20 $\mu\text{g/ml}$ and about 60 $\mu\text{g/ml}$, fibronectin is provided in a
20 concentration range between about 2 $\mu\text{g/ml}$ and about 5 $\mu\text{g/ml}$, and laminin is provided in a concentration range between about 10 $\mu\text{g/ml}$ and about 15 $\mu\text{g/ml}$.

28. The method of claim 26 wherein approximately 60 $\mu\text{g/ml}$ collagen IV, approximately 2 $\mu\text{g/ml}$ of fibronectin, and approximately 15 $\mu\text{g/ml}$ of laminin are
25 provided.

29. A method for proliferating and/or maintaining cell functionality of hepatocytes comprising: culturing hepatocyte or hepatocyte-like cells on a media extract comprising cellular matrix and non-matrix proteins isolated and purified from a cell-exposed
30 differentiation media comprising at least one soluble factor, at least one steroid and isobutylmethylxanthine.

30. The method of claim 29 wherein the cell-exposed differentiation media is exposed to at least one cell line of one or more preadipocytes.

31. The method of claim 29 wherein the soluble factor is selected from the group
5 consisting of rosiglitazone, troglitazone, thiazolidinedione, insulin and combinations thereof.

32. The method of claim 29 wherein the soluble factor is provided at a concentration between about 0.1 μ M and about 1.0 μ M.

10

33. The method of claim 29 wherein the steroid is selected from the group consisting of dexamethasone, cortisol, aldosterone, hydrocortisone, triiodothyronine, corticosterone, and combinations thereof.

15 34. The method of claim 29 wherein the steroid is provided at a concentration between about 0.1 μ M and about 1.0 μ M.

35. The method of claim 29 wherein the isobutylmethylxanthine is provided at a concentration between about 0.1 μ M and about 1.0 μ M.

20

36. The method of claim 29 wherein the media extract further comprises proteins of >100 kDa or > 10 kDa in size.

37. The method of claim 29 wherein the extracellular matrix proteins are selected
25 from the group consisting of collagen I, collagen IV, collagen V, collagen VI, fibronectin, laminin, hyaluronan, and combinations thereof.

38. The method of claim 29 wherein the non-matrix proteins are selected from the group consisting of chemokines, cytokines, lipoproteins, glycoproteins, receptor proteins,
30 ligands, signal transduction proteins, enzymes, hormones, and combinations thereof.

39. The method of claim 29 wherein the non-matrix proteins are selected from the group consisting of Coagulation factor III (CFIII), Dickkopf-1 (DKK-1), Dickkopf-3 (DKK-3), Developmental receptor tyrosine kinase (Dtk), Gremlin, Hepatocyte growth factor (HGF), Interleukin-1 receptor II (IL-1RII), Interleukin-4 (IL-4), Interleukin-5
5 receptor α (IL-5 α), Interleukin-20 (IL-20), Leptin receptor (Leptin R), Leukemia inhibitor factor (LIF), Tumor necrosis factor SuperFamily 14 (TNFSF14), Lymphotoctin, Macrophage inflammatory protein-1 α (MIP α), Osteopontin, Osteoporotegerin, Progranulin, P-Selectin, Resistin-like molecule β (RELM β), Secretory leukocyte peptidase inhibitor (SLPI), Tissue inhibitor of mettalloproteinases 1 (TIMP-1), Toll-like
10 receptor 4 (TLR4), Tomoregulin-1, Tumor necrosis factor SuperFamily 10 (TNFSF10), Ubiquitin, Vascular endothelial growth factor (VEGF), and combinations thereof.

40. The method of claim 29 wherein the extracellular matrix proteins are comprised of collagen IV, fibronectin, and laminin and the non-matrix proteins are
15 comprised of VEGF, HGF, and LIF.

41. The method of claim 29 wherein collagen IV is provided in a concentration range between about 20 $\mu\text{g/ml}$ and about 60 $\mu\text{g/ml}$, fibronectin is provided in a concentration range between about 2 $\mu\text{g/ml}$ and about 5 $\mu\text{g/ml}$, and laminin is provided in
20 a concentration range between about 10 $\mu\text{g/ml}$ and about 15 $\mu\text{g/ml}$.

42. The method of claim 41 wherein approximately 60 $\mu\text{g/ml}$ collagen IV, approximately 2 $\mu\text{g/ml}$ of fibronectin, and approximately 15 $\mu\text{g/ml}$ of laminin are provided.
25

43. A method for proliferating and/or maintaining cell functionality of endothelial comprising: culturing endothelial or endothelial-like cells on a media extract comprising cellular matrix and non-matrix proteins isolated and purified from a cell-exposed differentiation media comprising at least one soluble factor, at least one steroid and
30 isobutylmethylxanthine.

44. The method of claim 43 wherein the method stimulates angiogenesis.

45. The method of claim 43 wherein the cell-exposed differentiation media is exposed to at least one cell line of one or more preadipocytes.

5 46. The method of claim 43 wherein the soluble factor is selected from the group consisting of rosiglitazone, troglitazone, thiazolidinedione, insulin and combinations thereof.

47. The method of claim 43 wherein the soluble factor is provided at a
10 concentration between about 0.1 μ M and about 1.0 μ M.

48. The method of claim 43 wherein the steroid is selected from the group consisting of dexamethasone, cortisol, aldosterone, hydrocortisone, triiodothyronine, corticosterone, and combinations thereof.

15 49. The method of claim 43 wherein the steroid factor is provided at a concentration between about 0.1 μ M and about 1.0 μ M.

50. The method of claim 43 wherein the isobutylmethylxanthine is provided at a
20 concentration between about 0.1 μ M and about 1.0 μ M.

51. The method of claim 43 wherein the media extract further comprises proteins of >100 kDa.

25 52. The method of claim 43 wherein the extracellular matrix proteins are selected from the group consisting of collagen I, collagen IV, collagen V, collagen VI, fibronectin, laminin, hyaluronan, and combinations thereof.

53. The method of claim 43 wherein the non-matrix proteins are selected from the
30 group consisting of chemokines, cytokines, lipoproteins, glycoproteins, receptor proteins, ligands, signal transduction proteins, enzymes, hormones, and combinations thereof.

54. The method of claim 43 wherein the non-matrix proteins are selected from the group consisting of Coagulation factor III (CFIII), Dickkopf-1 (DKK-1), Dickkopf-3 (DKK-3), Developmental receptor tyrosine kinase (Dtk), Gremlin, Hepatocyte growth factor (HGF), Interleukin-1 receptor II (IL-1RII), Interleukin-4 (IL-4), Interleukin-5
5 receptor α (IL-5 α), Interleukin-20 (IL-20), Leptin receptor (Leptin R), Leukemia inhibitor factor (LIF), Tumor necrosis factor SuperFamily 14 (TNFSF14), Lymphotoctin, Macrophage inflammatory protein-1 α (MIP α), Osteopontin, Osteoprotegerin, Progranulin, P-Selectin, Resistin-like molecule β (RELM β), Secretory leukocyte peptidase inhibitor (SLPI), Tissue inhibitor of metalloproteinases 1 (TIMP-1), Toll-like
10 receptor 4 (TLR4), Tomoregulin-1, Tumor necrosis factor SuperFamily 10 (TNFSF10), Ubiquitin, Vascular endothelial growth factor (VEGF), and combinations thereof.

55. The method of claim 43 wherein the extracellular matrix proteins are comprised of collagen IV, fibronectin, and laminin and the non-matrix proteins are
15 comprised of VEGF, HGF, and LIF.

56. The method of claim 55 wherein collagen IV is provided in a concentration range between about 20 $\mu\text{g/ml}$ and about 60 $\mu\text{g/ml}$, fibronectin is provided in a concentration range between about 2 $\mu\text{g/ml}$ and about 5 $\mu\text{g/ml}$, and laminin is provided in
20 a concentration range between about 10 $\mu\text{g/ml}$ and about 15 $\mu\text{g/ml}$.

57. The method of claim 55 wherein approximately 60 $\mu\text{g/ml}$ collagen IV, approximately 2 $\mu\text{g/ml}$ of fibronectin, and approximately 15 $\mu\text{g/ml}$ of laminin are provided.
25

58. A method for proliferating and/or maintaining the phenotype of human embryonic stem cells comprising: culturing human embryonic stem cells on a media extract comprising cellular matrix and non-matrix proteins isolated and purified from a cell-exposed differentiation media comprising at least one soluble factor, at least one
30 steroid and isobutylmethylxanthine.

59. The method of claim 58 wherein the cell-exposed differentiation media is exposed to at least one cell line of one or more preadipocytes.

60. The method of claim 58 wherein the soluble factor is selected from the group
5 consisting of rosiglitazone, troglitazone, thiazolidinedione, insulin and combinations thereof.

61. The method of claim 58 wherein the steroid is selected from the group
10 consisting of dexamethasone, cortisol, aldosterone, hydrocortisone, triiodothyronine, corticosterone, and combinations thereof.

62. The method of claim 58 wherein the media extract further comprises proteins of >100 kDa.

15 63. The method of claim 58 wherein the extracellular matrix proteins are selected from the group consisting of collagen I, collagen IV, collagen V, collagen VI, fibronectin, laminin, hyaluronan, and combinations thereof.

64. The method of claim 58 wherein the non-matrix proteins are selected from the
20 group consisting of chemokines, cytokines, lipoproteins, glycoproteins, receptor proteins, ligands, signal transduction proteins, enzymes, hormones, and combinations thereof.

65. The method of claim 58 wherein the non-matrix proteins are selected from the
group consisting of Coagulation factor III (CFIII), Dickkopf-1 (DKK-1), Dickkopf-3
25 (DKK-3), Developmental receptor tyrosine kinase (Dtk), Gremlin, Hepatocyte growth factor (HGF), Interleukin-1 receptor II (IL-1RII), Interleukin-4 (IL-4), Interleukin-5 receptor α (IL-5 α), Interleukin-20 (IL-20), Leptin receptor (Leptin R), Leukemia inhibitor factor (LIF), Tumor necrosis factor SuperFamily 14 (TNFSF14), Lymphotoxin, Macrophage inflammatory protein-1 α (MIP α), Osteopontin, Osteoprotegerin,
30 Progranulin, P-Selectin, Resistin-like molecule β (RELM β), Secretory leukocyte peptidase inhibitor (SLPI), Tissue inhibitor of metalloproteinases 1 (TIMP-1), Toll-like

receptor 4 (TLR4), Tomoregulin-1, Tumor necrosis factor SuperFamily 10 (TNFSF10), Ubiquitin, Vascular endothelial growth factor (VEGF), and combinations thereof.

66. The method of claim 58 wherein the extracellular matrix proteins are
5 comprised of collagen IV, fibronectin, and laminin and the non-matrix proteins are
comprised of VEGF, HGF, and LIF.

67. The method of claim 66 wherein collagen IV is provided in a concentration
range between about 20 µg/ml and about 60 µg/ml, fibronectin is provided in a
10 concentration range between about 2 µg/ml and about 5 µg/ml, and laminin is provided in
a concentration range between about 10 µg/ml and about 15 µg/ml.

68. The method of claim 66 wherein approximately 60 µg/ml collagen IV,
approximately 2 µg/ml of fibronectin, and approximately 15 µg/ml of laminin are
15 provided.

69. A method for healing a wound in a subject comprising
administering to the wound site an extract comprising extracellular matrix and non-matrix
proteins that are isolated and purified from a cell-exposed differentiation media
20 comprising at least one soluble factor, at least one steroid and isobutylmethylxanthine.

70. The method of claim 69 wherein the administration of the extract results in a
1.5 fold increase in normalized cell numbers.

25 71. A method for treating aging skin of a subject comprising
administering to a dermal layer of a patient an extract comprising extracellular matrix and
non-matrix proteins that are isolated and purified from a cell-exposed differentiation
media comprising at least one soluble factor, at least one steroid and
isobutylmethylxanthine.

30

72. The method of claim 71 wherein administration of the extract results in enhanced expression of epidermal keratinocyte function and viability, as compared to untreated keratinocyte cells of approximately the same age.

5 73. A kit for proliferating or maintaining cell functionality comprising:
an extract comprising extracellular matrix and non-matrix proteins that are isolated and
purified from a cell-exposed differentiation media comprising at least one soluble factor,
at least one steroid and isobutylmethylxanthine; and
cellular growth media or a basement membrane composition.

10

74. Use of an extract comprising extracellular matrix and non-matrix proteins for
proliferating and/or maintaining cell functionality of hepatocytes, wherein the extract is
isolated from a cell-exposed differentiation media comprising at least one soluble factor, at
least one steroid, and isobutylmethylxanthine.

15

75. The use of claim 74, wherein the extract is an isolated cellular extract
according to any of claims 1-15.

20

76. The use of claim 74, wherein the extract is isolated by a method according to
any of claims 16-28.

25

77. Use of an extract comprising extracellular matrix and non-matrix proteins for
proliferating and/or maintaining cell functionality of endothelial or endothelial-like cells,
wherein the extract is isolated from a cell-exposed differentiation media comprising at
least one soluble factor, at least one steroid, and isobutylmethylxanthine.

78. The use of claim 77, wherein the extract is an isolated cellular extract
according to any of claims 1-15.

30

79. The use of claim 77, wherein the extract is isolated by a method according to
any of claims 16-28.

80. Use of an extract comprising extracellular matrix and non-matrix proteins for proliferating and/or maintaining the phenotype of human embryonic stem cells wherein the extract is isolated from a cell-exposed differentiation media comprising at least one soluble factor, at least one steroid, and isobutylmethylxanthine.

5

81. The use of claim 80, wherein the extract is an isolated cellular extract according to any of claims 1-15.

82. The use of claim 80, wherein the extract is isolated by a method according to
10 any of claims 16-28.

83. Use of an extract comprising extracellular matrix and non-matrix proteins for manufacture of a medicament for treatment of a wound in a subject, wherein the extract is isolated from a cell-exposed differentiation media comprising at least one soluble factor, at
15 least one steroid, and isobutylmethylxanthine.

84. The use of claim 83, wherein the extract is an isolated cellular extract according to any of claims 1-15.

20 85. The use of claim 83, wherein the extract is isolated by a method according to any of claims 16-28.

86. Use of an extract comprising extracellular matrix and non-matrix proteins for manufacture of a medicament for treatment aging skin in a subject, wherein the extract is
25 isolated from a cell-exposed differentiation media comprising at least one soluble factor, at least one steroid, and isobutylmethylxanthine.

87. The use of claim 86, wherein the extract is an isolated cellular extract according to any of claims 1-15.

30

88. The use of claim 86, wherein the extract is isolated by a method according to any of claims 16-28.

Hepatocyte Culture Technique

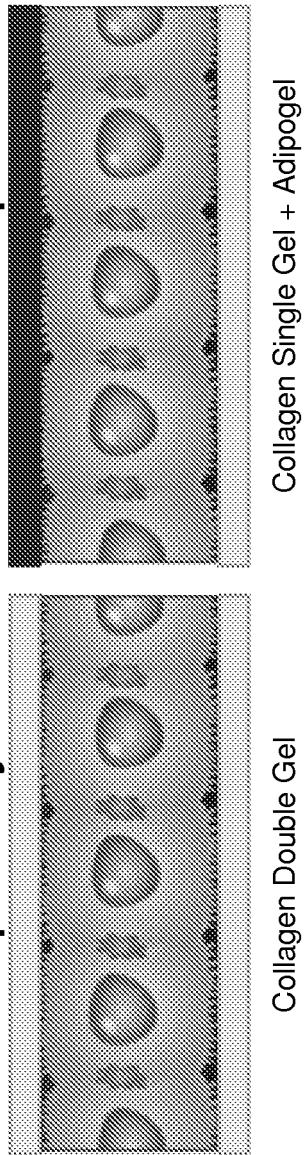


FIG. 1A

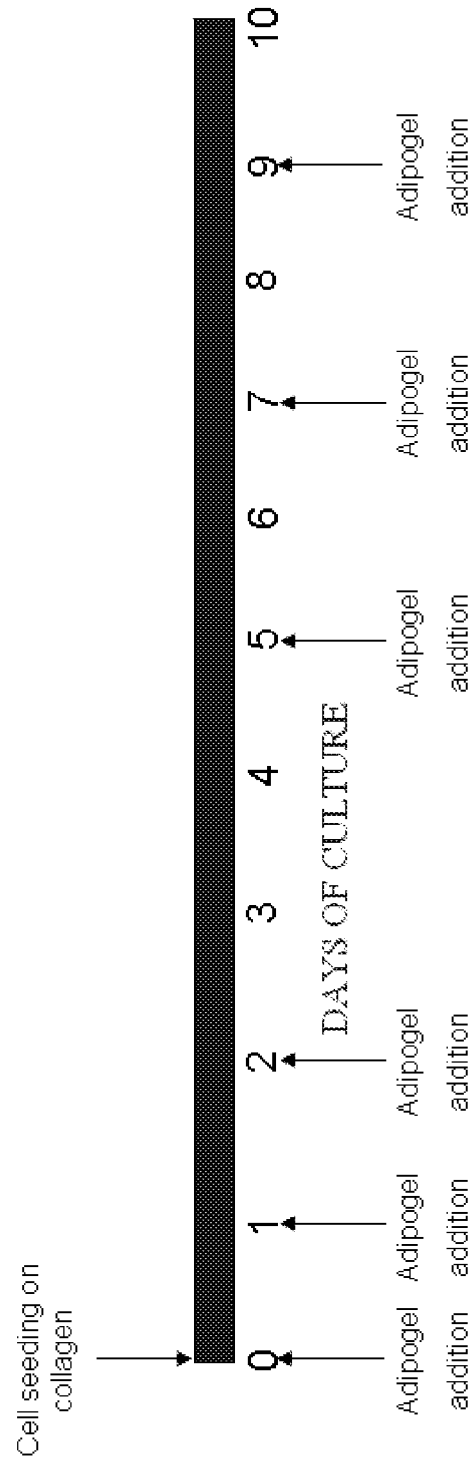


FIG. 1B

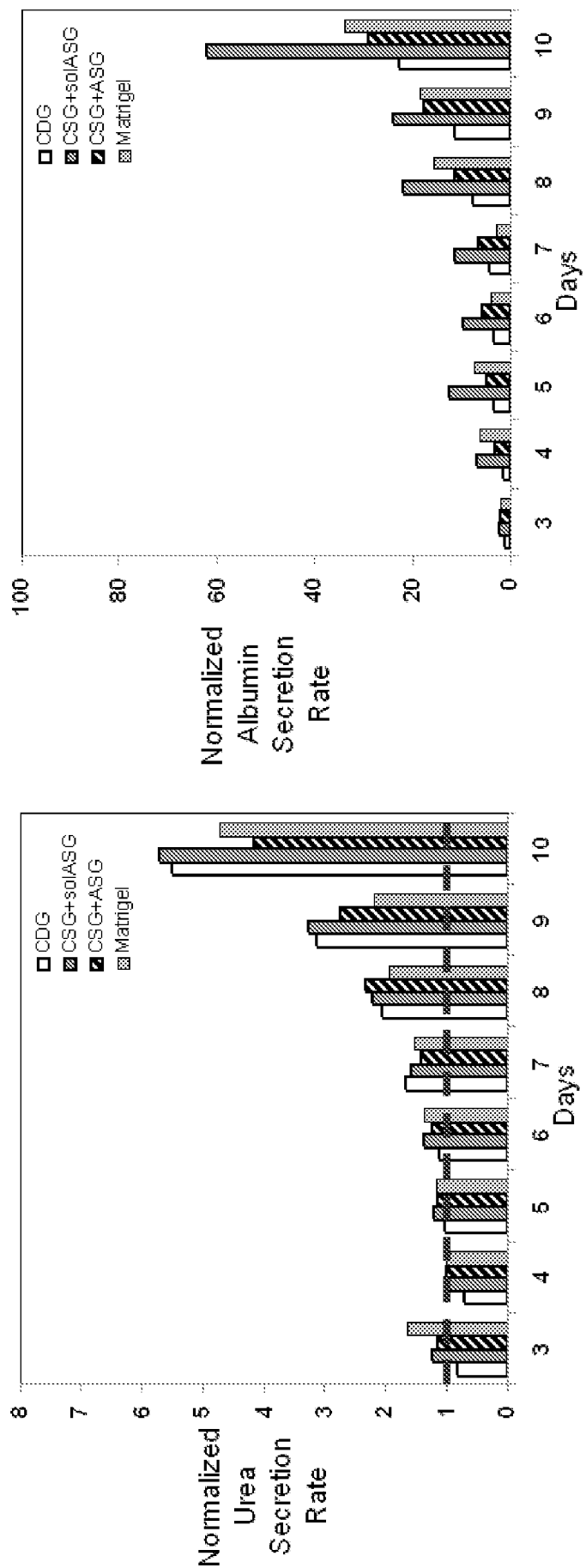
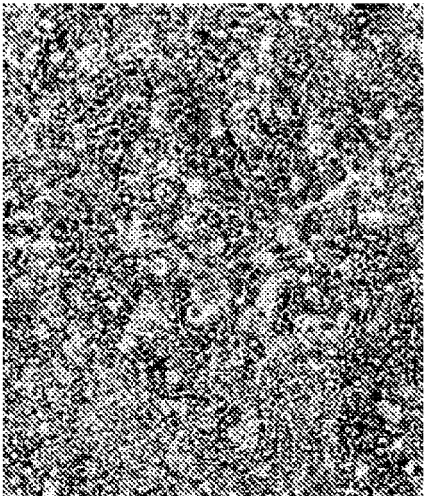
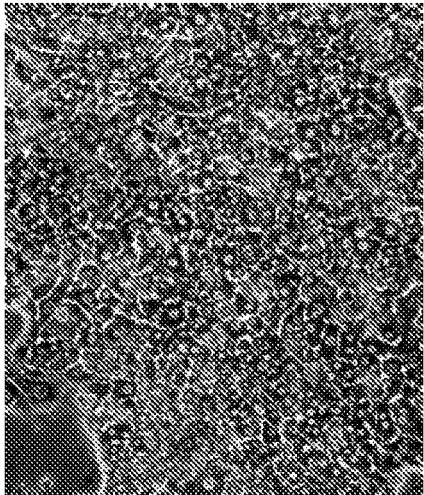


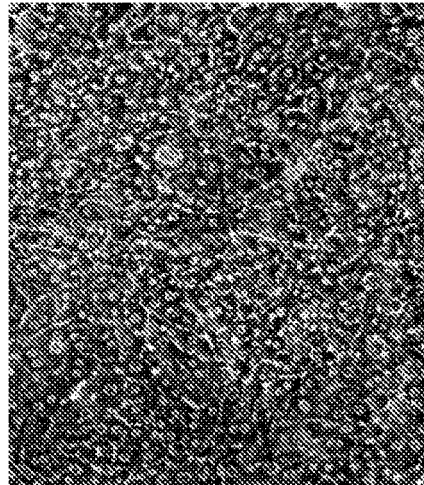
FIG. 2



Day 6

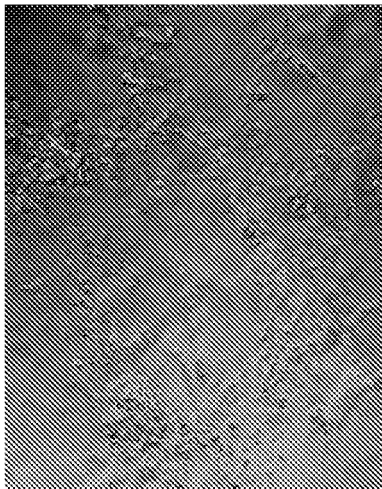


Day 8

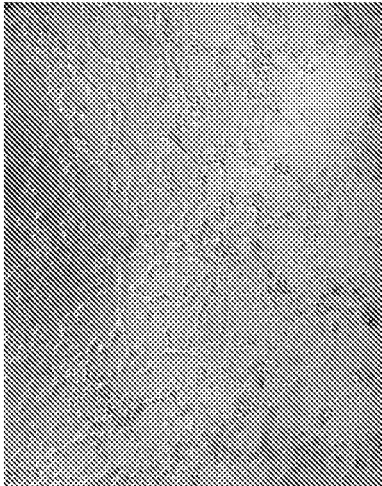


Day 9

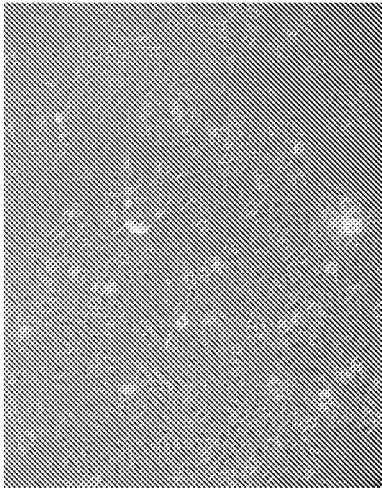
FIG. 3



HUVECs with Gel type 2



HUVECs with Gel type 1



HUVECs

FIG. 4

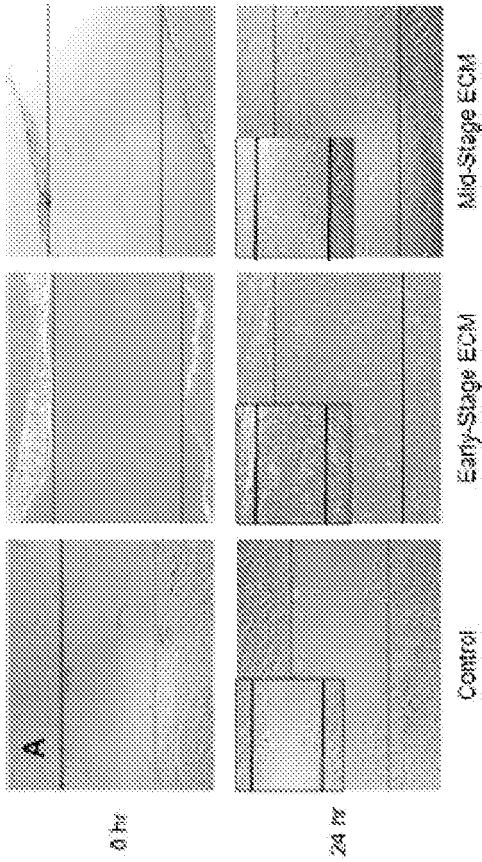
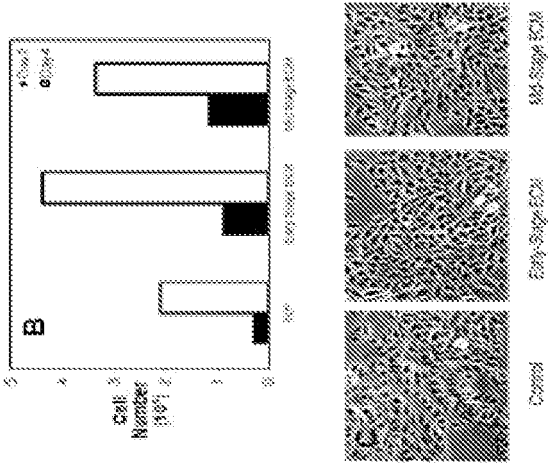


FIG. 5

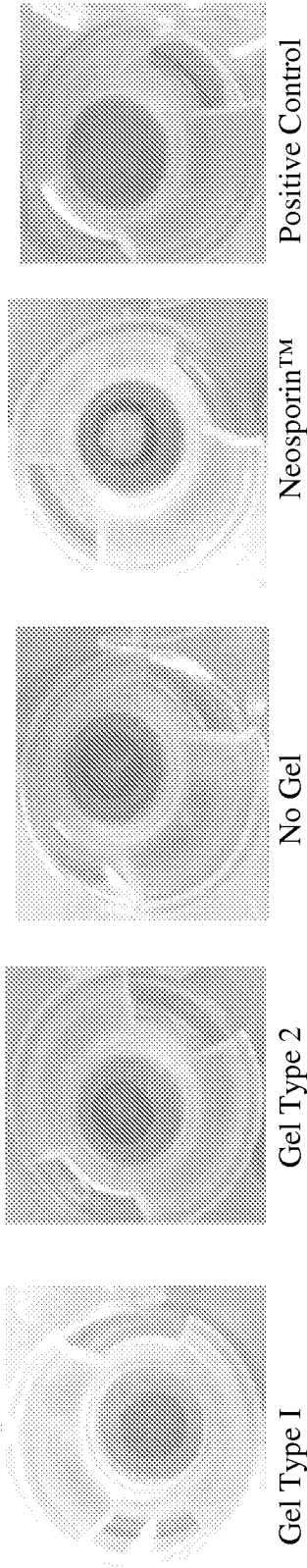


FIG. 6

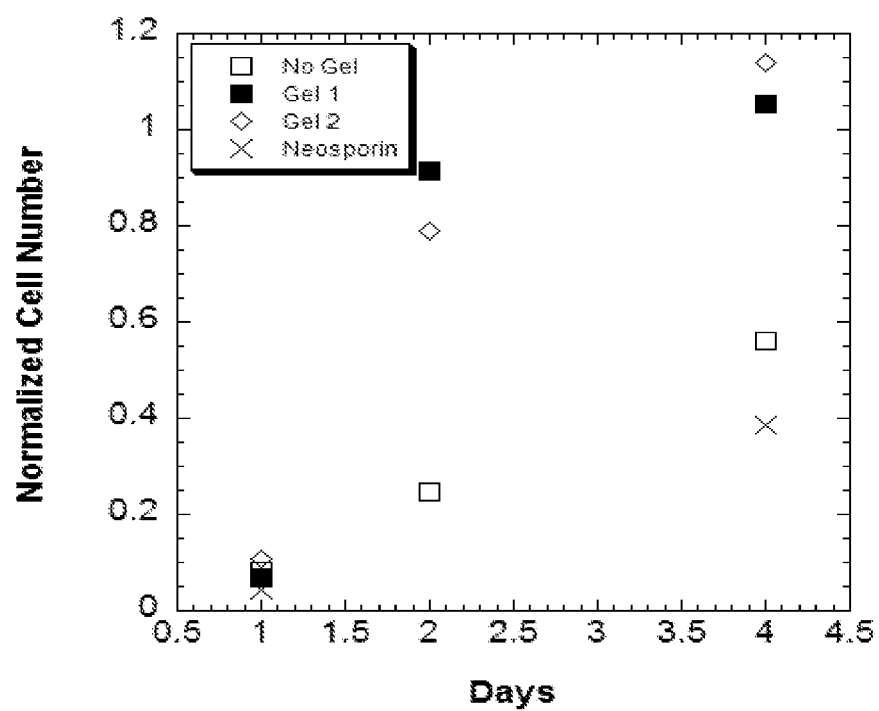


FIG. 7

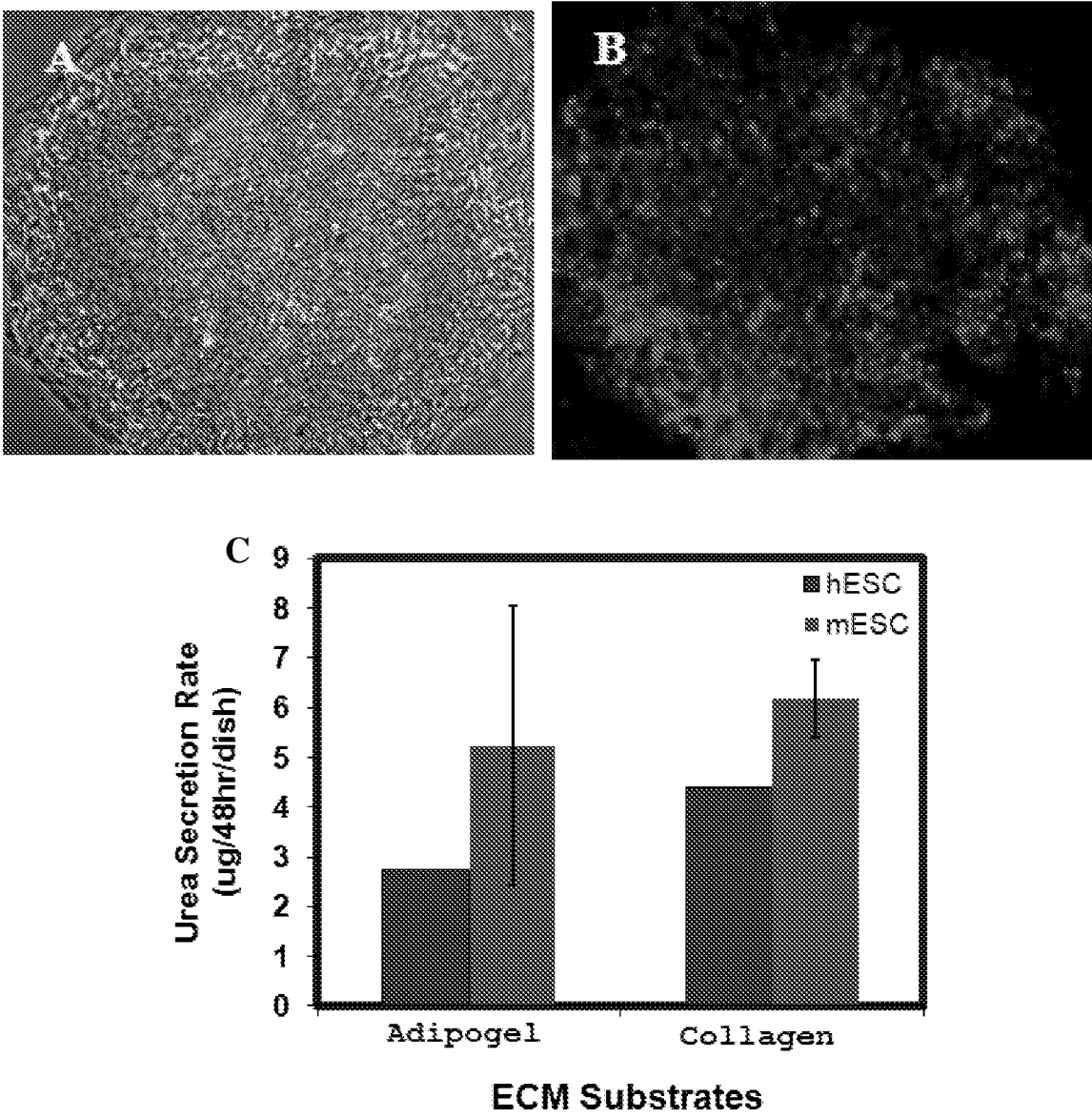


FIG. 8

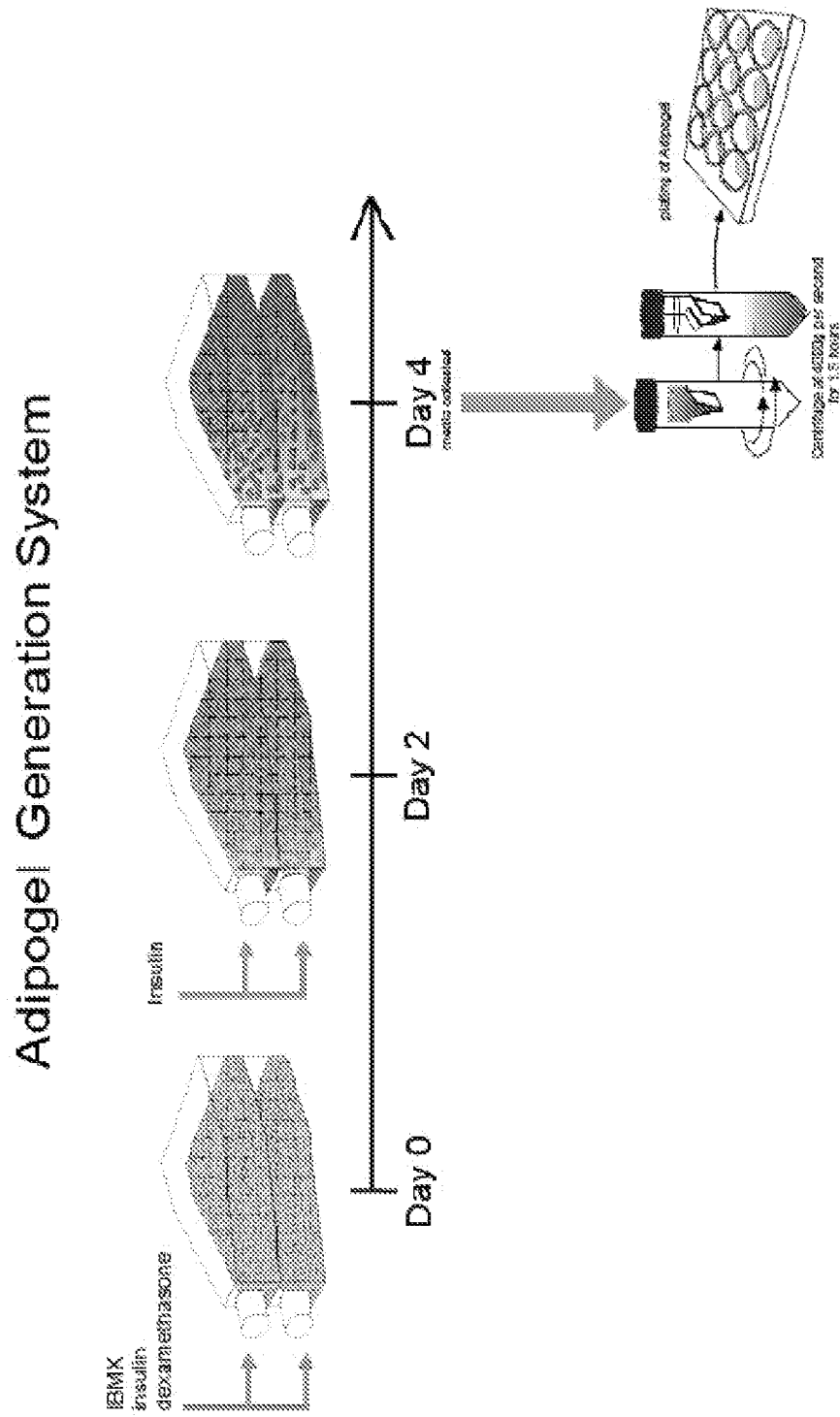


FIG. 9

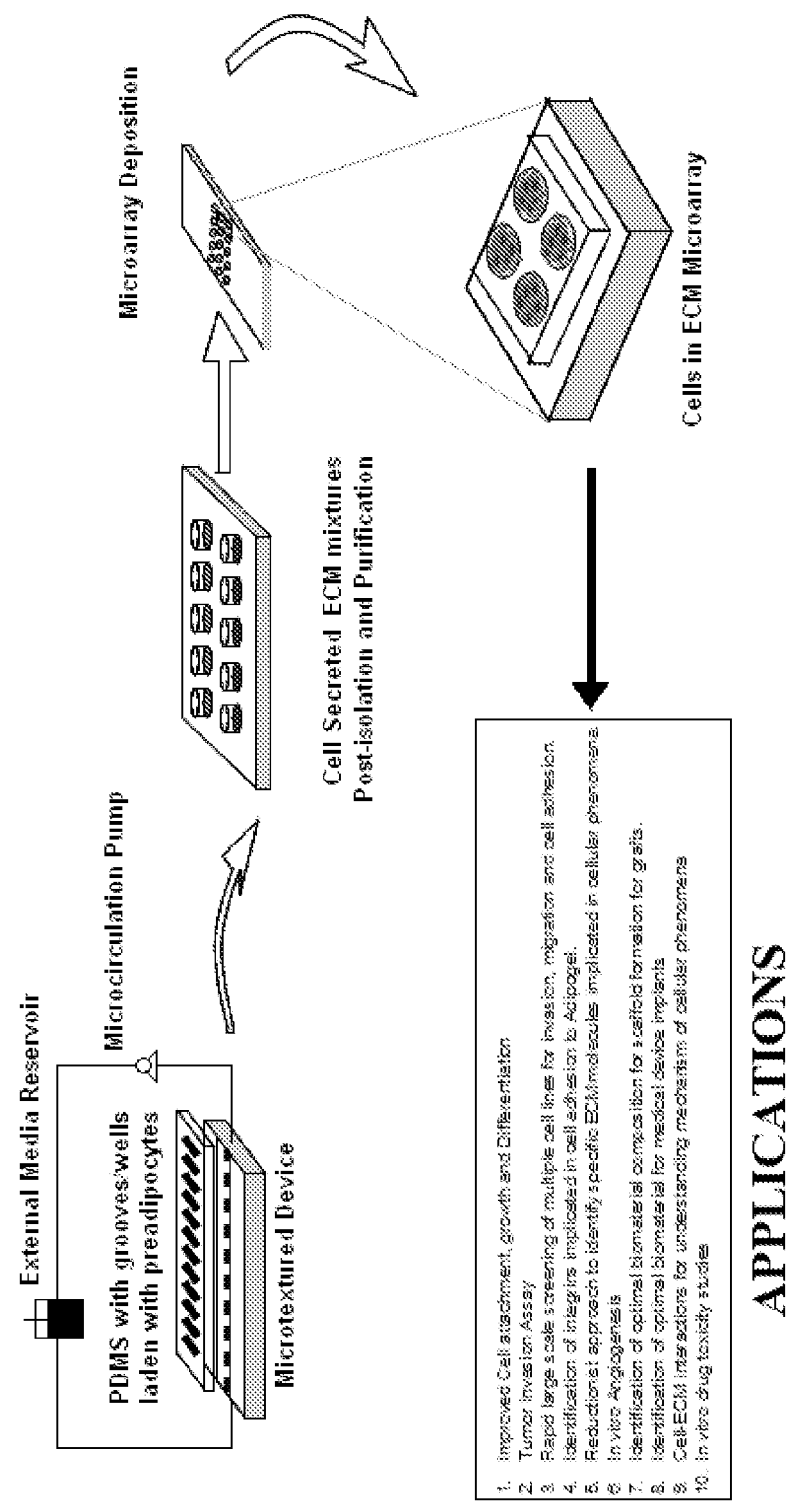


FIG. 10

MDA-MB-231 Cells

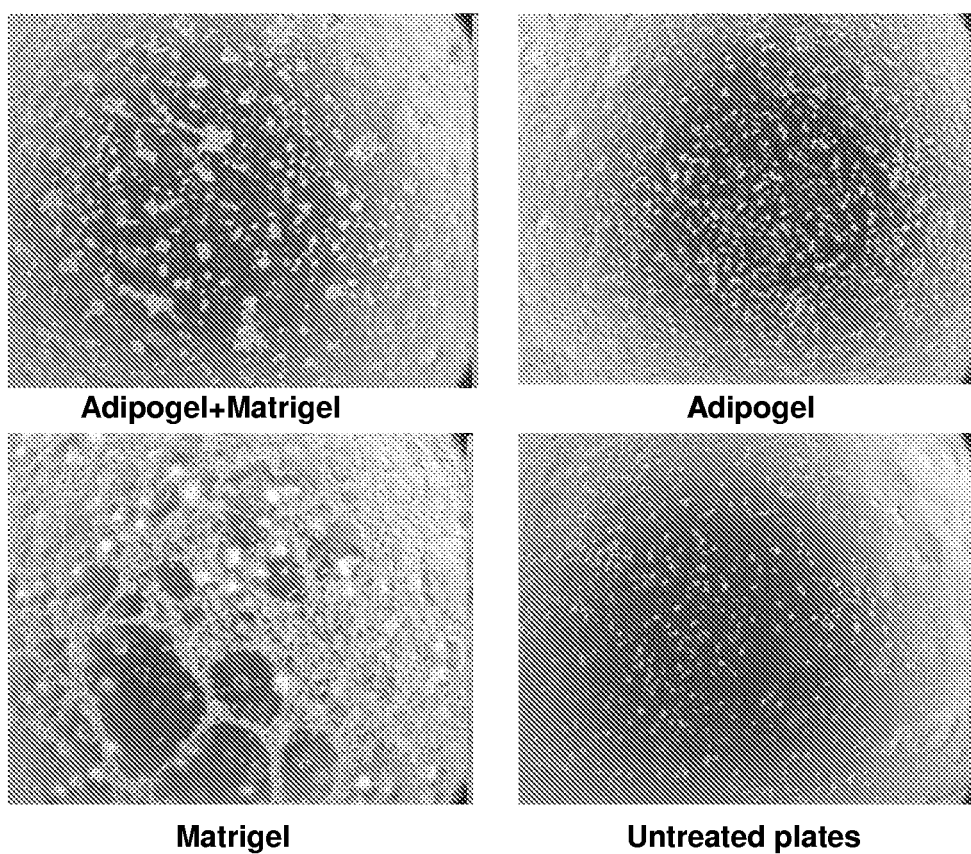


FIG. 11

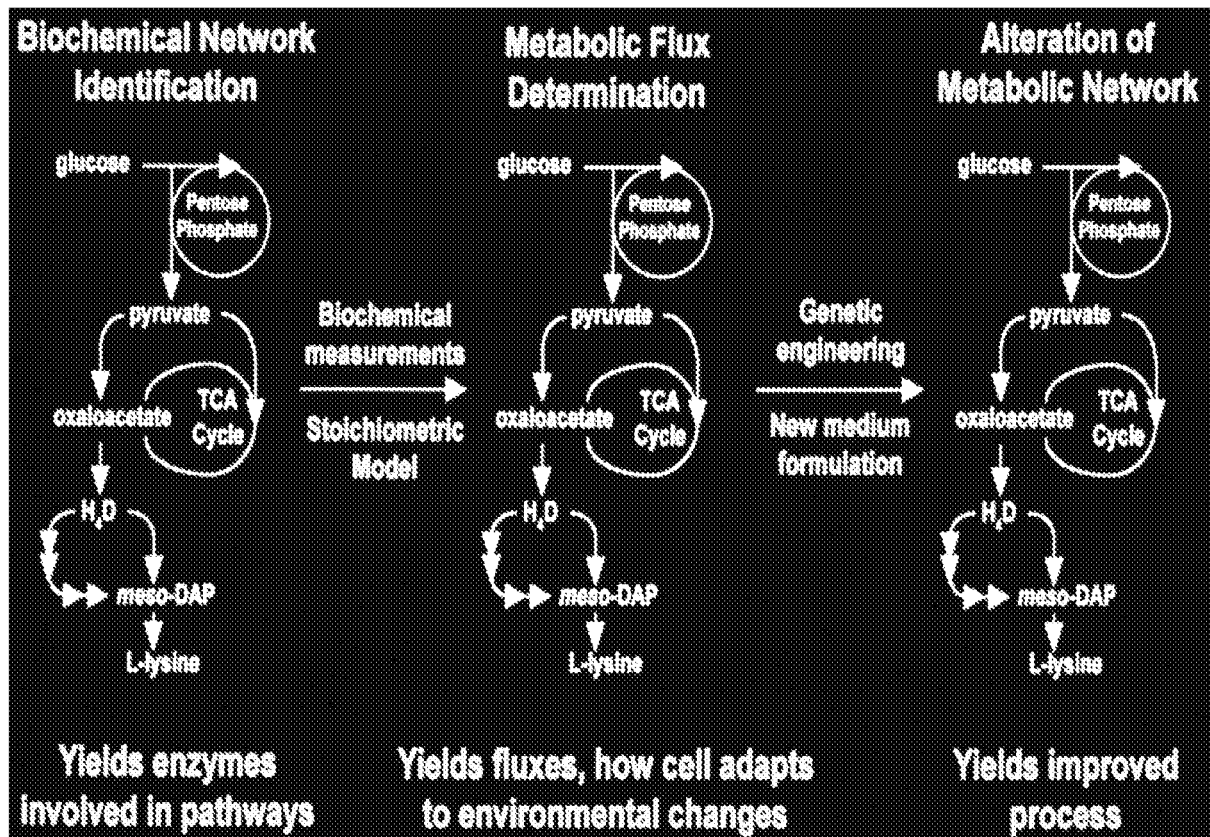
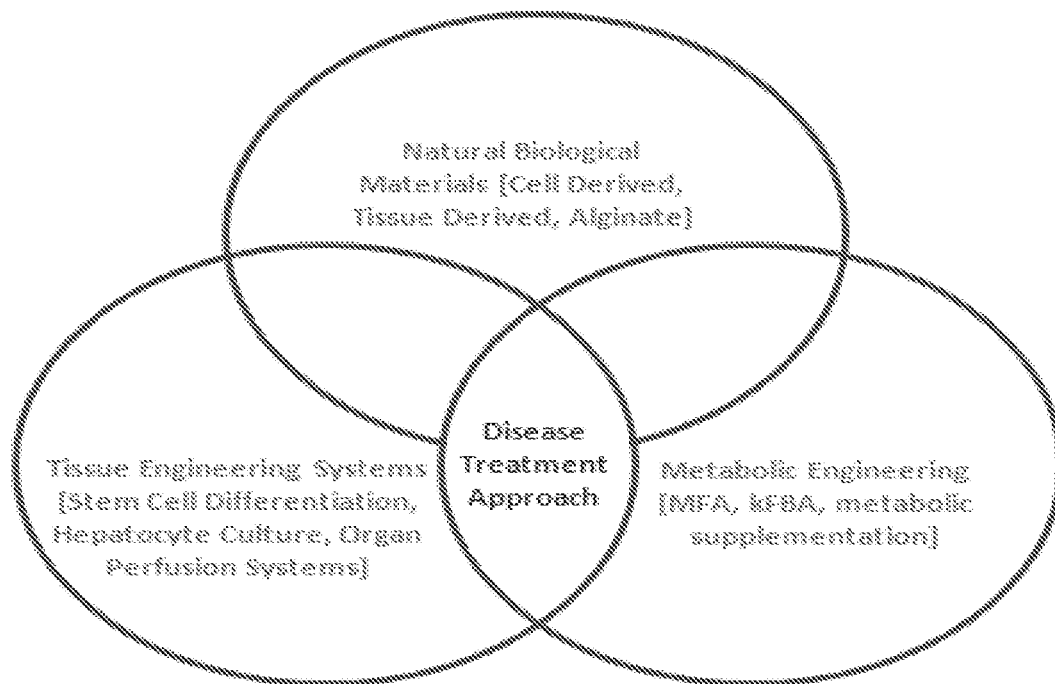


FIG. 12

11/23



Cellular Metabolism-Novel Biological Substrate Integrated Crosstalk to Elucidate Cell-ECM-Metabolism Interactions in Various Applications. 1. Stem Cell Differentiation 2. Primary Cell Culture 3. Tumor Invasion 4. Spinal Cord Injury 5. Obesity Research 6. High-Throughput Cell Screening Systems

FIG. 13

12/23

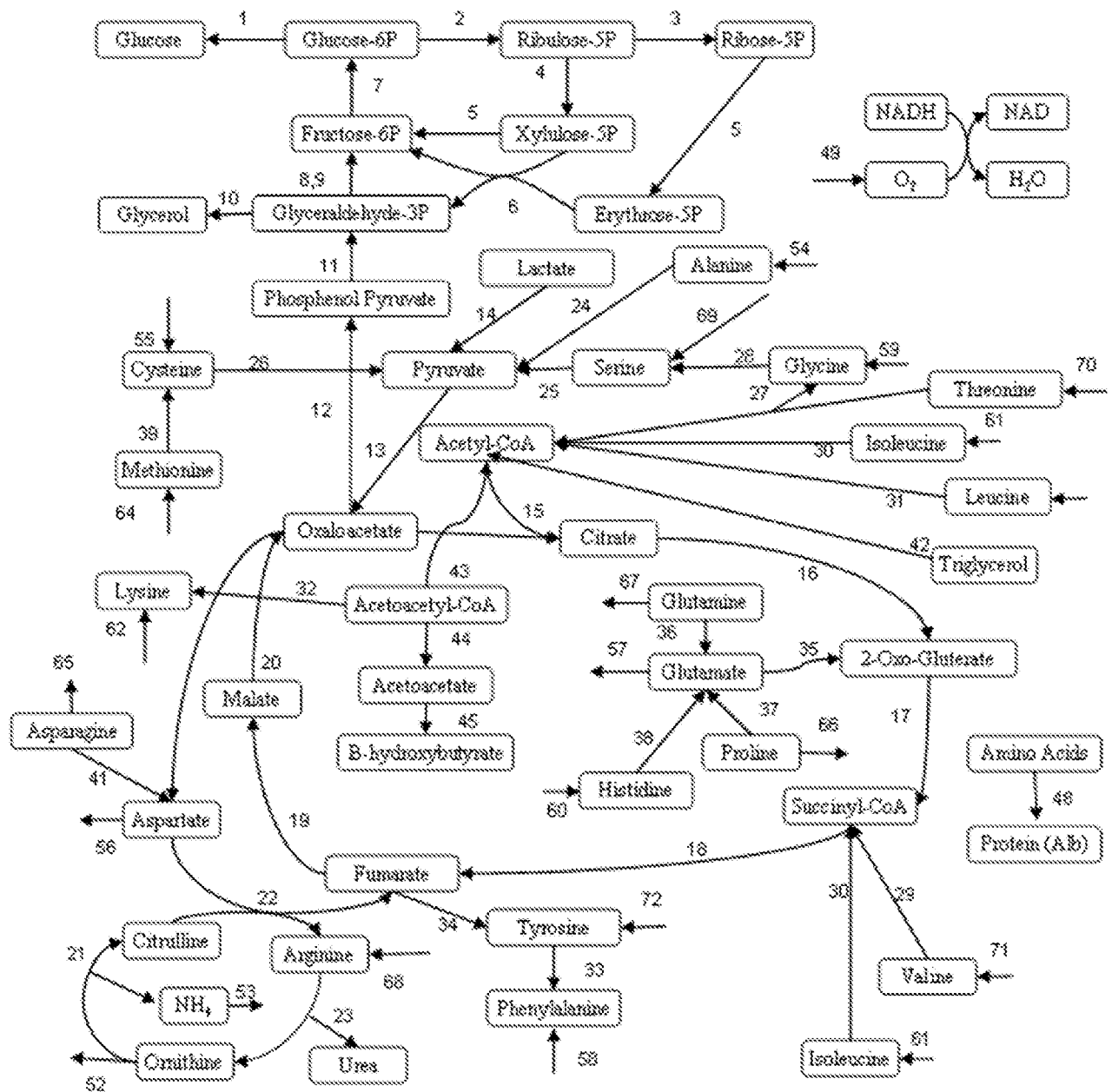


FIG. 14

13/23

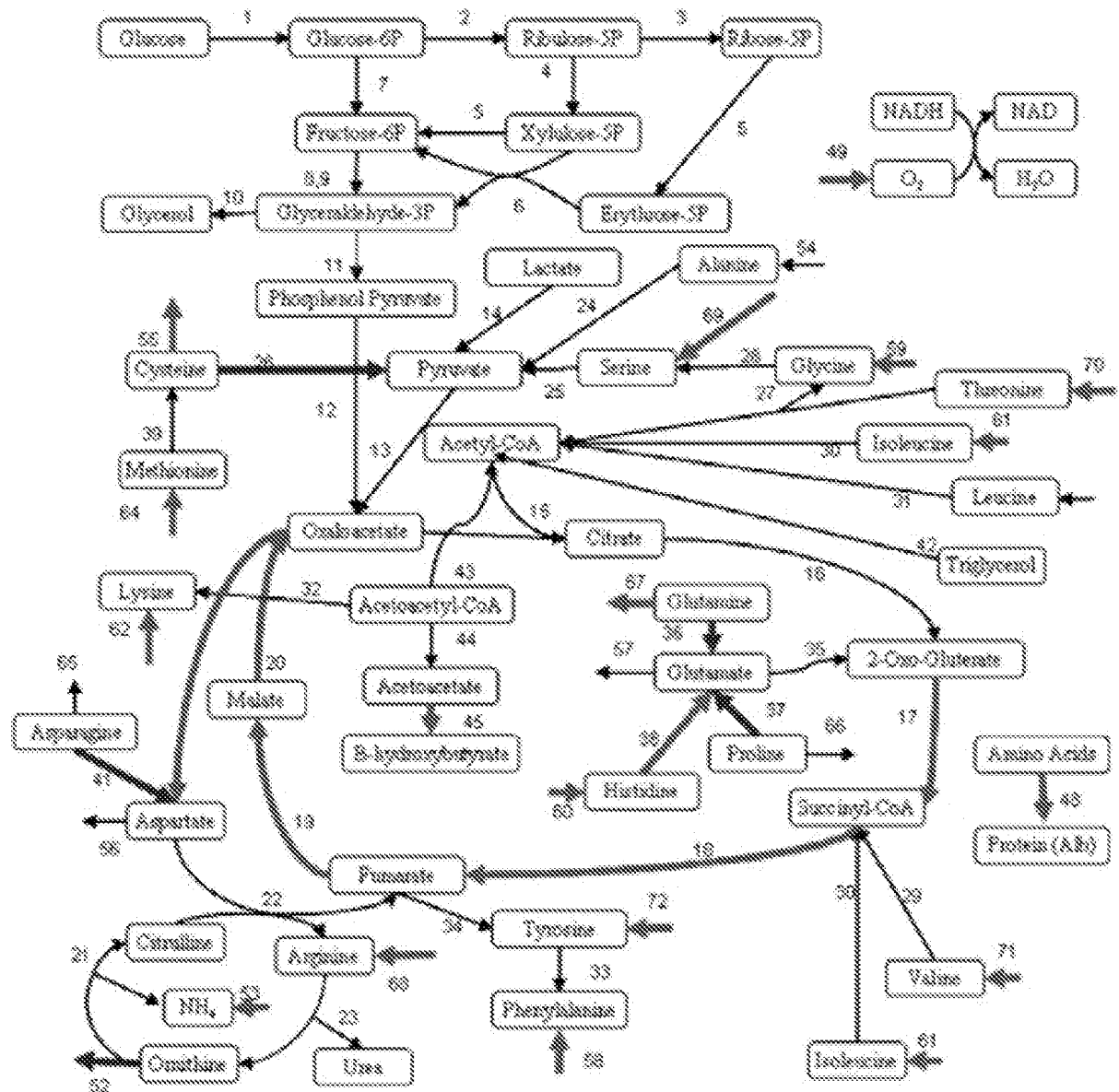
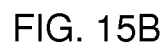
CDG vs CSGs/ASG Day 4

FIG. 15A



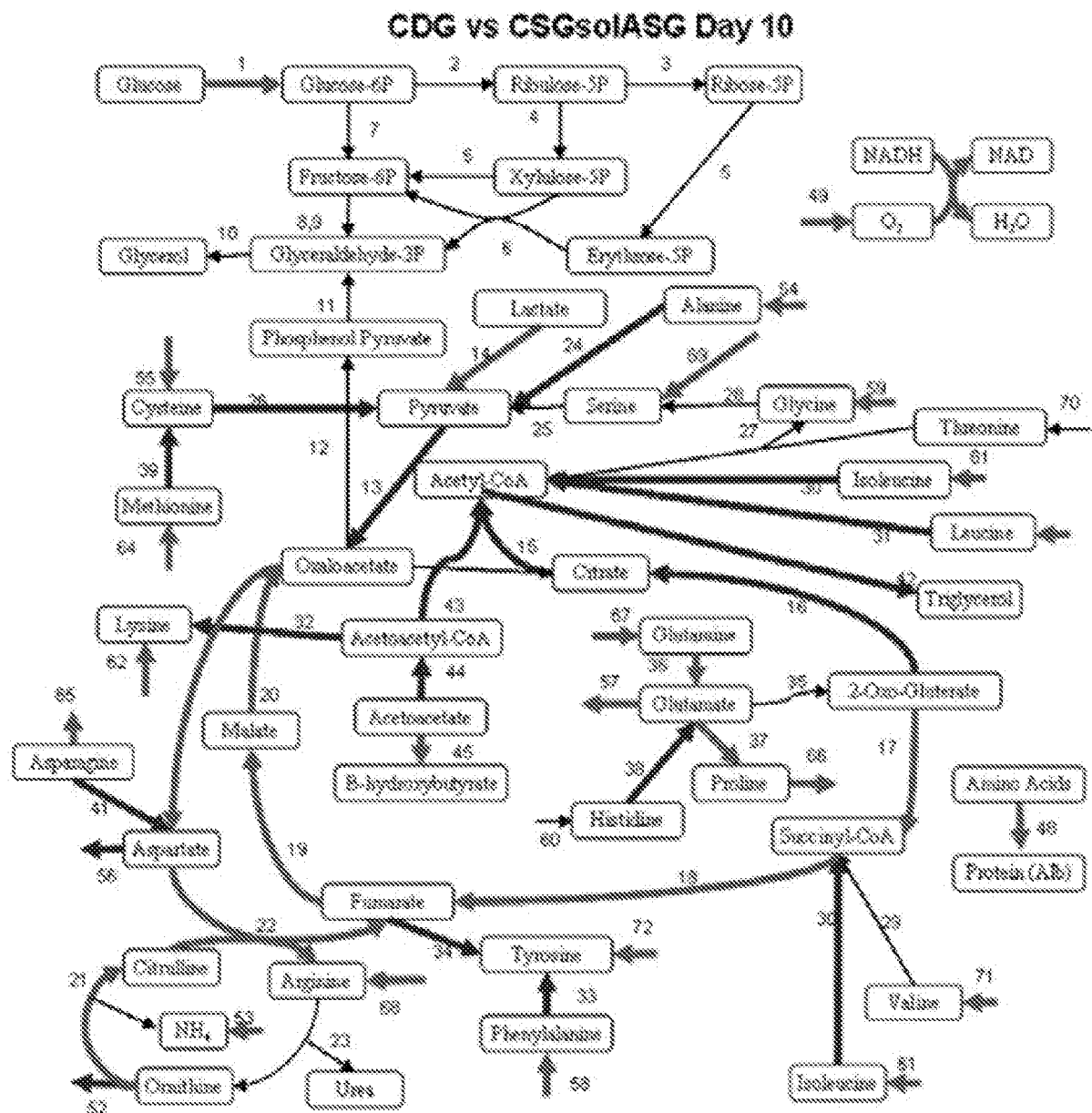


FIG. 15C

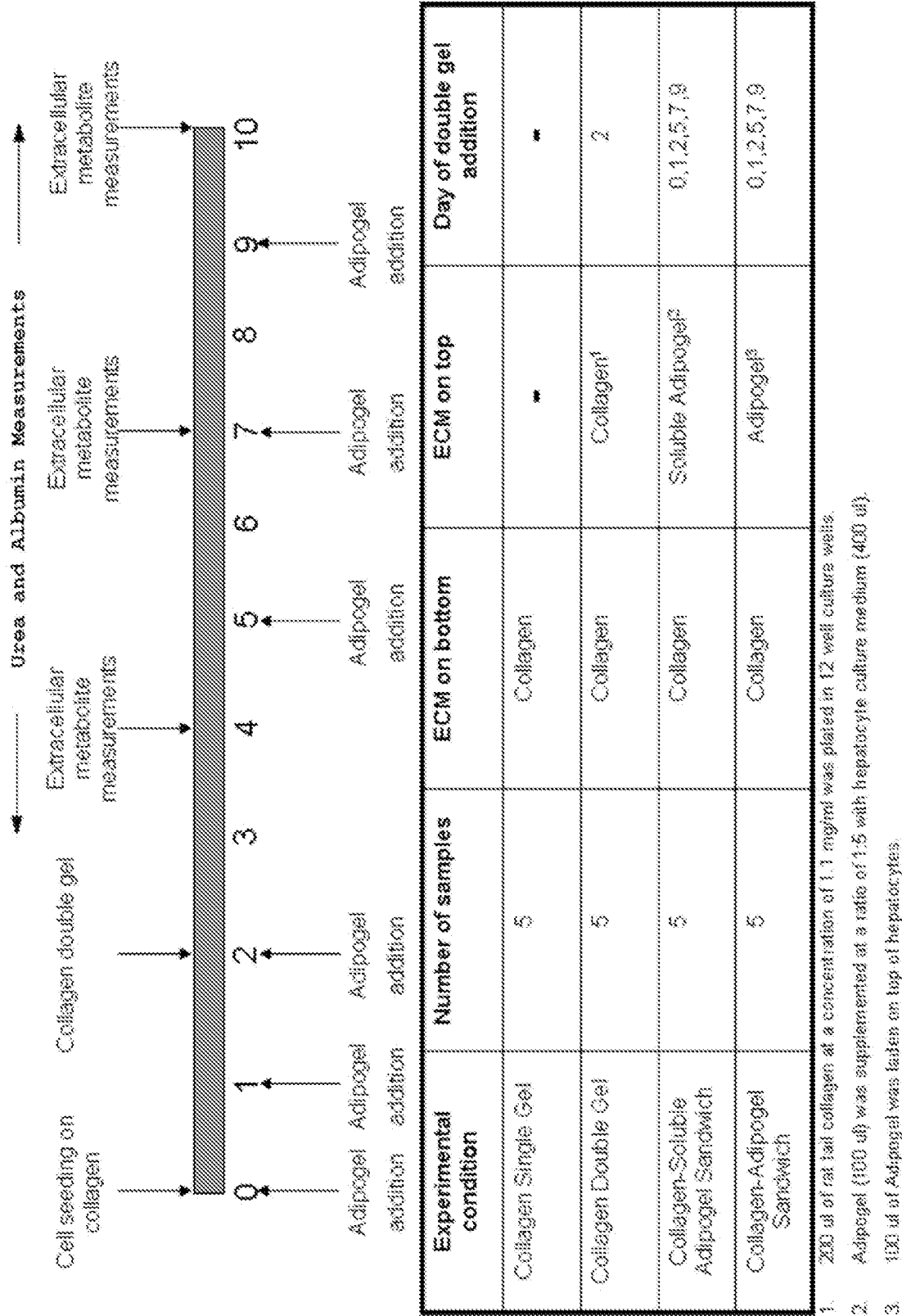
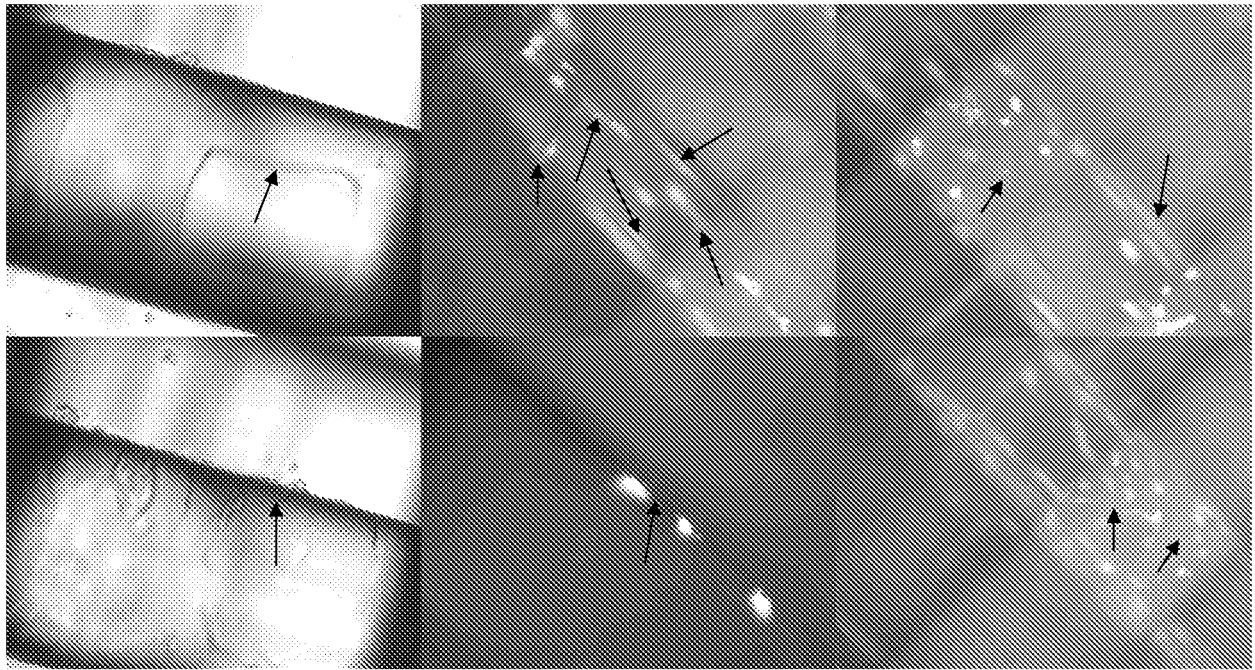


FIG. 16

17/23



Bright-field images of cells

Calcein AM staining of cytoplasm of viable cells

FIG. 17

18/23

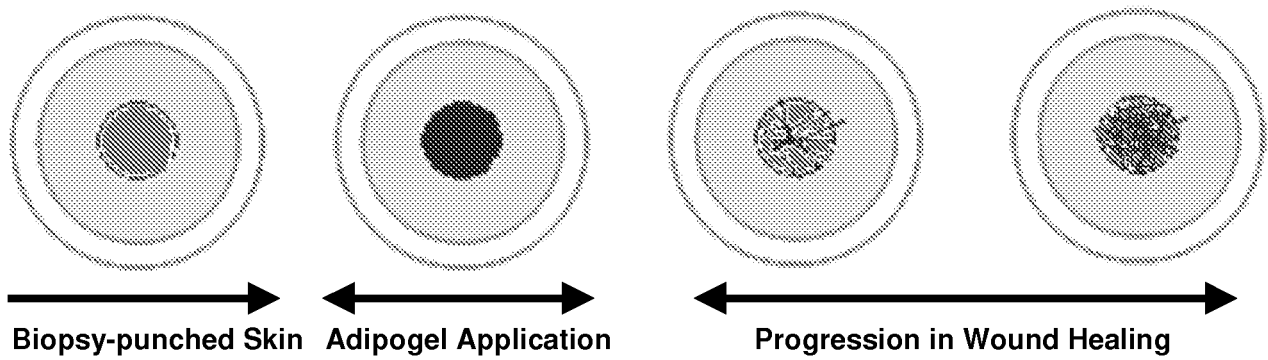
In Vitro System I

FIG. 18

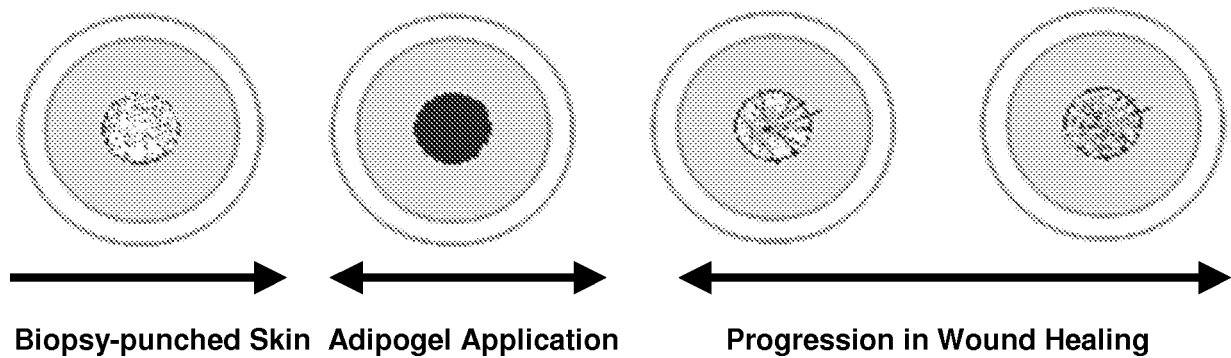
In Vitro System II

FIG. 19

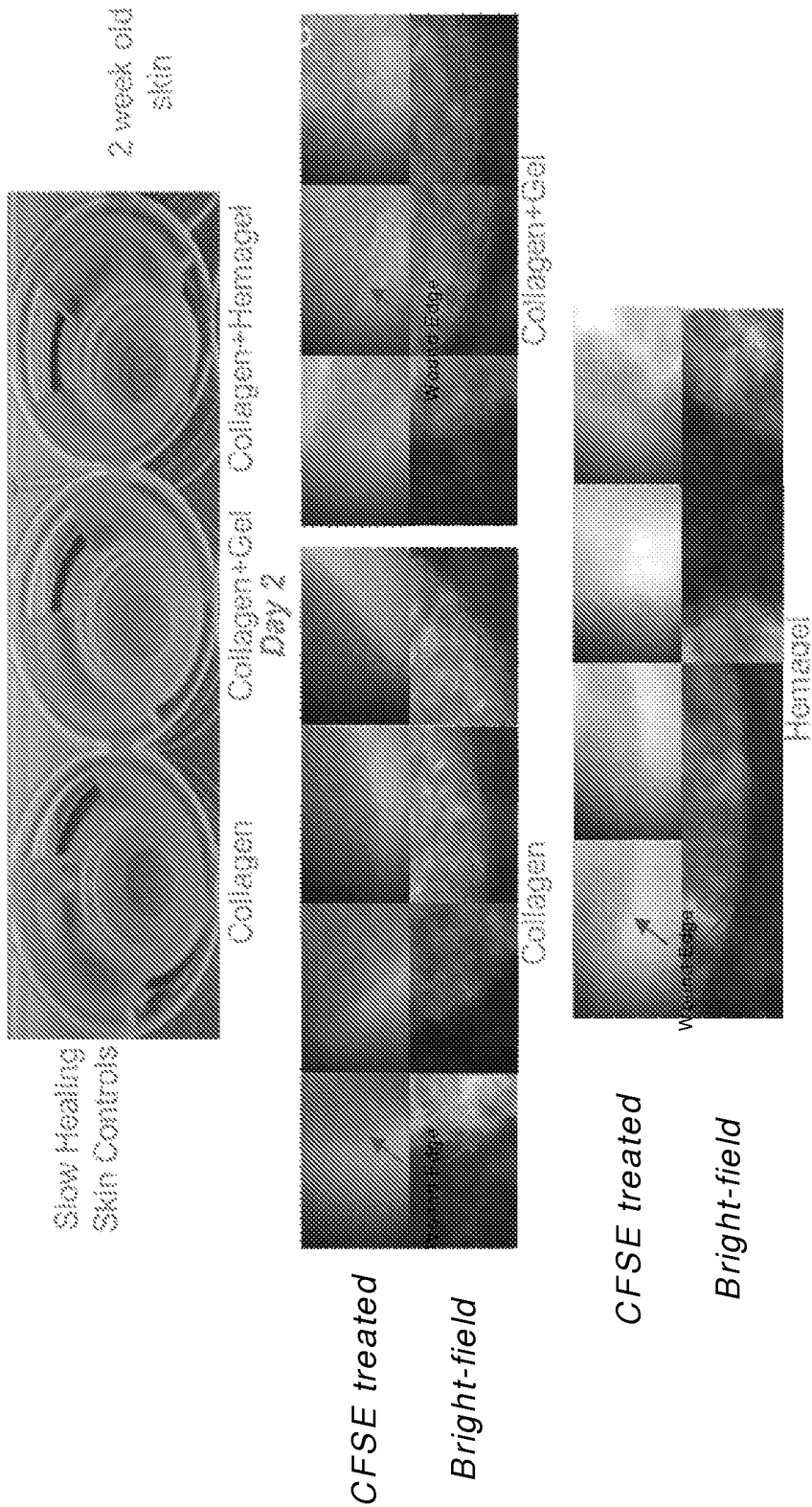


FIG. 20A

20/23

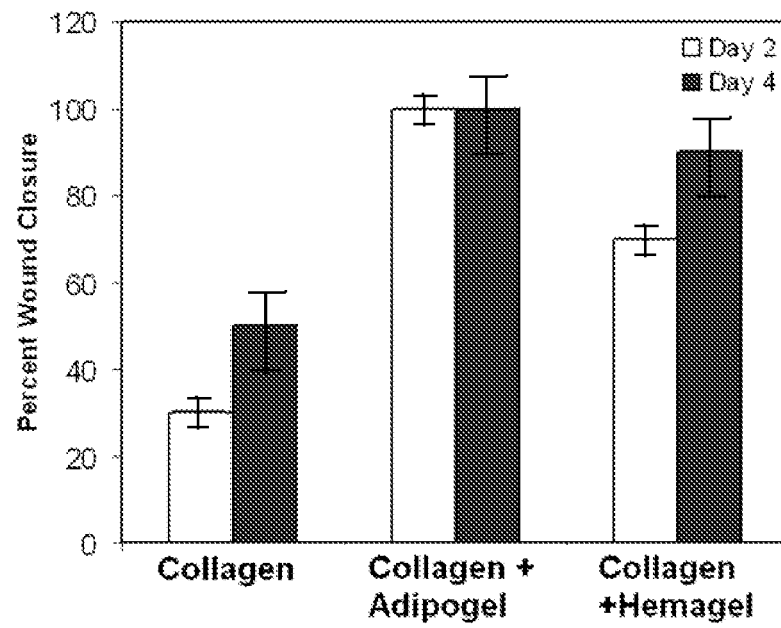


FIG. 20B

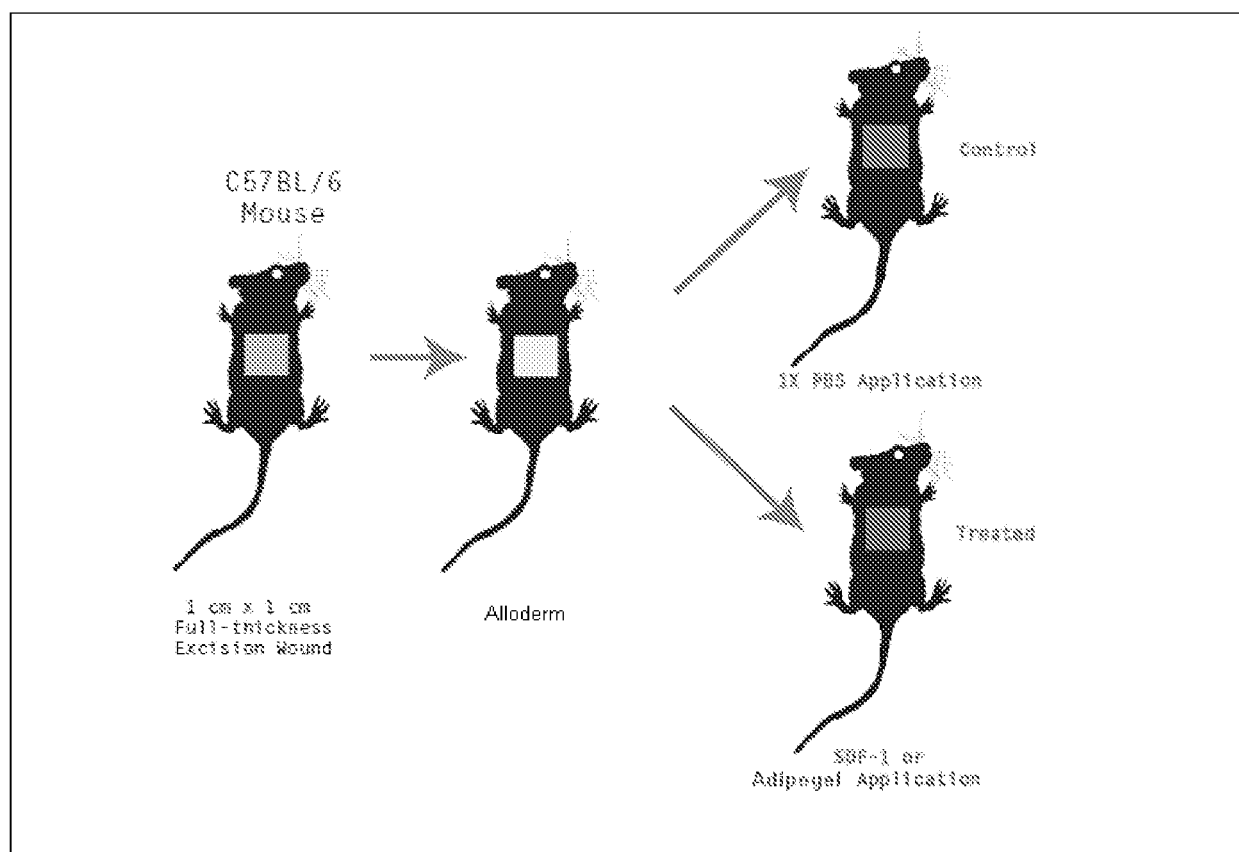
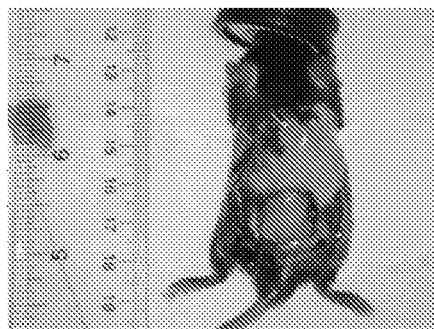
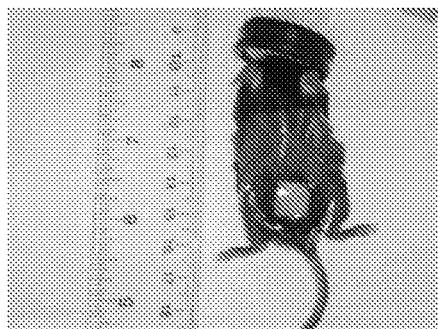
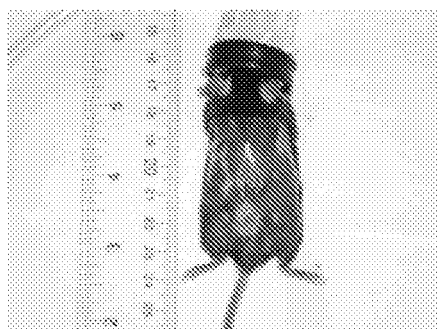


FIG. 21

Day 0



Day 7



Day 14

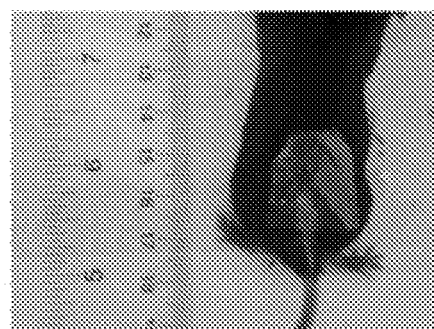
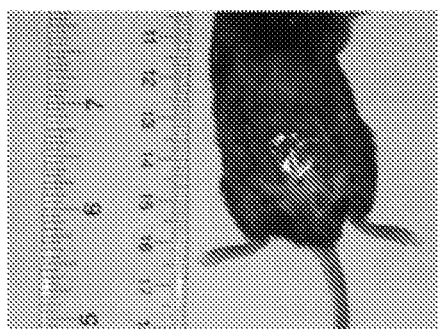


FIG. 22 (A)

23/23

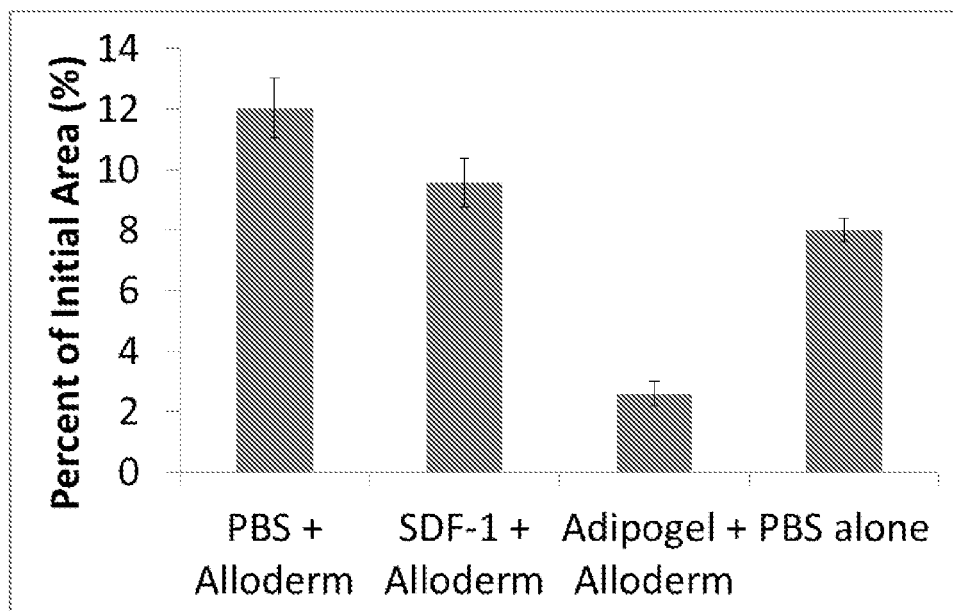
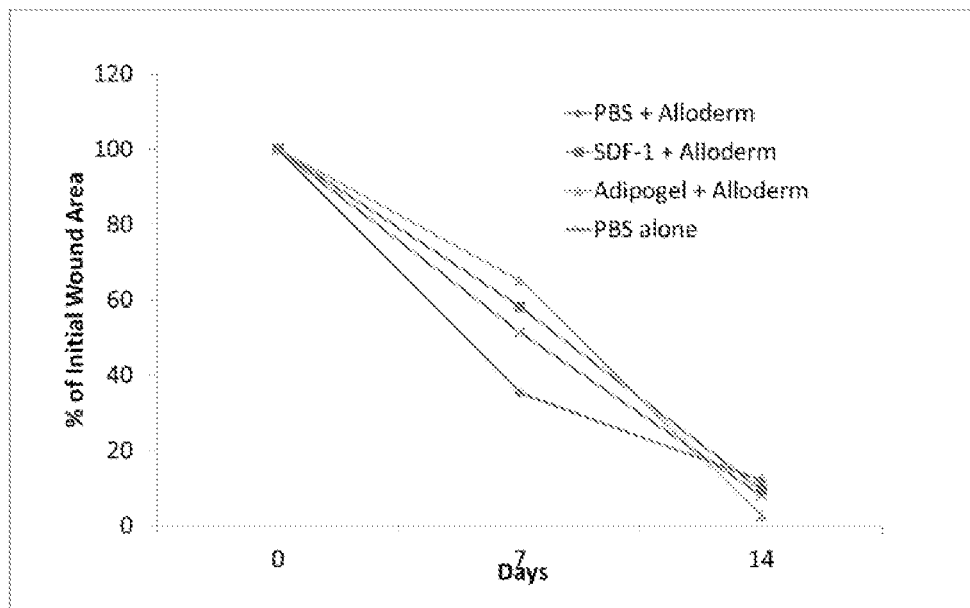


FIG. 22 (B)