Adipocyte Derived Basement Membrane Extract With Biological Activity: Applications in Hepatocyte Functional Augmentation *In vitro*.

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Abstract

Natural and synthetic biomaterials utilized in tissue engineering applications require 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. We have developed a mammalian adipocyte differentiation system for generating a natural basement membrane extract (Adipogel) comprising of ECM proteins (collagen IV and fibronectin) including relevant growth factors (hepatocyte growth factor, vascular endothelial growth factor and leukemia inhibitory factor). We have shown the effective utilization of the growth factor enriched extracellular matrix for enhanced albumin synthesis rate of primary hepatocyte cultures for a period of 10 days as compared to collagen sandwich cultures. A metabolic analysis of the cultures revealed that Adipogel in primary hepatocytes increased serine, glycine, threonine, alanine, tyrosine, methionine, lysine, isoleucine, leucine, phenylalanine, taurine, cysteine and glucose uptake rates to enhance hepatocyte function as compared to controls. The demonstrated synthesis, isolation, characterization and application of Adipogel provide immense potential for tissue engineering and regenerative medicine applications.

1. Introduction

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 [1]. The main challenges of developing an ideal biomaterial are 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. Both biologically derived and synthetic biomaterials have been extensively used in regenerative medicine and tissue engineering applications that require a dynamic interplay of complex macromolecular compositions of hydrated extracellular matrices, soluble growth factors and protein molecules expressed by cells. As of recently, cell and matrix biologists and bioengineers have used natural extracellular matrix (ECM) derived biomaterials for 3D scaffold formation for cell and tissue morphogenesis, growth, migration and differentiation [2-4]. The development and utilization of methodologies to modify synthetic biomaterials such as self-assembly oligopeptide nanofibers, RGD-grafted collagen and synthetic ECM analogs include enhancing protein-protein interactions using polymeric crosslinkers, modulation of fibrillar structures such as kinked, wavy or branched fibers that modify scaffold architecture, synthetic hydrogel modulation by presentation of cell adhesion ligands, proteolytic susceptibility and biologically relevant elasticity.

The main challenges of developing and utilizing synthetic ECMs is the effective control of dynamics and spatial organization of presentation of multiple signals 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 implicated in tissue morphogenesis. Thus, synthetic ECM analogs represent oversimplified mimics of natural ECMs lacking the spatial and temporal complexity.

Numerous techniques have been developed to isolate natural extracellular matrices from variety of sources such as decellularized sub-mucosal intestine [5]; urinary bladder [6, 7]; liver [8]; and skeletal muscle [9] for tissue engineering and regenerative medical applications. The novelty of development of mammalian cell derived natural extracellular matrix supersedes both previously established synthetic analogs and tissue based ECMs developed due to various advantages such as a. obviation of chemical and enzymatic procedures to isolate basement membrane extract (BME) and hence prevention of disruption of protein-protein interactions b. ease of generating basement membrane extract using a less cumbersome procedure, c. animal-free procedures and minimal batch to batch variability, d. reduction of pathogen transmission and e. ability to modulate the supramolecular composition of the BME utilizing various *in vitro* biochemical perturbations.

To develop a naturally cross-linked BME that meet the above criteria, a novel *in vitro* cell culture system has to be established that has the ability to generate substantial amounts of ECM with defined growth factor and extracellular matrix protein composition in an efficient manner. The synthesis, biomolecular, biophysical, biomechanical and optical characterization of the BME will be an important step to investigate the formation of scaffolds, gels and lyophilized powders for tissue engineering and regenerative medicine applications.

In the present work, we have developed a novel adipocyte cell differentiation system to derive naturally cross-linked extracellular matrix (hereof termed Adipogel) with growth factors, cytokines and hormones implicated in investigating cell adhesion phenomena. We have shown proof of concept of the BME by culture of hepatocytes and improvement of differentiated

function in the presence of soluble Adipogel as compared to conventional methods. In addition, we have investigated the effect of the soluble matrix supplementation on hepatic metabolism.

2. Materials and Methods

Materials
Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L glucose, fetal bovine serum (FBS), penicillin, streptomycin, and trypsin–EDTA were obtained from Invitrogen Life technologies (Carlsbad, CA). Unless otherwise noted, all other chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO).

Adipogel Generation using Preadipocyte Differentiation
3T3-L1 murine preadipocytes purchased from American Type Culture Condition (Manassas, VA) were cultured in T-175 cm^2 flasks in Dulbecco's Modified Eagle's media supplemented with 10% FBS, 2% Penicillin and Streptomycin till the cells attained confluency. 48 hours post confluency, the cells were differentiated in culture media supplemented with 1 µM dexamethasone, 0.1 µM isobutyl-methylxanthine and 10 µg/ml insulin agonist for 2 days with media changes every two days. On the second day post-differentiation, cells were exposed to culture medium supplemented with 10 µg/ml insulin agonist for 2 days. Media supernatant was collected on days 2 and 4 of differentiation and stored at 4^oC prior to further processing.

In order to purify the extracellular matrix rich material, the differentiated preadipocyte conditioned media was centrifuged at 4000g for 1.5 hr using an Amicon 100 kDa or 10kDa centrifugal filter. The concentrate, primarily composed of media constituents with molecular weight cut-off of 100 kDa and 10 kDa comprised the cell culture supernatant derived protein concentrate including extracellular matrix. About 250 µl of protein concentrate was obtained at the end of the purification step per 15 ml of conditioned media with a yield of ~60 fold concentrate. Since the concentrate was derived from adipocyte-related cell type and had a gel-like configuration, it was termed as 'Adipogel'.

SEM Analysis of Adipogel
Adipogel was plated at 100µl/well in a 96 well plate. After 24 hour incubation at 37^oC, Adipogel was fixed with 4 % paraformaldehyde for 15 mins followed by 1 hour incubation with 95 % alcohol and absolute alcohol. The post-fixed gels formed in 96 well plates were gold/palladium coated using a Balzers SCD004 Sputter Coating Unit followed by SEM imaging using an Amray 1830 I unit equipped with an EDAX 9800 X-ray system, a Robinson backscatter detector, an Acorn computer controlled stage and image analysis software.

Adipogel Composition Determination using Protein Arrays
The protein composition was determined using Biotin-Labeled Antibody Arrays (Ray Biotech, Norcross, GA) for simultaneous detection of 308 mouse proteins in Adipogel. Through a simple process, the diluted sample is biotinylated and then dialyzed overnight in preparation for incubation with the array. The newly 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 either by chemiluminescence or fluorescence. Protein concentrate purified from FBS supplemented basal medium was utilized as negative controls. A normalization of upregulated proteins in Adipogel vs. controls was performed to detect fold changes. Fold changes > 1.5 were identified and categorized into different protein sub-types.

Fibronectin, Collagen IV and Laminin ELISA Assays

Mouse fibronectin concentrations were determined using a quantitative sandwich enzyme immunoassay technique (AssayPro, St. Charles, MO). Fibronectin in standards and samples were sandwiched by the immobilized antibody specific for fibronectin precoated on microplates and biotinylated polyclonal antibody specific for fibronectin, which was recognized by a streptavidin-peroxidase conjugate. Unbound material was then washed away and a peroxidase enzyme substrate was added. The color development was stopped and the intensity of the color was measured and quantified against standards.

For detection of murine collagen IV, pre-diluted Adipogel samples, controls, assay standards and rabbit anti-collagen IV antibody were added to murine collagen IV coated wells (Exocell, Philadelphia, PA). The antibody interacts and binds with the collagen IV immobilized to the stationary phase or with the antigen in the fluid phase, hence the notion of competitive binding. After a suitable incubation period, the plates were washed, and an anti-rabbit -HRP conjugate was used to detect bound rabbit antibody. After washing, only the antibody-conjugate bound to the stationary phase remains in the well, and this is detected using a chromogenic reaction. Color intensity was inversely proportional to the logarithm of collagen IV in the fluid phase.

Mouse laminin was determined by the Laminin ELISA kit [Insight Genomics, San Diego, CA] as per manufacturer's instructions.

Primary Rat Hepatocyte Isolation

Female Lewis rats (Charles River Laboratories, Wilmington, MA) weighing 180 to 200 g (2 to 3 months 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 two-step collagenase perfusion method [10,11], which involves purification of the cell suspension by means of centrifugation over Percoll, approximately 100-200 million cells were routinely isolated from one rat with viability between 85 and 98%, as judged by trypan blue exclusion.

Hepatocyte Culture in Collagen Sandwich and Adipogel

Type 1 collagen was prepared by extracting acid-soluble collagen from Lewis rat-tail tendons [12]. 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 37ºC for 60 min to induce collagen gelation before cell seeding. Each well of the 12-well culture plates received $5x10^5$ primary hepatocytes in suspension in 0.5 ml standard hepatocyte culture medium, which consisted of DMEM supplemented with 14ng/ml glucagon, 7.5 ug/ml hydrocortisone, 0.5 U/ml insulin, 20ng/ml EGF, 200 U/ml penicillin, 200 ug/ml streptomycin, and 10 % FBS. Cultures were incubated in 90 % air/10% CO_2 at 37^{o}C. Cells were rinsed with 1X PBS to remove non-adherent cells 24 hours after seeding. For the double collagen gel culture configuration, a second layer of 250 µl collagen was put on top of the cells 48 hours post-seeding. Medium was changed every 24 hours and collected from day 3 onwards until day 10. Additional two culture conditions were utilized; for the soluble Adipogel condition, 100 µl of Adipogel was solubilized in 400 µl of culture medium by continuous pipeting. The supplemented media was added to cell cultures on days 0, 1,2,5,7 and 9 of cultures. 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^{o}C for 60 min followed by addition of culture medium.

Hepatocyte Functional Assessment
Albumin concentration in the collected medium samples was analyzed using a competitive enzyme-linked immunosorbent assay (ELISA) in triplicate. Albumin protein and it's antibody was purchased from MP Biomedicals. Urea concentration was determined via its specific reaction with diacetyl monoxime with a commercially available assay kit (Fisher Scientific, cat. #SB-0580-250). The absorbance was measured with a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA).

Biochemical Assays
The biochemical assays were performed on the collected medium. 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.) [13]. 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.

Statistical analysis
Each data point represents the mean of three experiments (each with three biological replicates), and the error bars represent the standard error of the mean. Statistical significance was determined using the Student's t-test for unpaired data. Differences were considered significant when the probability was less than or equal to 0.1.

3. Results

3.1 Determination of Morphological Characteristics and Composition of Adipogel

We have performed a preliminary characterization of the extracellular matrix components derived as a basement membrane extract from preadipocytes during the differentiation process. The preadipocytes are cultured in T-175 cm^2 flasks in Dulbecco's Modified Eagle's media supplemented with 10% FBS, 2% Penicillin and Streptomycin till the cells attain confluency. 48 hours post confluency, the cells are differentiated in culture media supplemented with 1 µM dexamethasone, 0.1 µM isobutyl-methylxanthine and 10 µg/ml insulin agonist for 2 days with media changes every two days. On day 2, the differentiation medium is supplemented with insulin agonist only.

During the differentiation process, cell exposed media is collected and processed further for generation of cell derived extracellular matrix. We has identified a highly viscoelastic material on days 2 and 4 of adipocyte differentiation resembling extracellular matrix 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. In order to purify the extracellular matrix-rich material, the cell exposed media is centrifuged at 4000*g* for 1.5 hr using an Amicon 100 kDa centrifugal filter. The concentrate, primarily composed of media constituents with molecular cut-off of 100 kDa and 10 kDa comprises the cell culture supernatant derived protein rich extracellular matrix [Figure 1].

We performed an SEM analysis of the gel to evaluate the structural and morphological characteristics. As shown in Figure 2 (A-E), both the day 2 and day 4 Adipogel comprise a dense, complex network with non-uniform peeling of edges in the cross-section. The texture of the biomaterial in general comprises of surfaces of two size ranges. The larger configuration

represents the size and orientation of the cross-section and the smaller configuration represents etched ridges and grooves on the surface.

The extracellular matrix has been characterized to compose of collagen IV and fibronectin. As shown in Figure 2, while the fibronectin concentration is comparable, the collagen IV concentration is ~3 fold higher for Mid Stage (Day 4 Adipogel) as compared to Early-Stage BME (Day 2 Adipogel). The laminin concentration is higher for Day 2 as compared to Day 4 Adipogel samples. In addition to characterization of extracellular matrix proteins, we have identified the protein composition of the Adipogel as shown in Figure 3. The protein content of the gel consists of 27 upregulated proteins as compared to controls (basal media protein concentrate). In addition, we have categorized the proteins in the pie chart; as shown, > 22 % of the upregulated proteins correspond to growth factors and receptor proteins each while ~19 % represent cytokines. The growth factors with applicability from a tissue engineering application perspective that were upregulated for Adipogel are HGF (hepatocyte growth factor), VEGF (vascular endothelial growth factor) and LIF (leukemia inhibitory factor). These growth factors have numerous biological functions ranging from cell proliferation, differentiation, migration to phenomena such as tissue-specific tumorigenesis, angiogenesis and wound healing, tumor suppression and embryogenesis. The ability to develop tunable matrix proteins specific to different applications is an exciting prospect with regards to the protein composition.

3.2 Improvement of Hepatic Differentiated Function

Routine culture of primary hepatocytes is difficult and cumbersome due to their ability to develop compromised function. We have developed a primary hepatocyte culture system that supersedes the traditional methodology of maintaining hepatocyte function and polarity in collagen double gel sandwich systems [14, 15]. As shown in Figure 4, hepatocytes when cultured on single collagen gel with a soluble matrix of Adipogel in the culture media showed comparable urea secretion rates but significantly higher albumin secretion rates from day 5 until day 10 of culture. As shown in Figure 5, a comparison of the morphology of the hepatocytes in culture shows that collagen single gel cultures have elongated morphology on day 7 of culture; on the other hand, collagen sandwich and collagen-Adipogel sandwich cultures result in uniform polygonal morphology. Hepatocytes cultured in collagen-Adipogel soluble matrix configuration results in morphologies that are intermediate to collagen single gel and collagen sandwich cultures.

3.3 Effect of Adipogel on Hepatic Metabolism

We have performed a metabolic analysis of the effect of 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). As shown in Table 1, while hepatocyes in CDG are gluconeogenic with lactate synthesis probably via glycogenolysis, there is a significant higher glucose consumption rate for the remaining experimental conditions.

Serine, glycine, threonine, alanine, tyrosine, methionine, lysine, isoleucine, leucine, phenylalanine, taurine, cysteine uptake rates are significantly higher for CSG+solASG condition vs. CDG condition with increased albumin synthesis rate while alanine, lysine, isoleucine, glutamine and cysteine are upregulated for the CSG+ASG condition vs. the CDG condition with no change in albumin synthesis. Thus, we can correlate increased amino acid uptake rates and changes in glucose metabolism to hepatocellular function without the development of a comprehensive metabolic network.

4. Discussion

Basement membranes are growth factor enriched specialized ECM protein complexes providing structural and functional support to cell monolayers separating from connective tissue. The most commonly studied tumor-derived BM complex, Matrigel, derived from Engelbreth-Holm-Swarm (EHS) mouse tumor is utilized for various cellular applications and differs from normal physiological BM in composition and applications. The ECM is a complex mixture of matrix molecules, including the glycoproteins fibronectin, collagens, laminins, proteoglycans and non-matrix proteins including growth factors. The composition of the ECM, rather than simply the presence of extracellular scaffold, is critical for regulating cell phenotype. ECMs determine body shape and stability, compartmentalization of organs and several cellular activities. These matrices include ubiquitously occurring basement membranes which are 20-200 nm broad deposits of specific proteins in close proximity to epithelial, muscle, fat and nerve cells.

BM compositions are extremely diverse, tissue specific and dynamic. BM provides structural support and organizes cell monolayers during tissue development. BM proteins possess multiple binding sites for cell adhesion molecules, and motifs that serve as ligands for cell surface receptors. BM components guide cellular differentiation, and inhibit or promote cell proliferation and migration. BM sequesters growth factors that influence cell behavior during BM remodeling. Stores of VEGF in vascular BM are released during vascular BM remodeling.

Cell-matrix adhesions are essential for cell migration, tissue reorganization and differentiation and as a result play central roles in embryonic development, tissue remodeling, and homeostasis of tissue and organ systems. These signals in synergy with other pathways regulate biological processes such as cell survival, cell proliferation, wound healing, and tumorigenesis. The elucidation of the structure and function of cell-matrix adhesions provides a critical vantage point for understanding regulation of eukaryotic cellular phenotypes in vivo. The profiles of proteins recruited to matrix adhesions specify the biochemical signals and biophysical properties of matrix adhesions. Principles such as roles of matrix composition, three dimensionality and rigidity as well as existence of distinct types of cell-matrix adhesions and bi-directional signaling responses provide a rationale foundation for development of novel approaches to tissue repair and intervention in disease processes.

To develop a naturally cross-linked BME that meet most of these criteria, a novel *in vitro* cell culture system has to be established that has the ability to generate substantial amounts of ECM with defined growth factor and extracellular matrix protein composition in an efficient manner. While various cell lines can be utilized to produce natural BMEs, a majority of the cells do not produce sufficient extracellular matrix. We have developed an adipocyte differentiation system that generates copious amounts of BME; this matrix termed Adipogel has numerous advantages over Matrigel, the most widely used tissue-derived basement membrane extract accounting for ~80 % of market share in the area of biomaterials as shown in Table 2.

Adipogel Texture and Composition

SEM provides rapid and easy evaluation of the morphological structure of materials. This technique can be utilized to determine the physical state and characteristics of the materials and obtain a qualitative analysis of the biomaterial surface texture, roughness with high resolution and in-depth field. In the current work, we have obtained SEM images of Adipogel in its native

format thus confirming the gel-like configuration [Figure 2]. This information provides us the basis for embedding cells into the gel in future work to evaluate the structure and interaction between the matrix composite and the cells.

The complex mixture of ECM molecules, 3-dimensional structural patterns and distribution of ECM constituents depending on the tissue source mediate structural and biological properties of the biological material. Elucidating the biochemical composition of extracellular matrices reveals its applicability in cell-biomaterial phenomena such as embryonic development, tissue remodeling and homeostasis of tissues and organ systems. As shown in Figure 2 and 3, the ECM and protein composition has been determined for Adipogel. Collagen IV, the most abundant ECM in basement membrane is present in Adipogel. Typically, collagen IV accounting for 50 % of BM proteins possessing both structural and functional properties is an integral component of basement membrane structures and provides ligands for cell adhesion.

While the heterogeneous molecular compositions and biochemical complexity of different organ BMs constitute biological function, the principal component comprising the BM composition is collagen IV. Collagen IV isolated from basement membranes has a size and amino acid composition similar is many ways to other collagen components. Collagen IV plays a very different role in histological structures, forming a sheet made by a meshwork of filaments rather than by linear fibrils. Fibronectin determination in Adipogel is also critical since this component is abundant in basement membranes. Fibronectin influences cell growth and differentiation through its effects on gene encoding cell cycle components. Also, fibronectin is required for self-assembly of ECM molecules such as collagen I fibril formation. Laminin is an essential component of basement membranes and is associated with numerous biological activities including promotion of cell adhesion, growth, proliferation and differentiation.

The main growth factor identified for tissue engineering applications were VEGF, HGF and LIF. VEGF is a potent pro-angiogenic factor that promotes formation of new blood vessels at site of injury also active in vasculogenesis and endothelial cell growth. VEGF also induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis and induces permeabilization of blood vessels. It binds to the VEGFR1/Flt-1 and VEGFR2/Kdr receptors, heparan sulfate and heparin (By similarity).

Hepatocyte growth factor/scatter factor (HGF/SF) is a paracrine cellular growth, motility and morphogenic factor. It is secreted by mesenchymal cells and targets and acts primarily upon epithelial cells and endothelial cells, but also acts on haemopoietic progenitor cells. It has been shown to have a major role in embryonic organ development, in adult organ regeneration and in wound healing. HGF regulated cell growth, cell motility and morphogenesis by activating a tyrosine kinase signaling cascade after binding to the proto-oncogenic c-Met receptor. HGF is secreted by mesenchymal cells and acts as a multi-functional cytokine of mesenchymal cells. 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 alpha chain and 34 kDa beta chain.

LIF derives its name from its ability to induce the terminal differentiation of myeloid leukaemic 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 and inflammation.

Adipogel and hepatocyte culture assessment

Various methodologies to maintain hepatocytes *in vitro* include effect of extracellular matrix topology and function, cellular environment and medium composition. The traditional technique for culturing rat hepatocytes *in vitro* is the collagen I sandwich configuration. While this system has been extensively characterized with expression of basolateral and apical markers, upregulation of differentiated function and maintenance of cell polarity, there is also evidence for the role of extracellular matrix composition on these cellular parameters. Variation in ECM compositions including addition of glycosaminoglycans and hepatic proteoglycans that promote formation of gap junctions; overlay of EHS biomatrix Matrigel as a substitute for collagen I that induces expression of cell adhesion molecules viz. connexins with maintenance of differentiated function comparable to double gel cultures. Matrigel, prepared from extract of murine EHS tumors is comprised primarily of collagen IV, laminin, perlecan, nidogen, FGF, EGF and IGF. While EHS tumor derived matrix composition viz. collagen IV, laminin and heparan sulfate proteoglycan is prevalent in the Space of Disse, utilizing a matrix that resembles the hepatic ECM will induce improved differentiated function similar to the *in vivo* microenvironment.

Adipogel laden on top of collagen single gel hepatocyte cultures have been shown to induce increased albumin differentiated function as compared to collagen double gel cultures and Matrigel cultures [based on literature evidence] in this work. Based on the comparison of the protein composition [Figure 2 and 3] and the differentiated function [Figure 4], we hypothesize that increased albumin synthesis in hepatocyte cultures as compared to controls may be due to increased levels of HGF, laminin and Collagen IV in Adipogel as compared to absence of these factors in double-gel sandwich configurations. Literature evidence show the effects of these components in augmenting albumin synthesis rates [16, 17], however, the combined effects of these components has not been thoroughly investigated. Nevertheless, since Adipogel BME has a complex composition that is not completely characterized, it is not trivial to identify the exact mechanism of the improvement of hepatic differentiated function. However, a complete characterization of the BME followed by comprehensive combinatorial single-protein component blocking experiments can provide vital information about previously unknown factors implicated in improved differentiated function.

Numerous researchers have investigated the intermediary metabolism of hepatocytes, the primary functional cells of the liver [18-21]. Since hepatic metabolism is directly linked to cellular energetic and functions, the effect of hepatocyte culture configurations on the biochemical pathways inherent in mature cells is critical. While mathematical programming tools have been used as an additional step to elucidate the effect of environmental perturbations on hepatic function [22-24], we have performed a metabolic analysis of the effect of the different culture configurations viz. the collagen single gel (CSG), collagen double gel (CDG), collagen-Adipogel sandwich (CSG+ASG), collagen single gel + soluble Adipogel (CSG+solASG). The analysis shows that the key amino acids implicated in increasing albumin synthesis rates for the CSG+solASG conditions are serine, glycine, threonine, tyrosine, methionine, lysine, leucine, phenylalanine, taurine and cysteine. This is in agreement with literature evidence that indicates that amino acid availability is imperative for increased albumin synthesis in perfused livers and cultured rat hepatocytes [25-28]. Also, supplementation of branched chain amino acids such as leucine and isoleucine has been shown to increase albumin synthesis [29] consistent with our findings.

5. Conclusion and Future Work

The development of a unique preadipocyte cell differentiation system has been utilized in this work for the generation of a natural basement membrane extract termed Adipogel with a unique extracellular matrix and growth factor composition. This methodology has numerous advantages over commonly used biomaterials such as Matrigel viz. presence of fibronectin in Adipogel thus promoting better cell attachment; obviation of enzymatic and chemical processing methods to generate the extract; animal-free procedure that is not cumbersome and relatively inexpensive procedure. We have shown that Adipogel can be utilized for augmenting hepatocyte differentiated function *in vitro* in combination with collagen over a 10 day period. In addition, the increase in amino acid and glucose metabolism in the presence of soluble Adipogel shows promise in the integration of cellular metabolism with cell-extracellular matrix interactions.

One of the major goals in the comprehensive characterization of basement membrane proteins is the understanding of principles that determine supramolecular organization. The self-assembly of individual collagen IV chains and other ECM proteins into supra-structures and networks are important for BM stability and function. Also, complex biochemical and molecular pathways activated or modified by integrin mediated adhesion provide insights into mechanisms that regulate adhesion dependent cellular processes. The shifting progression of integrin receptor expression may influence endogenous or exogenous tension or facilitate cell survival and migration in multiple tissues with different matrix compositions.

Future work will entail the mechanical characterization and optimization of the basement membrane extract for gel, scaffold and lyophilized powder formation. We will also evaluate cell-matrix adhesion molecules and their role in augmentation of hepatic function and effects on cell metabolism. Further, the determination of sub-chains of the collagen IV family along with interactions with other ECM proteins in Adipogel will be determined. In order to elucidate the effects of Adipogel on intracellular hepatic function, we will utilize metabolite measurements for development of a Metabolic Flux Analysis model. These methodologies will provide a better snapshot of the mechanistic implications of utilization of Adipogel for hepatocyte functional maintenance. Overall, Adipogel has the potential to be utilized as a basement membrane extract for numerous tissue engineering, regenerative medicine, cosmetics, medical device and drug delivery applications.

6. Figure and Table Legends

Figure 1. Schematic of Adipogel generation using a preadipocyte differentiation system. Murine 3T3-L1 preadipocytes were cultured to confluence in basal medium. 2 days post-confluency, cells were differentiated in the presence of IBMX, dexamethasone and insulin agonist. 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.

Figure 2. Scanning Electron Microscopy Images of Adipogel: (A, B) Day 2 Adipogel and **(C-E)** Day 4 Adipogel isolated from differentiating preadipocyte culture media. After 24 hour incubation at 37C, Adipogel is fixed with 4 % paraformaldehyde for 15 mins followed by 1hr incubation with 95 % alcohol and absolute alcohol. The post-fixed gels formed in 96 well plates are gold/palladium sputtered followed by SEM image acquisition and analysis.

Figure 3. Extracellular Matrix Characterization of Adipogel: (A) Fibronectin, **(B)** Collagen IV and **(C)** Laminin Concentration Determination using ELISA. As shown, the fibronectin concentration is comparable at Early stage (Day 2) and Mid stage (Day 4) derived extracellular matrices. The collagen IV concentration is significantly higher for the Day 4 Adipogel as compared to the Day 2 Adipogel. Laminin concentration is higher for Day 2 Adipogel as compared to Day 4 Adipogel.

* *indicates $p < 0.05$ for Day 4 Adipogel condition vs. Day 2 Adipogel condition.*

Figure 4. Protein Characterization of Adipogel: 10kDa MWCO Adipogel Upregulated Protein Composition as compared to 10 kDa cutoff DMEM basal media. Adipogel with > 10 kDa protein cut-off was isolated using Amicon Centrifugal Filters on day 4 of differentiation. The protein extract was isolated and protein composition was determined using Protein Array (Ray Biotech). Protein composition was determined using a five day process wherein biotinylated protein antibody was added to samples in a glass slide chip. Signal detection and quantitation was performed using the label antibody based protein array glass chip slides. The pie chart shows a distribution of proteins upregulated in Adipogel. The numbers in each section correspond to the percentage of proteins belonging to each category.

Figure 5. Functional Augmentation of Rat Hepatocytes *In vitro* Using Adipogel : (A) Urea (B) Albumin Secretion rate of Hepatocytes cultured in four 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 w Adipogel overlaid on top. Adipogel was utilized at a concentration of 30 mg/ml and media was changed on days 0, 1,2,5,7 and 9. While the urea secretion rates are similar for the CDG (positive control) and the CSG+solASG conditions, the albumin secretion rate is significantly higher for the CSG+solASG condition from day 5 to day 10 of culture period. * *indicates $p < 0.1$ for CSG+solASG condition vs. CDG. condition.*

Figure 6. Phase Contrast Images of cultured rat hepatocytes. (A) Collagen single gel, (B) collagen sandwich, (C) collagen single gel with soluble Adipogel and (D) collagen single gel with overlay of Adipogel on day 7 after seeding.

Table 1. Effect of Adipogel substrate on amino acid and glucose metabolism of day 10 hepatocyte cultures: Hepatocytes were cultured in four 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 w Adipogel overlaid on top. *Negative values correspond to metabolite synthesis rates and positive values correspond to metabolite consumption rates. Values are in umol/million cells/day. * indicates $p < 0.05$ for each experimental condition vs. CDG.*

Table 2. Comparison of Matrigel and Adipogel

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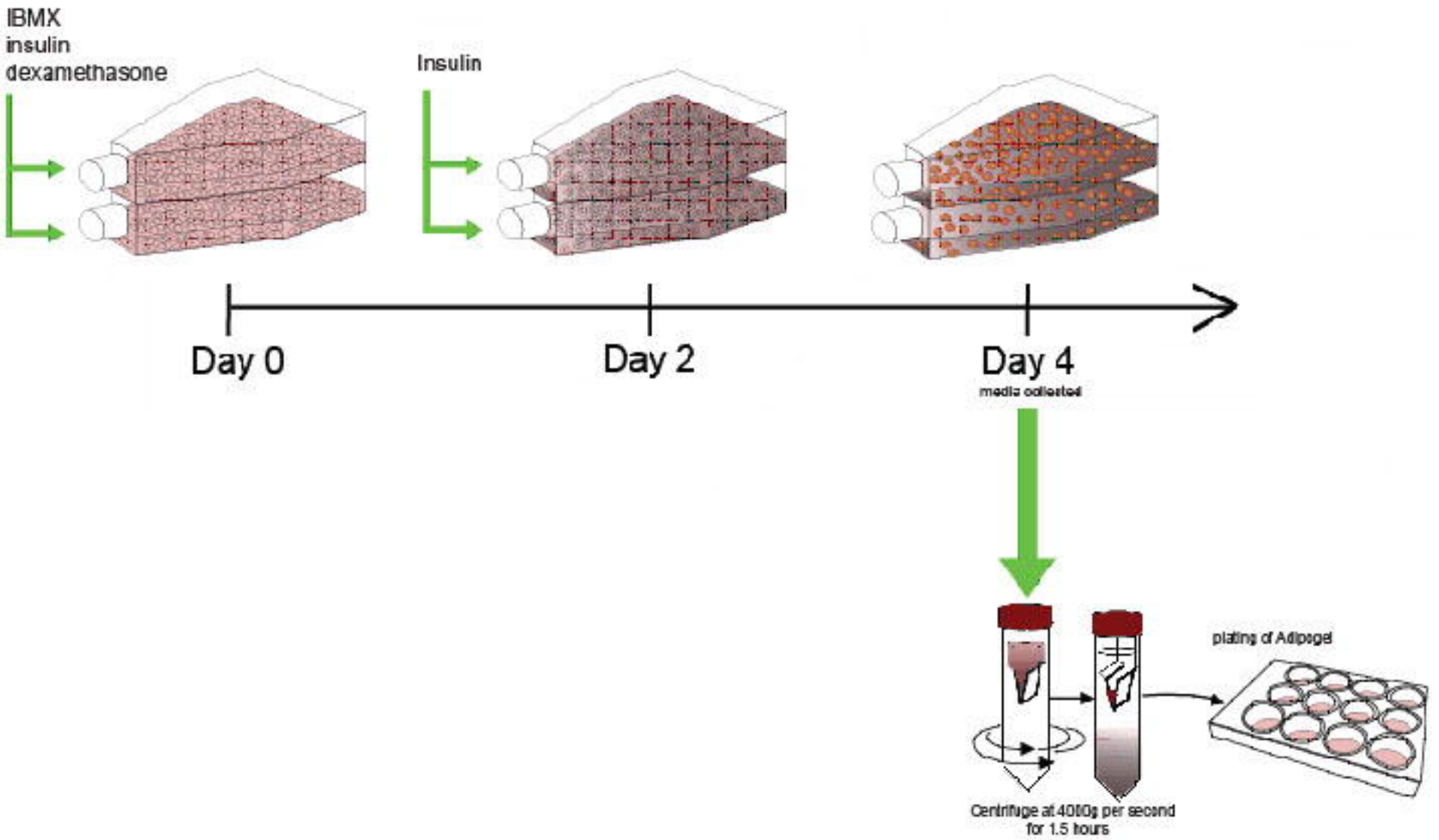
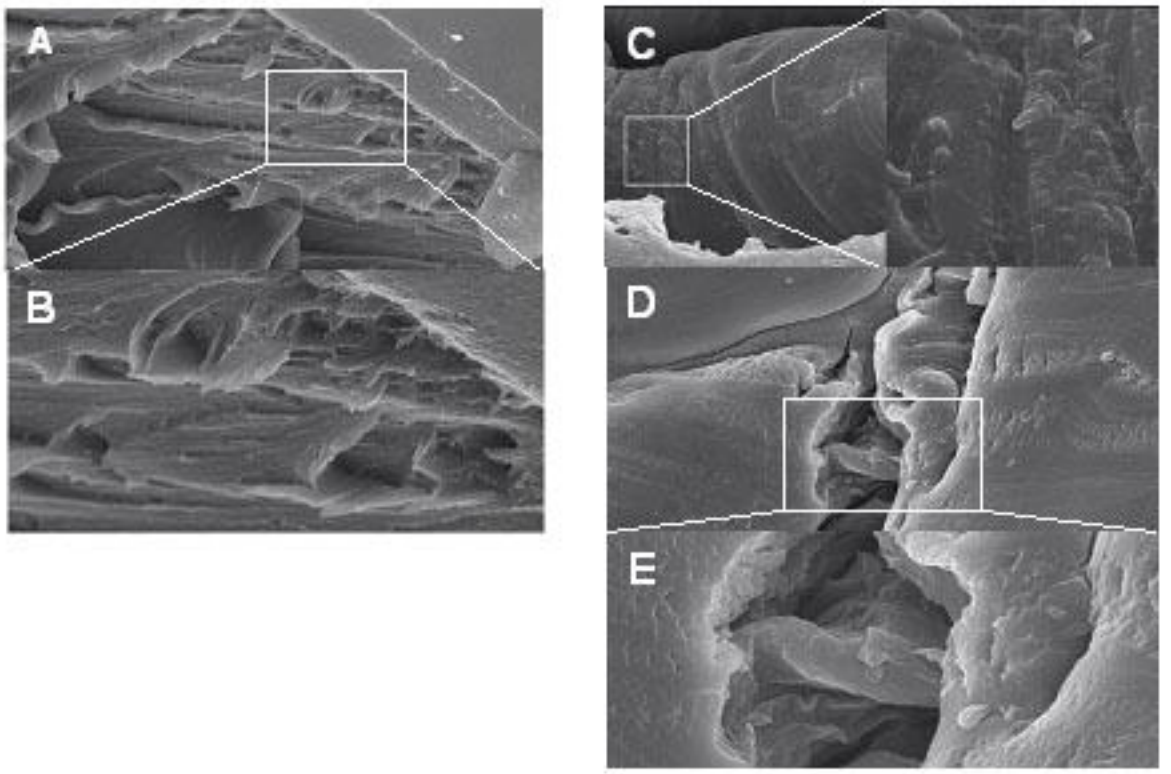
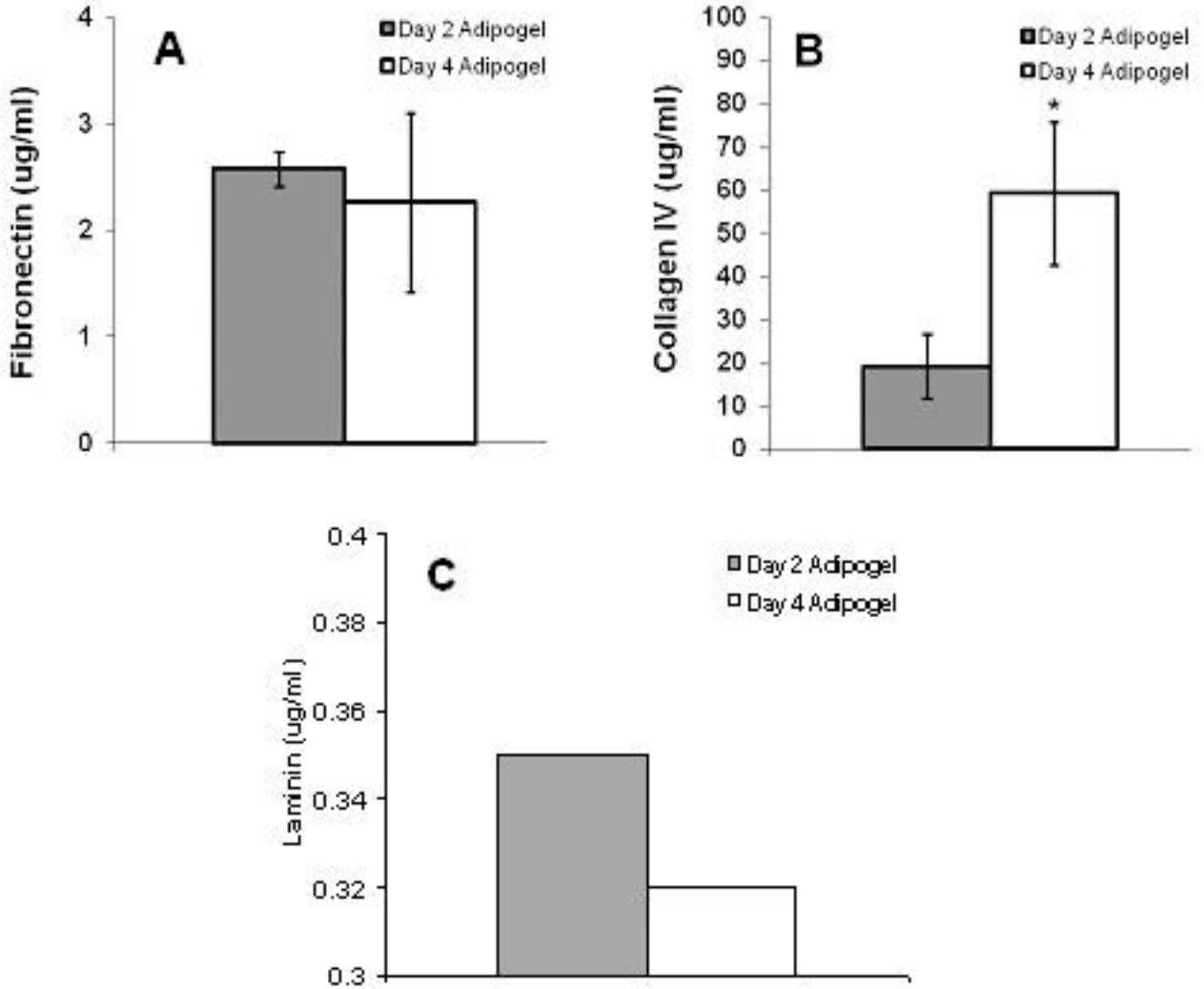
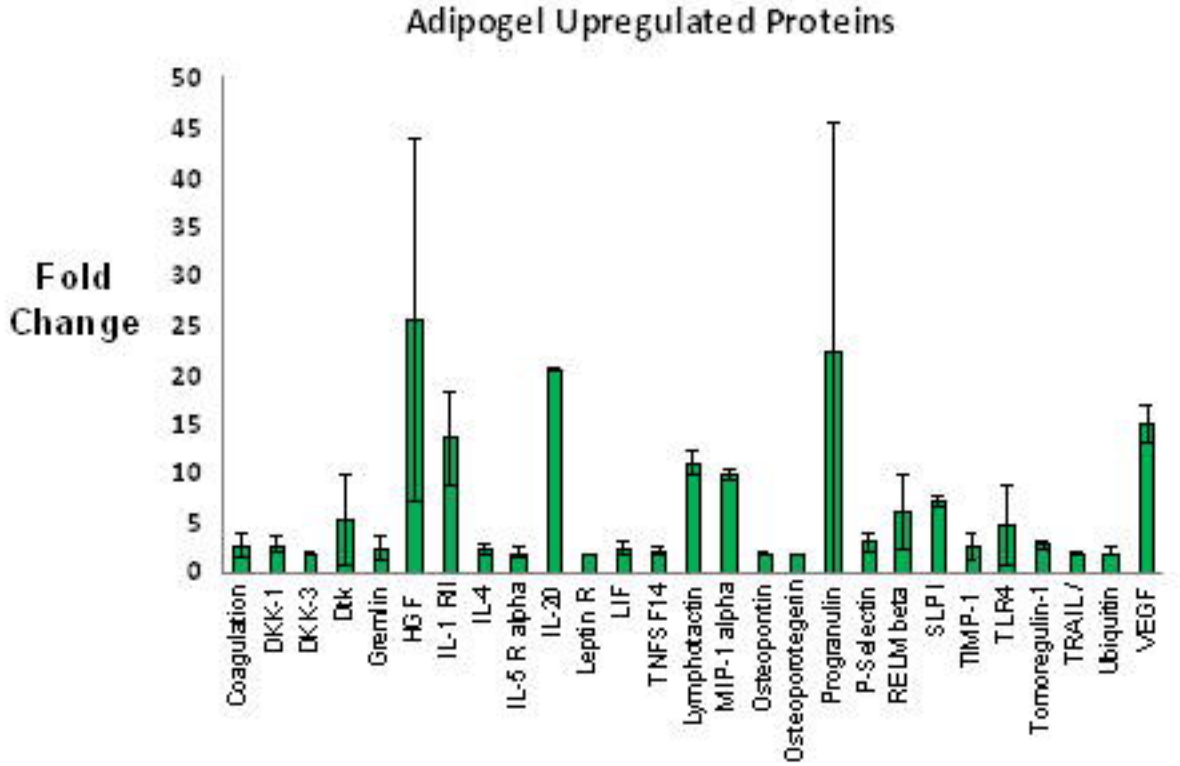
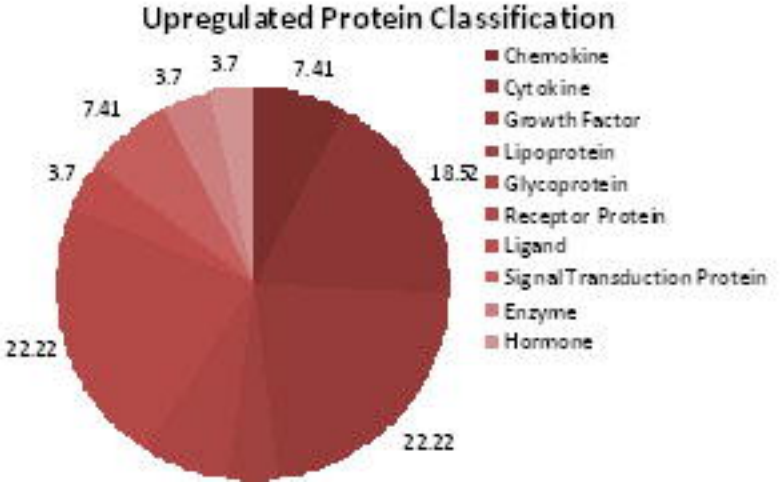
Adipogel Generation System

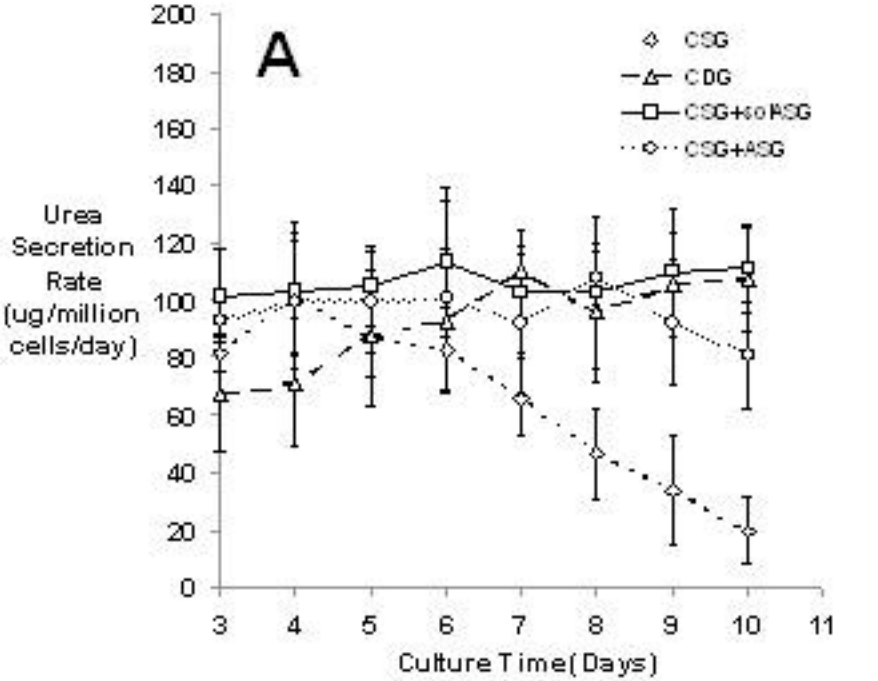


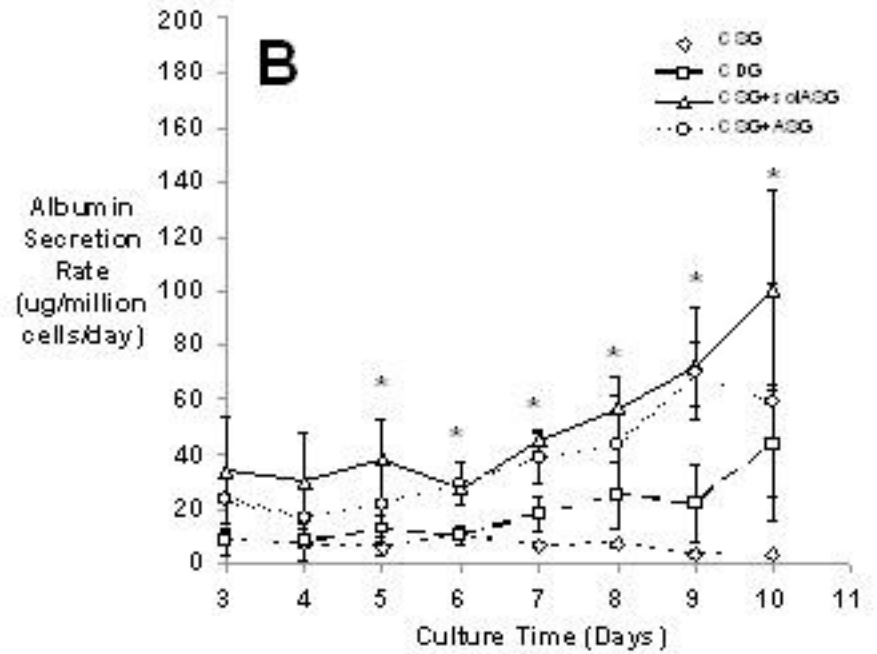

A
B
C
D
E

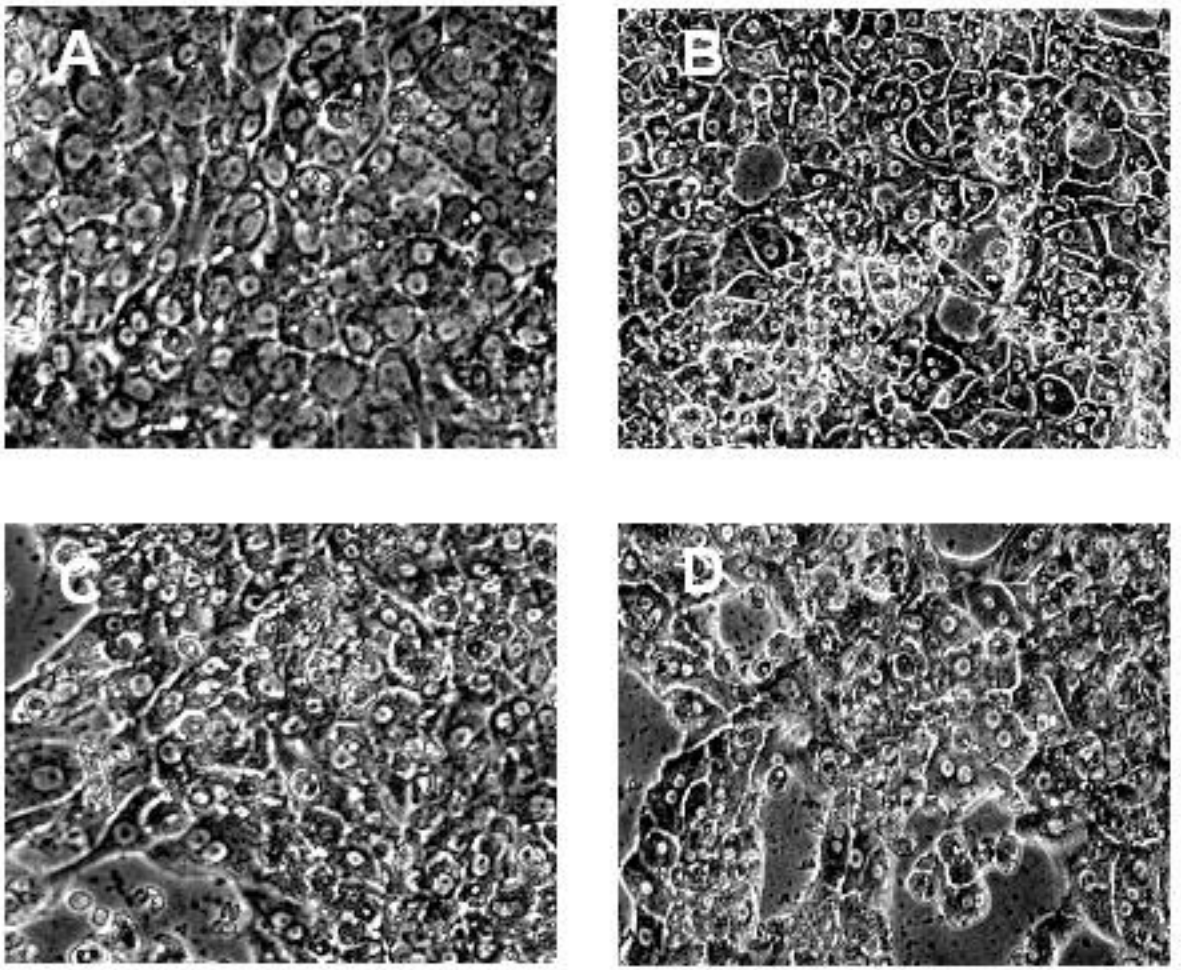

A
Day 2 Adipogel
Day 4 Adipogel
Fibronectin (ug/ml)
0
1
2
3
4
B
Day 2 Adipogel
Day 4 Adipogel
*
Collagen IV (ug/ml)
0
10
20
30
40
50
60
70
80
90
100
C
Day 2 Adipogel
Day 4 Adipogel
Laminin (ug/ml)
0.3
0.32
0.34
0.36
0.38
0.4


Adipogel Upregulated Proteins
Fold Change
50
45
40
35
30
25
20
15
10
5
0
Coagulation
DKK-1
DKK-3
Dtk
Gremlin
HGF
IL-1 RII
IL-4
IL-5 R alpha
IL-20
Leptin R
LIF
TNFSF14
Lymphotactin
MIP-1 alpha
Osteopontin
Osteoprotegerin
Progranulin
P-Selectin
RELM beta
SLPI
TIMP-1
TLR4
Tomoregulin-1
TRAIL/
Ubiquitin
VEGF


Upregulated Protein Classification
7.41
18.52
22.22
22.22
3.7
7.41
3.7
3.7
Chemokine
Cytokine
Growth Factor
Lipoprotein
Glycoprotein
Receptor Protein
Ligand
Signal Transduction Protein
Enzyme
Hormone


A
CSG
CDG
CSG+solASG
CSG+ASG
Urea Secretion Rate (ug/million cells/day)
200
180
160
140
120
100
80
60
40
20
0
3
4
5
6
7
8
9
10
11
Culture Time(Days)


B
CSG
CDG
CSG+solASG
CSG+ASG
Albumin Secretion Rate (ug/million cells/day)
200
180
160
140
120
100
80
60
40
20
0
3
4
5
6
7
8
9
10
11
Culture Time (Days)


A
B
C
D

Metabolites	CSG	CDG	CSG+solASG	CSG+ASG
ASP	-0.022±0.005	-0.015±0.002	-0.0085±0.002	-0.014±0.002
SER	0.0975±0.086*	0.189±0.0115	0.3095±0.0105*	0.152±0.0085*
GLU	0.2125±0.051*	-0.7095±0.017	-0.442±0.0225*	-0.458±0.06*
GLY	-0.081±0.215	0.1755±0.04	0.447±0.029*	0.0305±0.108
HIS	0.1885±0.046*	0.454±0.002	0.457±0.0025	0.4285±0.0005*
AMM	-0.6905±0.135*	0.0265±0.018	0.2125±0.015*	-0.027±0.0305
ARG	-0.0025±0.005*	0.0405±0.0015	0.0115±0.0035*	0.007±0.006*
THR	0.266±0.12*	0.9405±0.0065	1.024±0.0075*	0.9275±0.015
ALA	0.0695±0.1655	-0.053±0.0235	0.4965±0.019*	0.184±0.029*
PRO	-0.809±0.199*	-0.103±0.0175	-0.3315±0.032*	-0.313±0.016*
TYR	0.0675±0.1815*	-0.522±0.059	0.55±0.0545*	-0.1515±0.135*
VAL	-0.145±0.261*	1.0165±0.0395	0.4945±0.0605*	-0.0345±0.163*
MET	0.005±0.084*	0.408±0.019	0.4785±0.008*	0.245±0.0185*
LYS	-0.108±0.163	0.013±0.042	0.482±0.026*	0.203±0.0485*
ILEU	-0.113±0.278*	-0.86±0.0485	0.613±0.0785*	0.0945±0.1635*
LEU	0.0075±0.2385*	0.6725±0.0495	0.7775±0.069*	0.2235±0.1475*
PHE	0.0755±0.1855*	0.9895±0.0315	1.2445±0.0215*	0.834±0.0345*
ASN	-0.0375±0.008*	-0.0235±0.002	0±0	-0.0195±0.001*
GLN	-6.331±1.317*	-3.222±0.2155	-0.1935±0.073*	1.7145±0.1065*
TAU	-0.028±0.3295	0.3185±0.0155	0.86±0.062*	0.238±0.0395*
CYS	0.081±0.1495	-0.2095±0.014	0.327±0.0205*	0.04±0.0775*
ORN	0.2015±0.0315*	-0.409±0.0095	-0.0845±0.035*	-0.329±0.033*
GLC	-0.0677±0.008*	0.0008±0.023	-0.0605±0.02*	-0.072±0.02*
LAC	0.0695±0.0122	0.068±0.008	0.096±0.005*	0.103±0.022*

Features	Matrigel	Adipogel
Method of Synthesis	Prepared from Extract of Murine EHS Tumors	Purified from Differentiated Mammalian Preadipocyte Secretions
Composition	Collagen IV, Laminin, Perlecan, Nidogen, FGF,EGF,IGF	Collagen IV, Fibronectin, HGF,VEGF,LIF
Applications	Angiogenesis, Transplantation, Tissue Engineering	Primary Cells and Cell Line Culture, Functional Maintenance
Disadvantages	Batch to batch variability, cumbersome extraction procedure, animal derived, complex mixture , chemical digestion; limited cell proliferation; not completely characterized	Not completely characterized, complex mixture, animal cell line derived, relatively nascent technology
Advantages	Tested in multiple applications, basement membrane-like complex, gelation procedure easy	Naturally crosslinked; no chemical, enzymatic procedures; less-cumbersome; relatively cheap, basement membrane-like complex; extensive cell proliferation due to high fibronectin content