Video Article

Engineering a Bilayered Hydrogel to Control ASC Differentiation

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Abstract

Natural polymers over the years have gained more importance because of their host biocompatibility and ability to interact with cells *in vitro* and *in vivo*. An area of research that holds promise in regenerative medicine is the combinatorial use of novel biomaterials and stem cells. A fundamental strategy in the field of tissue engineering is the use of three-dimensional scaffold (e.g., decellularized extracellular matrix, hydrogels, micro/nano particles) for directing cell function. This technology has evolved from the discovery that cells need a substrate upon which they can adhere, proliferate, and express their differentiated cellular phenotype and function [2-3]. More recently, it has also been determined that cells not only use these substrates for adherence, but also interact and take cues from the matrix substrate (e.g., extracellular matrix, ECM)[4]. Therefore, the cells and scaffolds have a reciprocal connection that serves to control tissue development, organization, and ultimate function. Adipose-derived stem cells (ASCs) are mesenchymal, non-hematopoetic stem cells present in adipose tissue that can exhibit multi-lineage differentiation and serve as a readily available source of cells (i.e. pre-vascular endothelia and pericytes). Our hypothesis is that adipose-derived stem cells can be directed toward differing phenotypes simultaneously by simply co-culturing them in bilayered matrices[1]. Our laboratory is focused on dermal wound healing. To this end, we created a single composite matrix from the natural biomaterials, fibrin, collagen, and chitosan that can mimic the characteristics and functions of a dermal-specific wound healing ECM environment.

Video Link

The video component of this article can be found at https://www.jove.com/video/3953/

Protocol

1. Isolating Adipose-Derived Stem Cells (ASCs) [1, 5]

Note: All procedures were performed at room temperature unless otherwise noted.

1. Isolate rat perirenal and epididymal adipose and wash with sterile Hank's buffered salt solution (HBSS) containing 1% fetal bovine serum (FBS) as previously described [5]. This study has been conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations and in accordance with the principles of the Guide for the Care and Use of Laboratory Animals.
2. Mince the tissue and transfer 1-2 g into 25 mL of HBSS containing 1% FBS into a 50-mL tube and centrifuge at 500 g for 8 min at room temperature.
3. Collect the free-floating adipose tissue layer and transfer to 125-mL Erlenmeyer flask and treat with 25 mL of collagenase type II (200 U/mL) in HBSS for 45 min at 37 °C on an orbital shaker (125 rpm).
4. Carefully remove the liquid fraction (below oil and adipose layer) by pipeting and filter it sequentially through a 100- µm and 70- µm nylon mesh filter. Centrifuge the filtrate at 500 g for 10 min at room temperature, aspirate the supernatant, and wash the pellet twice with 25 mL of HBSS.
5. Resuspend the cell pellet in 50 mL of growth medium (MesenPRO RS Basal Medium) supplemented with MesenPRO RS Growth Supplement, antibiotic-antimycotic (100 U/mL of penicillin G, 100 µg/mL of streptomycin sulfate, and 0.25 µg/mL of amphotericin B), and 2 mM of L-glutamine and pipette cells into two T75 flasks (25 mL/flask).
6. Culture the ASC in a 5% CO_2-humidified incubator at 37 °C (passage 2-4 ASCs are used for all experiments).

2. Preparing Chitosan Microspheres (CSMs)

Note: All procedures were performed at room temperature unless otherwise noted.

1. CSMs are prepared by a water-in-oil emulsification process along with an ionic coacervation technique using our previous protocol [5]. Emulsify an aqueous solution of chitosan (6 mL of 3%w/v chitosan in 0.5 M of acetic acid) in a 100 mL of an oil phase mixture consisting of soya

oil, n-octanol (1:2 v/v) and 5% sorbitan-mono-oleate (span 80) emulsifier, using overhead (1700 rpm) and magnetic stirring (1000 rpm) simultaneously in opposite directions. This dual method of mixing ensures that micelles formed early on before cross-linking occurs can remain in solution and do not to settle to the bottom. Furthermore, the magnetic stir bar aids in de-aggregating chitosan during micelle formation and rigidization.
2. Stir the mixture continuously stirred for approximately 1 hour until a stable water-in-oil emulsion is obtained. Initiate ionic cross linking with the addition of 1.5 mL of 1% w/v potassium hydroxide in n-octanol every 15 min for 4 h (24 mL total)
3. After completion of the cross linking reaction, slowly decant the oil phase of the mixture containing CSM and immediately add the 100 mL of acetone, the solution will become cloudy, due to the oil residue. After 5 minutes, decant, add an additional 100 mL of acetone and incubate overnight at room temperature. The next day, re-wash the beads with acetone for five minutes. After performing these serial washes the solution should have turned clear, if not continue washing until all oil is removed and the acetone solution is clear.
4. Dry the recovered spheres in a vacuum desiccator and analyze without further processing. You can determine the average CSM particle size, surface area per milligram, and the unit cubic volume by using a particle size analyzer.
5. For subsequent experiments, wash the CSM three times with sterile water to remove residual salts and sterilize by washing overnight with 5 mL of absolute ethanol.

3. Determining the Number of Free Amino Groups in CSMs

Note: All procedures were performed at room temperature unless otherwise noted.

1. Determine the number of free amino groups present in CSMs after ionic cross linking by using the trinitro benzenesulfonic (TNBS) acid assay of Bubnis and Ofner[6]. Incubate 5 mg of microspheres with 1 mL of 0.5% TNBS solution in a 50-mL glass tube for 4 h at 40 °C and hydrolyze with the addition of 3 mL of 6N HCl at 60 °C for 2 h.
2. Cool the samples to room temperature and extract the free TNBS by adding 5 mL of deionized water and 10 mL of ethyl ether.
3. Warm a 5-mL aliquot of the aqueous phase to 40 °C in a water bath for 15 min to evaporate any residual ether, cool to room temperature, and dilute with 15 mL of water.
4. Measure the absorbance at 345 nm with a spectrophotometer using TNBS solution without chitosan as blank and the chitosan used for CSM preparation to determine the total number of amino groups. Estimate the number of free amino groups of the CSM relative to chitosan.

4. Loading ASC in CSM

Note: All procedures were performed at room temperature unless otherwise noted.

1. Equilibrate 5 mg of sterilized CSMs from section 2.5 in sterile HBSS overnight and add to an 8- μm pore size membrane culture plate insert (24-well plate).
2. After the CSMs have settled onto the membrane, carefully aspirate the HBSS and add 300 μL of growth medium to the inside of the insert and 700 μL of growth media to the outside of the insert.
3. Resuspend ASCs at the appropriate concentration (1×10^4 to 4×10^4) in 200 μL of growth medium and seed over the CSM inside the culture plate insert. The final volume of medium within the culture insert, after seeding, is 500 μL.
4. Incubate the ASC seeded on CSMs for 24 h in a 5% CO_2 humidified incubator at 37 °C.

5. Determining the Percentage of ASC Loading and Cell Viability in CSMs

Note: All procedures were performed at room temperature unless otherwise noted.

1. After incubation, pipet the ASC-loaded CSM in a sterile 1.5-mL microcentrifuge tube without disturbing the cells that have migrated into the insert membrane.
2. Remove the residual medium and add 250 μL of fresh growth medium to the tube.
3. To each tube, add 25 μL of MTT [3-(4,5-dimethylthiozole-2-yl)-2,5-diphenyltetrazolium bromide] solution (5 mg/mL) and incubate for 4 h in a 5% CO_2 humidified incubator at 37 °C.
4. After incubation, remove the medium, add 250 μL of dimethyl sulfoxide, and vortex the mixture for 2-5 min to solubilize the formazan complex.
5. Centrifuge the CSM at 2700 g for 5 min, pipet off the supernatant and determine its wavelength absorbance at 570 nm and 630 nm using a standard plate reader, according to MTT manufacturer's specifications.
6. Determine the cell number associated with the CSMs relative to the values obtained from defined ASC numbers cultured to develop a standard curve.

6. Preparation and Characterization of ASC-CSM Embedded in PEG-fibrin Gels

Note: All procedures were performed at room temperature unless otherwise noted.

1. Polyethylene glycol (PEG) fibrin (PEG-fibrin) hydrogel prepared by Suggs *et al.*[7] by dissolving the succinimidyl glutarate modified polyethylene glycol (PEG; 3400 Da) using 4 mL of tris-buffered saline (TBS, pH 7.8) and filter sterilize with a 0.22-μm filter just before starting the experiment. Dissolved PEG is only effective in this application for the first 3-4 hours.
2. Mix 500 μl of fibrinogen stock (40 mg/mL in TBS, pH 7.8) and 250μl of PEG stock in a culture well of a 6-well plate and incubate for 20 minutes in a 5% CO_2 humidified incubator at 37 °C. This mixture constitutes a molar concentration ratio of 1:10, SG-PEG-SG: fibrinogen.
3. Take 250 μl of ASC-CSM at a concentration of 5 mg of CSM, (≈ 2×10^4 cells) and mix with the PEGylated fibrinogen solution.
4. Immediately add 1 ml of thrombin stock (25U/mL) and quickly triturate once or twice with the pipette. After mixing the thrombin with the PEG-fibrinogen, immediately place the cell-gel mixture in a 12-well plate and incubated in a 5% CO_2 humidified incubator at 37 °C for 10 min to

allow for complete gelation. Since the gelation time is fast, do not try and hold the gel solution within the pipette tip for more than 5 seconds. The PEGylated fibrinogen is cleaved by thrombin and forms a PEGylated fibrin hydrogel. As such, the final gel product is referred to as PEG-fibrin.
5. Wash the PEG-fibrin gels twice with HBSS and incubate with alpha minimal essential media (α-MEM) supplemented with 10% FBS in a 5% CO_2 humidified incubator at 37 °C.
6. Observe the migration of cells from CSM into the gel over an 11-day period using standard light microscopy techniques.

7. Preparation and Characterization of ASC-CSM Embedded in Collagen Gels

Note: All procedures were performed at room temperature unless otherwise noted.

1. Mix ASC-CSM (5 mg containing ≈ 2×10^4 cells) with type 1 collagen (7.5 mg/mL) extracted from rat tail tendons according to the method of Bornstein[8] and fibrillate after adjusting the pH to 6.8 using 2N NaOH.
2. Add the fibrillated collagen-ASC-CSM mixture to a 12-well plate and incubate for 30 min in a 5% CO_2 humidified incubator at 37 °C.
3. After complete fibrillation, incubate the collagen-ASC-CSM gels for up to 11 days in a 5% CO_2 humidified incubator at 37 °C.
4. Observe the migration of cells from CSM into the gel over an 11-day period using standard microscopy techniques.

8. Development of Bilayered PEG-fibrin-(ASC-CSM)-Collagen Gel Constructs

Note: All procedures were performed at room temperature unless otherwise noted.

1. To develop the bilayer construct, prepare both the collagen and the PEG-fibrin gels as described above, with slight modifications. Briefly, to study the migratory and co-induction properties of a single source of stem cells using two bioscaffolds, "sandwich" the ASC-CSMs between the collagen and the PEG-fibrin scaffolds by using a four-step process: 1) Load ASC onto CSM, 2) cast fibrillated collagen gel and layer the ASC-CSM beads over the collagen gel, 3) cast PEG-fibrin gel over the ASC-CSM-collagen gel and allow gel to solidify, and 4) add medium to well and insert to study cells *in vitro* or remove from insert for *in vivo* applications (see **Figure 1**).
2. Prepare a 1-mL type 1 collagen (7.5 mg/mL) mixture as described above in 7.1, without adding ASC-CSM to the mixture. Place the mixture in a 6-well tissue culture insert (8-µm pore size) and incubate for 30 min in a 5% CO_2 humidified incubator at 37 °C.
3. After complete fibrillation, layer over the collagen surface 5 mg of ASC-CSM (10, 000 cells/mg) suspended in culture media (200 µl). After the microspheres have settled over the gel, prepare the PEG-fibrin gel as described in section 6.0, without adding ASC-CSM to the mixture, and layer the PEGylated fibrinogen/thrombin solution over the ASC-CSM- collagen layers. When preparing the PEG-fibrin gel, use 250 µl of cell culture medium in place of the 250 µl of medium containing the cells.
4. Once completed, incubate the constructs for 30 min in a 5% CO_2 humidified incubator to achieve complete gelation before feeding the construct with culture medium.
5. After complete gelation, place 1 mL of medium in the upper chamber over the construct and 3 mL of medium in the lower chamber.

9. Making Stock Solutions

Note: All procedures were performed at room temperature unless otherwise noted.

- **Calcium chloride stock (40 mM):** Use only $CaCl_2.2H_2O$. Dissolve 588.4 mg of $CaCl_2.2H_2O$ with 100 ml of deionized water. Sterilize using a 0.22-µm filter.
- **Polyethylene glycol:** PEG is highly reactive with oxygen and can become oxidized when exposed to room air. As such, PEG should be stored under nitrogen (N_2) atmosphere. Accurately weigh 32 mg in a 2-ml centrifuge tube (make sure to purge the tubes with N_2 before use) and store at -80 °C until ready for use. Dissolve 32 mg of PEG in 4 mL of TBS solution before use.
The following solutions must be made fresh before every experiment.
- **TBS solution:** (pH 7.75-7.77 at 25 °C; pH at 25 °C is very critical). To prepare 15 ml of TBS solution, carefully dissolve one buffer tablet in filter-sterilized deionized water and adjust to the required pH. Do not store the stock solution—prepare it fresh every time.
- **Fibrinogen solution:** (40 mg/ml). Dissolve fibrinogen powder in TBS to make the fibrinogen concentration 40 mg/ml. Dissolve it overnight using a magnetic stirrer at 4 °C. It is easier to dissolve the entire 1-g quantity with the required amount of TBS. The following day, remove the fibrinogen from 4 °C (usually cloudy) and allow it to warm in a water bath until a homogenous solution is obtained. Finally, filter sterilize the fibrinogen using a 0.45-µm filter.
- **Thrombin solution (25 Units/ml):** To make the thrombin solution, weigh out the required amount and dissolve with the prepared 40 mM $CaCl_2.2H_2O$. It's always a good practice to use the entire vial of thrombin to make the stock. Example: Dissolve a bottle of 5 kU thrombin with 200 mM of $CaCl_2.2H_2O$, aliquot and store at -20 °C.

10. Representative Results

The overall goal of the technique presented here is to demonstrate the potential of simultaneous matrix-driven differentiation of ASC into multiple phenotypes using CSM as a delivery vehicle. We demonstrate an *in vitro* strategy to deliver stem cells from CSMs into a bilayered collagen-PEG-fibrin scaffold. Characterization of ASC embedded within this scaffold revealed that ASC-loaded CSMs can be "sandwiched" in between a layer of collagen and PEG-fibrin simultaneously and differentially take cue from both extracellular environments to thrive under their new conditions. We first characterized the ability for the model system to maintain cell viability and migratory capacities. Collagen supported the ability of ASCs to maintain their "stemness," as was demonstrated by their expression of Stro-1 and their fibroblast-like morphology (**Figure 2D and 2F**). In contrast, PEG-fibrin induced the ASCs to differentiate toward a vascular phenotype, as is demonstrated by their tube-like structure morphology, their endothelial cell-specific expression of von Willebrand factor (**Figure 2E and 2G**), and pericyte-specific expression of NG2 and platelet-

derived growth factor receptor beta (PDGFRβ) (data not shown). Furthermore, these observed phenotypes appeared to occur early in culture and were maintained over 11 days, as is demonstrated in **Figure 3**.

Tables and Figures

Benefits of Bilayer Construct:

- The scaffold alone without cells can perform as a bioactive scaffold.
- PEG-fibrin can induce stem cells to differentiate without the addition of growth factors.
- Collagen can assist in maintaining the stem cell phenotype of ASCs.
- A bilayer construct can be used as an active substrate for other cell types to migrate and proliferate (e.g., endothelial cells, fibroblast, keratinocytes, smooth muscle cells, pericytes).
- It can be used with hard-tissue engineering scaffolds like hydroxyaptite or demineralized bone to regenerate hard and soft tissue.
- It can be used to develop a multi-layered, diverse tissue-engineered construct (like dermal-vascular, vascular-epithelial, dermal-vascular-hypodermal, etc.).
- It uses a single-cell source to simultaneously develop multicellular compartments.
- It has the potential to integrate with the host tissue since it is of natural origin.
- CSM within the gel construct provides a platform for cells to migrate from.
- Chitosan, used to prepare CSM, is a well-known active chemo-attractant.
- The overall concept applied in this protocol of "matrix-driven stem cell differentiation" may be applied to other stem cells types. However, further investigation is warranted to determine the feasibility of matrix-driven differentiation. The bilayered gel scaffold can act as a reservoir to deliver the cells in a sustained and controlled manner.
- After reconstruction, the gels can still be separated into individual components.

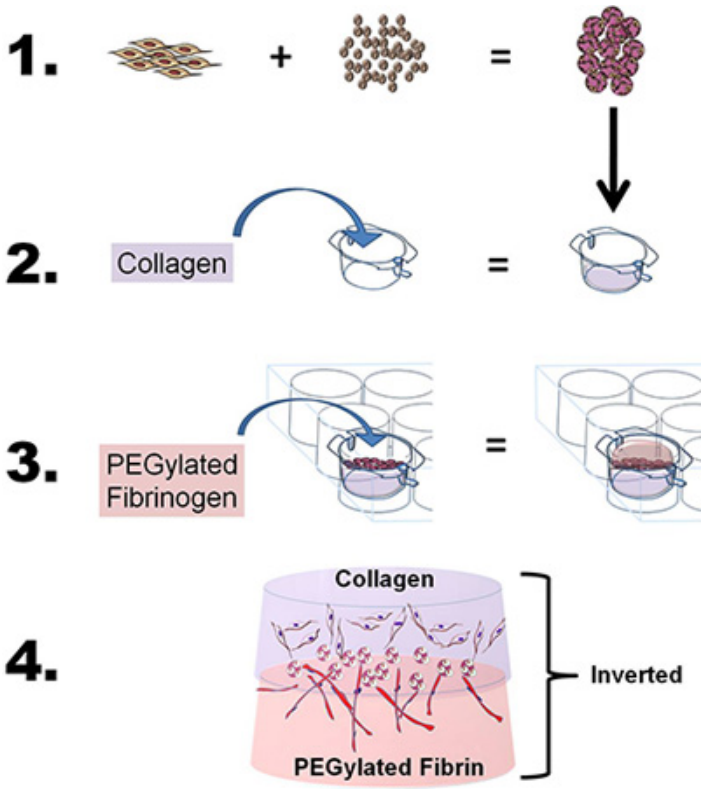

Figure 1. Schematic depicting the overall goal and process of the technique. 1) Adipose-derived stem cells (ASCs) are loaded onto chitosan microspheres. 2) Collagen is then poured into a 6-well insert, the pH adjusted to fibrillate the collagen, and the insert placed into a 6-well plate chamber. The ASC-loaded CSM spheres are then layered over the collagen. 3) The PEGylated fibrinogen is then poured over the collagen (ASC-CSM) and gelled by the addition of thrombin. 4) The final bilayer construct can then be removed from the culture insert and used for *in vitro* or *in vivo* analysis.

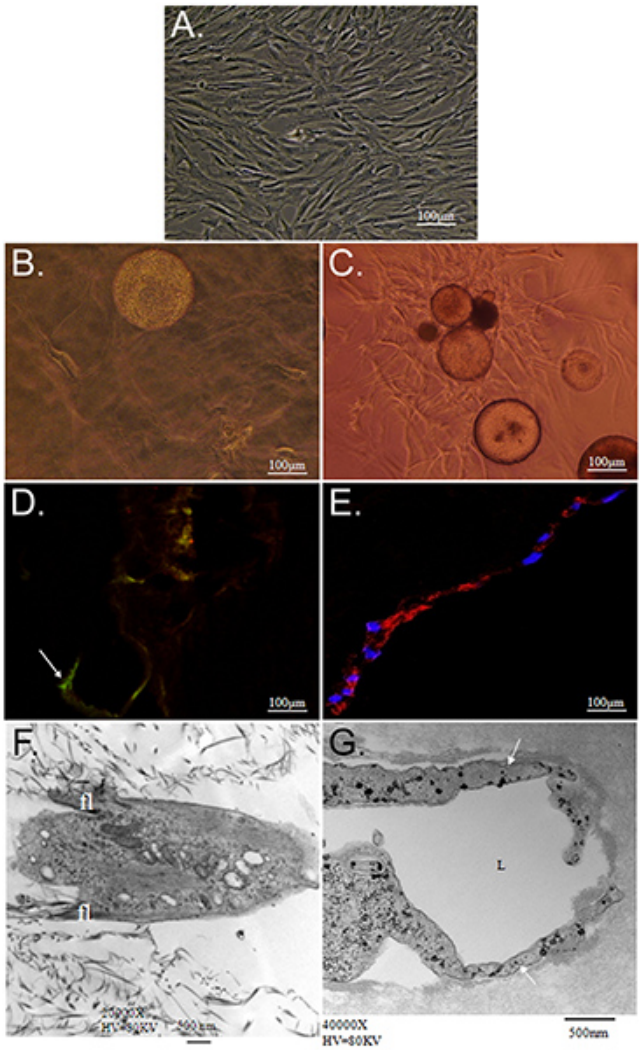

Figure 2. Characterization of ASC cultured within collagen and PEG-fibrin 3D matrices. A) Phase-contrast photomicrograph of isolated ASC passaged and maintained using routine 2-dimensional cell culture techniques. Photomicrographs B, D, and F depict ASC-CSM cultured within a 3-dimensional collagen gel; whereas C, E, and G show ASC-CSM cultured within a 3-dimensional PEG-fibrin gel, both at day 12. In B and C), ASCs are shown migrating away from the CSM sphere in both scaffold types. ASCs appear to have a flattened, spindle-like morphology in collagen (B), while maintaining their expression of the stem cell marker Stro-1 (D; arrow). When cultured in PEG-fibrin ASCs exhibit more tube-like structures and are induced to express such vascular cell markers as von Willebrand Factor (E). Transmission electron microscopy depicts the typical morphology demonstrated by ASCs within each scaffold. ASCs in collagen gel appear to have smaller filopodia (fl) extending from the body of the cell (F), whereas ASCs typically formed lumenal (labeled L) structures (G; arrow).

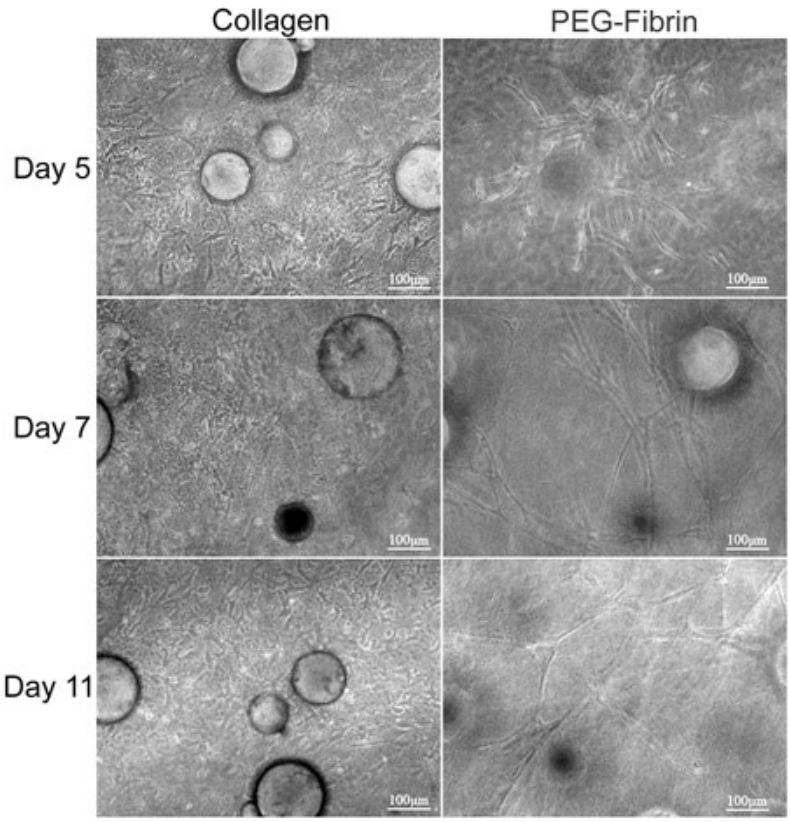

Figure 3. Morphological analysis of ASC-CSMs between bilayers of collagen and PEG-fibrin gels. ASC-CSMs were "sandwiched" between collagen and PEG-fibrin gels and maintained in culture for 11 days. The left column depicts ASCs migrating and proliferating within the collagen matrix and appear to take on a spindle-like morphology. The right column depicts ASCs migrating away from the CSMs and forming tube-like structures throughout the PEG-fibrin gel.

Discussion

ASCs are well-known for their ease of isolation and ability to differentiate toward various cell types. With the techniques described in this manuscript, we are able to exploit the plasticity of ASCs by exposing these cells to multiple biomatrices simultaneously. As cells migrate away from their CSM base and enter their surrounding extracellular environment, the cells take cue from the scaffold and can either maintain "stemness" (collagen) or be induced to differentiate toward vascular- and vascular-supportive cell types (fibrin). Since our lab is interested in skin and soft-tissue wound healing, we strategically implemented a bilayer of collagen and fibrin since collagen supports dermal and epidermal regeneration by the host, whereas fibrin naturally induces vascular network formation [9]. Furthermore, it is now understood that cross-talk occurs between proliferating skin keratinocytes, fibroblast, and the underlying vascular network; and this complex mechanism is highly critical for proper wound healing [10]. Without an underlying vascular network, tissue-engineered skin grafts have difficulty inosculating with host tissue. Therefore, our overall hypothesis is that collagen and fibrin, when used as a bilayer in combination with ASCs, will decrease wound healing times by improving one or more stages of blood vessel, dermal, and re-epithelialization processes.

Fibrin is a versatile biopolymer formed after thrombin-mediated cleavage of fibrinopeptide A derived from monomeric fibrinogen. Fibrin has been used clinically as a hemostatic agent (approved by the U.S. Food and Drug Administration) and as a sealant in a variety of clinical applications, including procedures such as soft-tissue dissection. Fibrin hydrogels from commercially purified allogeneic fibrinogen and thrombin have been used widely in the last decade in a variety of tissue-engineering applications. However, some major disadvantages in using a fibrin hydrogel can be 1) the potential for the scaffold to shrink, 2) low mechanical stiffness, and 3) rapid degradation before proper formation of the tissue-engineered structures. To overcome these problems, fibrin needs to be modified before use to serve as a better three-dimensional tissue-engineering scaffold. One such approach is copolymerizing the fibrin with polyethylene glycol (PEG-Fibrin). Our preliminary work has demonstrated several unique features of PEG-fibrin that make it advantageous in wound healing over other hydrogel dressings, including unmodified fibrin. PEGylated-fibrin exhibits unique features of both synthetic hydrogels and natural materials. Specifically, the presence of PEG provides a highly hydrated (>90% water) moist environment for managing exudates. Second, the presence of fibrin confers biodegradability to the material; however, prior results show that PEGylated fibrin is significantly more stable *in vitro* than unPEGylated fibrin[11]. Third, the inherent biologic activity of fibrin encourages healing by stimulating tissue and blood vessel in-growth.

The second component of our bilayer is collagen. Collagen is a natural biomaterial ubiquitously expressed in mammals and serves as a direct site of attachment for various types of cells. Different tissues express different types of collagen (type 1-type29), depending on the type of functional need of the tissue. Other inherent properties of collagen that make it highly attractive in our model are that 1) it is non-inflammatory, 2) both whole and degraded components of collagen are biocompatible, 3) it is a highly porous hydrogel, 4) it supports cell infiltration and migration, 5) it maintains multipotency of stem cells, 6) it is tunable by modulating formulation, and 7) it readily inosculates with host tissue. For our studies

in skin and soft-tissue regeneration, a bilayer composite consisting of collagen is a natural choice since it is highly expressed throughout the skin, including deep dermal regions, and can be used as an immunohistochemistry marker to assess the depth of a wound.

In this protocol, we demonstrate a technique to simultaneously maintain "stemness" of ASCs while differentiating ASCs toward various vascular cell types. For the study of skin and its underlying soft tissue, collagen and PEG-fibrin are directly applicable. However, other bioscaffold materials can be implemented to study their explored unique abilities to induce ASC into other cell types. The technique implemented here helps to highlight the importance of the extracellular matrix in controlling stem cell phenotypes and lends credence to the exploration of layered composites for skin regeneration.

Summary

Critical Steps

- Collagen should be adjusted to its correct isoelectric pH (pH6.8-7.1) to induce fibrillation and get a stable gel structure.
- PEG should be prepared freshly before mixing with fibrinogen solution since the shelf life of aqueous PEG is short (4-5 hours).
- Incubation time of fibrinogen-PEG mixture should not exceed 25 minutes (at 37 °C) since over-incubation may cause a turbid opaque precipitation during gelation.
- After adding thrombin to the PEGylated fibrinogen solution, the mixture should be dispensed to culture inserts immediately (after gentle mixing with a pipette). Holding the solution mixture in the pipette tip for a longer time (>30 sec) may cause gelation within the pipette tip and difficulty in obtaining evenly poured gels.
- Using uniformly sized microspheres and evenly distributed cells over the CSM bed is critical for optimal cell loading. CSM size variation in diameter directly influences surface area available for cell attachment per milligram of microspheres, whereas evenly distributed ASC-CSM directly influences cell migration into the scaffolds. The cell-loaded microspheres (ASC-CSM) should be distributed evenly on top of the collagen gel to ensure even migration of ASCs within the scaffold. If the distribution of ASC-CSMs is too dense, the cell migration will get concentrated to a particular area within the gels and may limit cell-cell interaction post-migration.
- Ensure that the volume of media added to the outer well of the cell culture insert is higher than the meniscus of the inner well. Typically, an inner:outer volume ratio of 1:2 is appropriate, as this facilitates cells attaching to the microspheres.

Limitations

- This method is limited to the total surface area available on the microspheres. If a higher cell number is desired, more microspheres are required to increase the surface area.
- Since the cells are intended to migrate from microspheres, a time-lag may be observed for the initial migration of cell onto and within the gels.
- The microspheres are in the interface of two gels (collagen and PEG-fibrin), and this may limit/take longer time for the migration of cells to distal ends of the gels.
- During construction of the bilayer, PEGylated fibrinogen-thrombin mixture has to be added quickly over a CSM-ASC bed on top of collagen. This may cause uneven re-distribution of ASC-CSMs. In case of uneven re-distribution of ASC-CSMs at the interface during PEG-fibrin gel casting, the gel construct may be slowly agitated manually to quickly redistribute the microspheres before complete gelation.
- This method is limited to cells that have migratory properties and may not be suited to deliver anchorage independent cell types.
- The collagen solution before fibrillation remains in acidic pH and limits the direct mixture of cells within collagen gels. As such, if not adjusted within a reasonable timeframe (~30 min), cell viability may be compromised.
- Since the gels used in this construct are individually layered, a possible disruption or separation of the gels may occur during handling process. Hence, it may limit ease in handling during *in vivo* studies.

Possible Modifications

- ASC-CSMs can be distributed evenly within the individual gels instead of layering them at the interface.
- ASCs can be directly mixed within individual gels rather than delivering by CSM, but this may limit development of highly organized cell-patterned tissue constructs.
- To avoid uneven re-distribution of ASC-CSMs during PEG-fibrin casting, the ASC-CSMs may be suspended in a small volume of aqueous chitosan solution (1% w/v) and distributed evenly over collagen gels. This will ensure firm attachment of ASC-CSMs over the collagen gel layer and will prevent dislodging of ASC-CSMs from the collagen surface.
- The construct by itself can be utilized in an inverted manner; i.e., PEG-fibrin gels may be cast first and ASC-CSMs can be seed on top of PEG-fibrin gels followed by collagen gels. Doing so allows the user to cast the fibrillated collagen solution over the (ASC-CSM)-PEG-fibrin layer more cautiously since, unlike PEG-fibrin gels, fibrillated collagen solution requires extra time (30 minutes) to solidify. By adopting this process one can ensure to maintain even distribution of ASC-CSM.
- The gels (collagen and/or PEG-fibrin gels) can be incorporated with mitogens, like growth factors (e.g., vascular endothelial growth factor, fibroblast growth factors), so as to induce faster cell migration and proliferation.

Troubleshooting

- During fibrillation of collagen solution, if the pH exceeds the isoelectric pH of >7.2, the pH should be readjusted by either adding more collagen stock solution, lowering the pH by using weak acid solution.
- When preparing the CSM, if a large variation in size occurs, then a number of parameters can be adjusted to yield desired CSM size (viscosity of chitosan stock solution, volume of surfactant, stirring speed, etc.).
- In case of slow cell migration when populating the gels, a higher number of ASC-CSMs can be used to boost the number of cells migrating out of the CSM into gels.
- The process of PEG-fibrinogen gelation can be retarded by cutting the volume of thrombin added to the mixture (typically 900 µL instead of 1 mL) or preparing a lower concentration stock of thrombin (20 U/mL in place of 25 U/mL). This allows slower gelation of PEG-fibrinogen mixture and hence gives extra time for cautious casting of PEGylated-fibrinogen and thrombin mixture over the ASC-CSMs.

- During incubation, if a phase separation occurs (between collagen and fibrin gel), a sterile Teflon 'O' ring can be placed on the top construct to ensure fastening of the gel phases.

Disclosures

No competing financial interests exist.

Disclaimers

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the U.S. Government. The authors are employees of the U.S. Government, and this work was prepared as part of their official duties. All work was supported by the U.S. Army Medical Research and Materiel Command. This study was conducted under a protocol reviewed and approved by the U.S. Army Medical Research and Materiel Command Institutional Review Board and in accordance with the approved protocol.

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