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Peptide Nanofiber Scaffolds for Multipotent Stromal Cell Culturing

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Abstract

Self-assembled peptide nanofibers are versatile materials providing suitable platforms for regenerative medicine applications. This chapter describes the use of peptide nanofibers as extracellular matrix mimetic scaffolds for two-dimensional (2D) and three-dimensional (3D) multipotent stromal cell culture systems and procedures for in vitro experiments using these scaffolds. Preparation of 2D and 3D peptide nanofiber scaffolds and cell culturing procedures are presented as part of in vitro experiments including cell adhesion, viability, and spreading analysis. Analysis of cellular differentiation on peptide nanofiber scaffolds is described through immunocytochemistry, qRT-PCR, and other biochemical experiments towards osteogenic and chondrogenic lineage.

Keywords: Peptide nanofibers, Self-assembly, Hydrogels, Multipotent stem cells, Nanofiber networks

1 Introduction

Multipotent stromal cells (MSCs) hold great potential for regenerative medicine applications due to their ease of isolation from bone marrow and other tissues such as adipose, periosteum, synovial membrane, and synovial fluid (1). These cells are more prone to generate particular tissue types such as cartilage, bone, and adipose, and have potential to escape from immune system (2). Due to these reasons, use of MSCs is highly preferable in regenerative medicine applications.

MSC fate decision can be fine-tuned by a number of signaling molecules residing in the microenvironment of these cells. The dynamic interactions of cell–cell, cell–extracellular matrix, and cell–soluble factors that take place in stem cell niche determine the differentiation patterns of stem cells. In regenerative medicine studies, artificial scaffolds are engineered by exploiting these interactions to mimic stem cell milieu. By this way engineered scaffold system on which MSCs reside provides control over cell fate. Therefore,

mechanical, chemical, and biological signals incorporated within artificial scaffold systems must be precisely controlled in a spatio-temporal fashion (3).

Self-assembled peptide nanofibers are versatile nanostructures that can trigger and control cellular behaviors such as adhesion, proliferation, migration, and differentiation through functional epitopes. These nanofibers are usually composed of peptide molecules that have bioactive groups and β-sheet-forming peptide segments in addition to hydrophobic groups. Hydrophobic part of the peptide molecules triggers self-assembly process through hydrophobic collapse in water. Hydrogen bonding between the β-sheet-forming peptide segments induces formation of well-defined and long cylindrical fibers instead of spherical micelles. Self-assembly through charge screening can also be triggered by addition of charged amino acids in peptide sequence followed by mixing with oppositely charged peptides or electrolytes (4). Peptide nanofibers are versatile structures so that many bioactive peptide epitopes can be presented on the design in high density and this characteristic provides a variety of functional properties that can be utilized in nanotechnology and regenerative medicine applications. This characteristic is especially important for mimicking extracellular matrix components, which are highly important in regenerative medicine applications. Figure 1 illustrates the nanofiber formation through self-assembly of

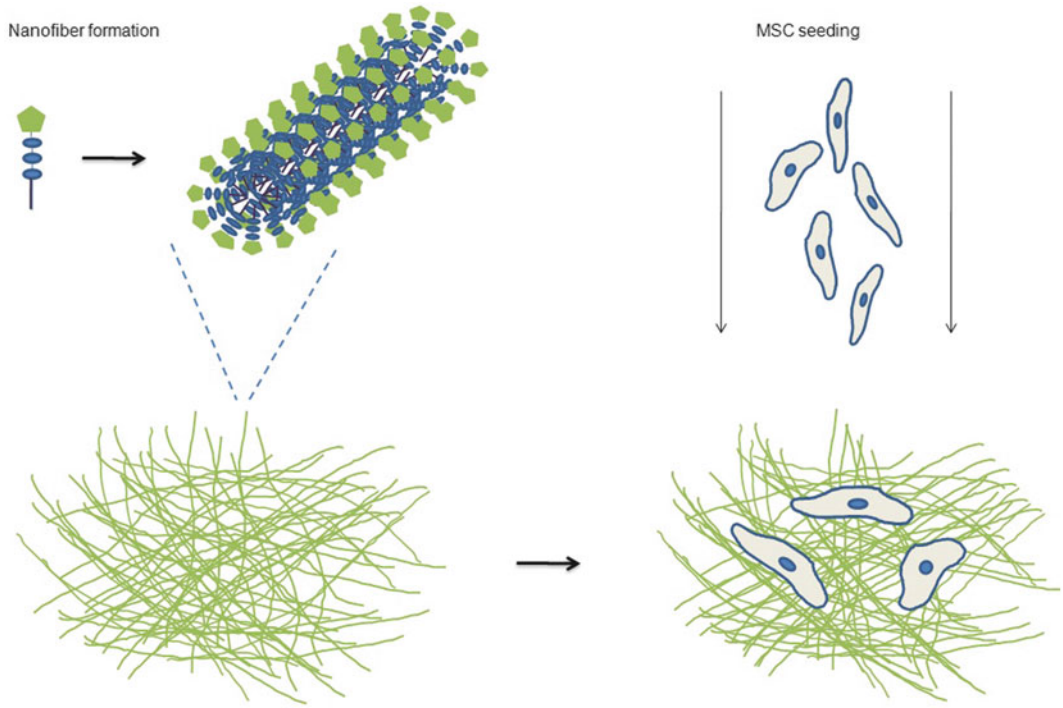

Fig. 1 Schematic representation of nanofiber formation through self-assembly and MSC seeding on the peptide nanofiber scaffolds

monomers and MSC seeding on the scaffolds. So far, researchers have used a number of bioactive epitopes on the peptide nanofiber systems to alter cellular behaviors by mimicking functional ligands found in the extracellular matrix that interact with cell surface components, such as growth factor receptors, integrins, and glycosaminoglycans. For example, cellular adhesion can be triggered through commonly used peptide ligands such as RGDS (5), REDV (6), or KRSR (7) and differentiation can be induced by IKVAV (8) epitope for neural cells and DGEA (9) for osteogenesis.

In this chapter, we describe protocols used to develop 2D and 3D self-assembled peptide nanofiber scaffolds and their use for MSC culture and differentiation in detail. Specifically, MSC culturing on 2D and 3D peptide nanofibers including analysis of cellular characteristics such as adhesion, spreading, viability, and differentiation by using immunocytochemistry, qRT-PCR, and osteogenic or chondrogenic specific biochemical assays is discussed (see Note 1).

2 Materials

Materials used in peptide synthesis, purification, and characterization were previously described (6–8).

2.1 Cell Culture

1. Pipettes and tissue culture plates (Corning).
2. 1× PBS.
3. Expansion medium: Dulbecco's Modified Eagle's Medium-Glutamax, Low (1.5 g/L) Glucose (Invitrogen) supplemented with 10% MSC qualified FBS and 1% penicillin–streptomycin (Invitrogen).
4. 0.25% Trypsin–EDTA (Invitrogen).
5. Osteogenic differentiation medium: Dulbecco's Modified Eagle's Medium-Glutamax, Low (1.5 g/L) Glucose (Invitrogen) supplemented with 10% MSC qualified FBS and 1% penicillin–streptomycin (Invitrogen), 10 mM β-glycerophosphate (Alfa Aesar), 50 μg/mL L-ascorbic acid (Alfa Aesar), and 10 nM dexamethasone (Sigma).
6. Chondrogenic differentiation medium: Dulbecco's Modified Eagle's Medium, High (4.5 g/L) Glucose (Invitrogen) supplemented with 10 ng/mL TGF-β1 (Invitrogen), 1×10^{-7} M dexamethasone (Sigma), 100 μM L-ascorbic acid (Alfa Aesar), 1% sodium pyruvate (Invitrogen), 0.5 mg/mL BSA, 10 μg/mL insulin, 6 μg/mL transferrin, and 3×10^{-8} M sodium selenite.

2.2 Adhesion, Spreading, Viability, and Proliferation

1. Adhesion medium: Dulbecco's Eagle modified medium with Low (1.5 g/L) Glucose supplemented with 4 mg/mL BSA (Invitrogen), 50 μg/mL cyclohexamide (AppliChem), and 1% penicillin–streptomycin.
2. FITC-conjugated phalloidin (Sigma).
3. 2% glutaraldehyde (Sigma) and 4% osmium tetroxide (Sigma).
4. Increasing concentrations of EtOH.
5. Calcein AM (Invitrogen) diluted to 1 μM in PBS.

2.3 ALP Activity

1. M-PER Protein Extraction Solution (Thermo)
2. Protease inhibitor cocktail
3. BCA Protein Assay Kit (Thermo)
4. NaOH (0.25 M)
5. *p*-nitrophenol (Sigma)
6. *p*-nitrophenylphosphate substrate solution (Sigma)

2.4 Alizarin Red Staining

1. Alizarin Red-S solution: Prepare 40 mM Alizarin Red-S solution by dissolving appropriate amount of Alizarin Red-S (Sigma) in ddH_2O and adjust pH to 4.2 by titrating with 1 M NH_4OH.
2. Ethanol (70%).
3. Cetylpyridinium chloride (CPC) solution: Prepare 10% (w/v) CPC solution by dissolving appropriate amount of CPC (Merck) in Na_3PO_4 buffer (10 mM, pH 7).

2.5 Papain Digestion

1. PBE buffer: First prepare 100 mL 500 mM Na_2EDTA by adding 18.6 g Na_2EDTA to 80 mL ddH_2O. Adjust pH to 8. Then prepare 100 mM sodium phosphate buffer by dissolving 6.53 g Na_2HPO_4 and 6.48 g NaH_2PO_4 in 900 mL and add 10 mL of 500 mM Na_2EDTA. Adjust pH to 6.5, and then complete volume to 1 L. Sterilize with 0.22 μm filter.
2. Papain digestion buffer: Prepare first 10 mM L-cysteine solution by dissolving 47.25 mg L-cysteine hyrdrochloride in 30 mL PBE buffer. Then transfer 20 mL of PBE–cysteine solution to a sterile tube and add papain (Sigma) to get 125 μg/mL concentration of papain solution (see Note 2).

2.6 Dimethylmethylene Blue Assay

1. 1 L DMB solution: Prepare 40 mM Glycine (Sigma), 40 mM NaCl by dissolving 2.37 g NaCl and 3.04 g glycine in 900 mL ddH_2O. Then dissolve 16 mg dimethylmethylene blue (DMMB) (Sigma) in 5 mL absolute EtOH for 16 h with a magnetic stir bar. Add dissolved DMMB to NaCl–glycine solution. Then adjust pH to 3. Complete to 1 L with ddH_2O. Then sterilize with 0.22 μm filter (see Note 3).
2. Papain digests.
3. Chondroitin sulfate salt standard solutions.

2.7 RNA Isolation and qRT-PCR

RNA Isolation:

1. Trizol Reagent (Invitrogen)
2. DNAse/RNase free water (Gibco)
3. DNAse/RNase free Eppendorf tubes
4. Temperature-controlled centrifuge
5. RNase-free solvents: Chloroform, isopropanol, and increasing concentrations of EtOH

qRT-PCR:

1. SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen)
2. PCR thermal cycler (Bio-Rad)
3. Quick spin
4. Vortex
5. RNA samples

2.8 Immunocytochemistry

1. 1× PBS
2. Fixative; 4% paraformaldehyde/PBS
3. Blocking solution: 1% BSA–PBS
4. 0.1% Triton X-100
5. Prolong Gold antifade reagent (Invitrogen)

2.9 Fixation, Dehydration, Embedding, and Sectioning of 3D Cultures

1. Fixative; 4% paraformaldehyde/PBS
2. Coverslips
3. Glass slides
4. Xylene (Sigma)
5. Graded EtOH: 70, 80, 95, 100%
6. Paraffin
7. Embedding cassettes
8. Embedding molds
9. Microtome (Leica SM 2010 R)
10. Hybridization oven

3 Methods

3.1 Peptide Synthesis, Purification, and Characterization

Peptide synthesis is performed by using a standard Fmoc-protected solid-phase peptide synthesis method. Purification and characterization steps are carried out as described previously (6–8). Alternative peptide synthesis protocols can be accessed from here (10).

1. After collection of peptide from high-performance liquid chromatography (HPLC) purification, remove the organic solvent

by using rotary evaporator. Then, freeze the peptide samples at 80°C for 4–5 h and lyophilize to obtain peptide powder.

2. Dissolve lyophilized peptide samples in ultrapure water. In order to completely dissolve peptide samples, add appropriate amount of HCl or NaOH according to the p*K*a values of peptides. Sonication might be required to prevent the small peptide aggregate formations in the solution by using ultrasonicator.
3. Carry out circular dichroism (CD) experiment to reveal the secondary structure of peptides. Dissolve peptides in ultrapure water at 10^{-5} M concentration separately. (Peptide concentrations can be prepared in the range of 1×10^{-6} and 5×10^{-4}). The details of the measurement procedure can be found here (6–8, 11). Analysis of the secondary structure of peptide nanofibers is important for determining the effects of structural properties of the nanofiber system on the cells.
4. In order to analyze mechanical characteristics of peptide nanofiber hydrogel system, perform rheology experiments by using a rheometer. According to the plate diameter and gap size, mix peptides in a way that the gap is full with peptide mixture and wait for gelation for 10 min. Then, perform the analysis by using various measurements depending on the purpose, such as frequency sweep or time-dependent measurements. Gel formation is described with the equation $G' > G''$. Further details can be found here (6–8, 11).
5. Characterize self-assembled peptide nanofibers and their network formation by using various microscopy techniques such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM) as described here (12).
6. After characterizing the peptide molecules and self-assembled peptide nanofibers, cell culture experiments on peptide nanofibers are performed.

3.2 Cell Culture

3.2.1 Cell Culture on 2D Scaffold

Self-assembly of two oppositely charged peptide molecules at neutral pH has been previously defined (13). Peptide gels have the ability to mimic the native extracellular matrix due to their nanofibrous structure and hydrogel characteristics. In order to construct 2D scaffolds for MSC culturing applications, negatively charged peptides and positively charged peptides are used to form gels on tissue culture plates. Figure 2 illustrates gel formation and sterilization steps before cell seeding.

1. Dissolve the peptides in water to obtain 0.1 mM peptide solution and adjust pH to 7.4 using 1 mM HCl or NaOH, depending on the sequence and the p*K*a of peptide (see Note 4).
2. Sonicate peptide solutions in ultrasound bath for 10–30 min, depending on the volume of your peptide solution.

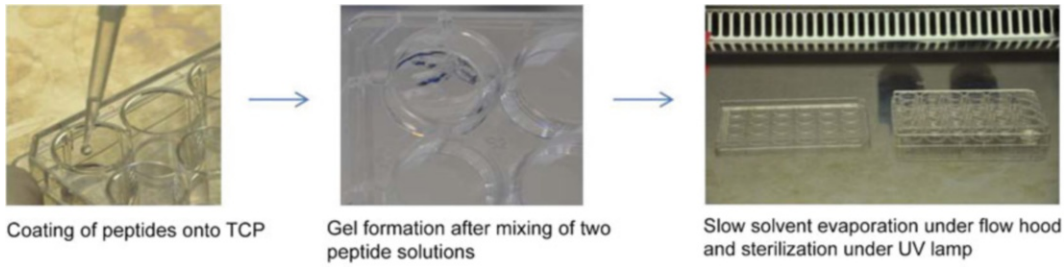

Fig. 2 Steps of 2D scaffold preparation before cell culture. *Left*: Peptide solutions are added one by one. *Middle*: After mixing gel formation immediately occurs. *Right*: Peptide gels are kept in fume hood for drying overnight and sterilized under UV afterwards

3. Add negatively and positively charged peptide solutions at a final concentration of 200 μL/cm^2. After adding the first peptide solution, drip second peptide solution onto the first one and stir for mixing.
4. Incubate at 37°C for 30 min.
5. To get 2D coating, leave gels under laminar flow hood overnight in order to evaporate the solvent.
6. Sterilize coated plates under UV lamp for 30 min to 1 h. After sterilization, keep coated plates sterile.
7. Prepare MSC suspension in expansion medium.
8. Seed 500 μL of cell suspensions onto each coating. In order to distribute cells homogeneously gently tap well plate. Place the plates in a 37°C humidified incubator with 5% CO_2. Change media every 3–4 days until the date of analysis.

3.2.2 Cell Culture on 3D Scaffold

1. Dissolve peptides in HEPES buffer to obtain 10 mM peptide solution (see Note 5).
2. Sonicate peptide solutions in ultrasound bath for 10–30 min, depending on the volume of your peptide solution.
3. Sterilize peptide solutions under UV lamp for 30 min to 1 h. After sterilization keep peptide solutions sterile.
4. Prepare MSC suspension at 2 × 10^4 cells/μL concentration in differentiation medium.
5. Place coverslips into each well of 24-well plate.
6. Add the first peptide solution onto coverslip.
7. Add 10–20 μL of cell solution onto first peptide solution by mixing slightly. Be careful not to disturb the convex shape of peptide solution drop while adding cell suspension.
8. Then add the second peptide solution onto the mixture of cell and first peptide solution. Be careful not to disturb the convex shape of the cell–first peptide mixture while adding the second peptide solution. This method is called the sandwich method

and provides a powerful tool, especially if you would like to analyze the migration or spreading patterns of cells.
Alternatively, you can first mix the first peptide solution with cell suspension in an Eppendorf tube prior to placing the mixture in wells. Then place the mixture onto coverslip and add the second peptide solution onto that. Be careful not to disturb the convex shape of mixture. This method results in a more homogenous cell–scaffold mixture.

9. Incubate gel at 37°C for 30 min.
10. Add 750 μL of culture medium onto gel carefully. Change culture medium every other day.
11. The cells should be cultured for at least 3 weeks.

3.2.3 Fixation, Dehydration, Embedding, and Sectioning of 3D Cultures

1. At determined time points remove the medium from the 3D cultures and wash with 1× PBS, taking care not to disturb constructs.
2. Fix 3D constructs in 4% paraformaldehyde for 24–36 h in the same culture plates.
3. Then first aspirate out paraformaldehyde, dehydrate samples in graded EtOH solutions, and clear them in xylene as follows:
   (a) 70% EtOH—two changes—1 h each
   (b) 80% EtOH—two changes—1 h each
   (c) 95% EtOH—two changes—1 h each
   (d) 100% EtOH—two changes—1 h each
   (e) Xylene—two changes—1 h each
4. Place embedding cassettes into molds and label them.
5. Place 3D constructs onto embedding cassettes and pour warm paraffin gently. Use forceps to hold the glass coverslips that 3D constructs are placed on while transferring them (see Note 6).
6. Cool paraffin blocks at RT for 30 min to 1 h, and then trim and place blocks to microtome.
7. Cut 5 μm sections of paraffin-embedded constructs using microtome. Transfer paraffin ribbon to water bath at 40°C for 2–3 s. Take sections on glass slides.
8. Leave slides at RT to dry and then bake them in 45–50°C oven overnight.
9. Deparaffinize and rehydrate sections in graded EtOH solutions as follows:
   (a) Xylene—2 changes—10 min each
   (b) 100% EtOH—two changes—3 min each
   (c) 95% EtOH—1 min
   (d) 80% EtOH—1 min
10. Then rinse in distilled water.

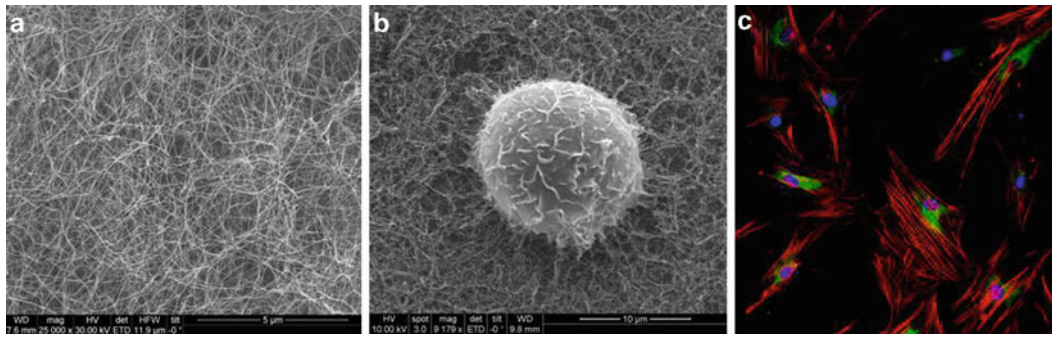

Fig. 3 ECM-mimetic peptide nanonetwork provides mechanical support to the MSCs in addition to bioactive cues presented by the nanofibers. (**a**) Peptide nanofiber scaffold imaged by SEM. (**b**) After 2-h seeding of cells, cells start to adhere on scaffold and extend their protrusions. (**c**) After 24 h, cells spread and gain their native morphology (*Red* : actin filaments; *green*: CD44, a stem cell marker; *blue* : cell nuclei)

3.3 Biological Characterizations

3.3.1 Cell Adhesion Tests

1. Prior to the experiment, replace the medium of stem cells in the flask with serum-free DMEM supplemented with 4 mg/mL BSA and 50 μg/mL cyclohexamide for 1 h at standard cell culture conditions (37°C humidified chamber with 5% CO_2).
2. After trypsinization and resuspension of cells in serum-free medium, count the cell number with hemocytometer.
3. Seed cells on peptide nanofiber-coated surfaces at 5×10^4 cells/cm^2 density.
4. After 1-h incubation remove medium containing unbound cells and wash the wells twice with PBS.
5. Add 200 μL of 2 μM calcein AM in PBS per well and incubate cells for 30 min at standard culture conditions.
6. After incubation, take images of stained cells from at least five random points per well by using fluorescence microscope.
7. Count adhered cells using Image J and normalize data to polystyrene tissue culture plate surface.

3.3.2 Spreading

One of the early cellular responses upon seeding on a material is cell spreading. In order to monitor spreading characteristics of MSCs after seeding on scaffold systems, cells are stained with TRITC-conjugated phalloidin dye that binds to F-actin stoichiometrically. Moreover, spreading characteristics of cells are also analyzed via SEM imaging. Figure 3 illustrates the adhesion and spreading of cells that are cultured on peptide nanofiber scaffolds.

1. For phalloidin staining, remove medium from cultures 3 and 48 h after seeding cells, and wash wells with 1× PBS.
2. Fix cells in 4% paraformaldehyde/PBS for 10 min at room temperature, and then wash extensively with 1× PBS.

3. In order to permeabilize cells, treat cells with 0.1% Triton X-100 in PBS, and wash again with PBS.
4. Stain cells with 50 μg/mL phalloidin solution/PBS for 20 min and wash with PBS extensively.
5. Mount cover glasses with Prolong Gold Antifade Reagent (Invitrogen), seal with nail polish, and then analyze.
6. For SEM imaging, after 3 and 48 h of incubation, remove the medium from cultures and wash cells with 1× PBS.
7. Fix attached cells with 2% glutaraldehyde/PBS solution for 2 h and wash with 1× PBS briefly.
8. Then treat fixed cells with 4% osmium tetroxide for 30 min and wash briefly with 1× PBS.
9. Dehydrate cells in graded EtOH solutions starting with 20% and continuing up to absolute EtOH for 10 min at each step.
10. Dry samples using critical point dryer.
11. Coat samples with 6 nm Au/Pd using a sputter coater.
12. Analyze samples under scanning electron microscope and take images by using an ETD detector at high vacuum mode at 10 keV beam energy.

3.3.3 Viability

Viability of cells on scaffolds is determined using calcein AM staining. Calcein is a membrane-permeable dye. When it enters the cells, intracellular esterases cleave the acetoxymethyl (AM) ester group resulting in the membrane-impermeable calcein fluorescent dye. Dead cells cannot retain calcein dye due to their impaired membrane integrity.

1. On days 1, 2, and 3, remove medium from the cultures and wash wells with PBS in order to remove dead cells.
2. Add 200 μL of 2 μM calcein AM per well and incubate cells for 30 min at standard culture conditions.
3. After incubation, take images of viable cells from at least five random points per well by using fluorescence microscope.
4. Count viable cells using Image J and normalize data to polystyrene tissue culture plate surface.

3.4 Differentiation Studies

3.4.1 Osteogenic Differentiation

Seed MSCs in growth medium (DMEM-Glutamax/10% FBS/1% penicillin) to the peptide nanofiber-coated wells at 10^4 cells/cm^2 ratio. After reaching confluency (1–2 days), exchange growth medium with osteogenic medium as described in Section 2.

ALP Activity

Alkaline phosphatase (ALP) is an enzyme that hydrolyzes inorganic pyrophosphate to provide inorganic phosphate source and its activity is used as an early marker of osteogenic differentiation.

1. At predetermined time intervals (3, 7, 10, 14, 21, and 28 days) remove the osteogenic medium from wells.
2. Wash with 1× PBS once.
3. Add 100 μL of protein extraction solution to each well and incubate on shaker for 30 min at room temperature.
4. Collect the protein from each well and transfer them into chilled 1.5 mL sterile Eppendorf tubes. Pipetting and scratching the surfaces could be beneficial to gather proteins efficiently.
5. Centrifuge the tubes at 14,000 × *g* for 10 min at 4°C.
6. Transfer the supernatants (including proteins) from each tube into clean chilled tubes. Take care not to take pellet, which is composed of cellular debris.
7. Perform protein assay in 96-well plates in order to measure protein concentration of each sample by using protein assay kit.
8. Prepare *p*-nitrophenol standards by serial dilution with 0.25 M NaOH. Use 0.25 M NaOH alone for blank.
9. Add 50 μL of protein solution for each sample to the wells of 96-well plate. Bring the total volume up to 200 μL by adding 150 μL *p*-nitrophenylphosphate substrate solution.
10. After incubating for 30 min on shaker at RT, measure absorbance at 405 nm by using microplate reader.

Alizarin Red-S Staining

Alizarin Red-S staining is used to determine calcium deposition and mineralization of cells through binding of Alizarin Red-S to calcium ions.

1. At predetermined time points (7, 14, 21, and 28 days) discard osteogenic medium from the wells.
2. Wash the cells with 1× PBS once.
3. Add 200 μL of ice-cold ethanol (70%) to each well and incubate them for 1 h at room temperature.
4. Wash with ddH_2O twice (5–10 min).
5. Add 200 μL of Alizarin Red-S solution on top of the cells and incubate them on shaker for 30 min at room temperature.
6. Wash with ddH_2O four to five times to get rid of nonspecific Alizarin Red-S binding.
7. Add enough PBS to cover wells and use optical microscope for imaging.
8. Discard PBS and add 200 μL CPC solution to extract Alizarin Red-S/Ca^{2+} complex from the surface by incubating on shaker for 30 min.
9. Measure absorbance at 562 nm by using microplate reader.

RNA Isolation and qRT-PCR

All tubes and tips should be RNase free and RNA isolation is performed under Class I airflow hood.

RNA Isolation

1. At least 1.5×10^5 cells are required for sufficient RNA isolation per experimental group.
2. Remove the medium from the wells and wash briefly with PBS.
3. Add 500 μL Trizol reagent on cells, mix extensively, and then transfer cell lysates into 2 mL RNase-free Eppendorf tubes. At this step, cell lysates could be stored in a −80°C freezer.
4. Add 300 μL of chloroform onto cell lysates, shake tubes vigorously to dissolve lipids, and leave at RT for 2–3 min.
5. Centrifuge samples at 15,000 rpm for 15 min at 4°C. Transfer 600–700 μL of upper clear phase into a clean tube. Be careful not to disturb bottom layer; otherwise RNA will be contaminated by DNA and phenol extracts.
6. Add equal volume of isopropanol to the clear solution and mix. Incubate for 10 min at RT. In order to increase RNA yield you can incubate samples at −20°C.
7. Centrifuge samples in order to precipitate RNA at 15,000 rpm for 12 min at 4°C.
8. After centrifugation discard supernatant. Add 1 mL of 70% EtOH. Be careful not to dissolve the pellet; just detach it from the tube wall.
9. Centrifuge samples at 8,000 rpm for 8 min at 4°C.
10. Discard all supernatant and air-dry the pellet until there is no EtOH left. Add 30 μL DNase/RNase-free water and dissolve the pellet.
11. Assess yield and purity of RNA using NanoDrop.

qRT-PCR

Osteogenic and chondrogenic differentiation are indexed by analysis of bone- or cartilage-specific gene expression, respectively. For gene expression analysis, comparative Ct method with primer efficiency correction is used.

1. The cDNA synthesis from RNA and qRT-PCR reactions are performed using SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit according to manufacturer's instructions.
2. Reaction conditions are as follows: a cDNA synthesis step at 55°C for 5 min and 95°C for 5 min and 40 cycles of 95°C for 15 s, 60°C for 30 s, and 40°C for 1 min, followed by a melting curve to confirm product specificity.
3. Before assessment of target genes, determine reaction efficiencies of each primer set. Prepare fivefold serial dilution of total RNA and generate a standard curve with slope that defines primer efficiency.

4. The efficiency of the reaction can be calculated by the following equation: Eff $= 10^{(-1/\text{slope})} - 1$. The efficiency of the PCR should be 90–110%.
5. After determining primer efficiencies, perform gene expression analysis using different primers corresponding to different target genes.
6. Normalize resulting gene expression data to the expression level of a housekeeping gene.

Immunofluorescence

1. At predetermined time intervals (1, 14, and 28 days) discard the differentiation medium from the wells.
2. In the fume hood, fix the cells with 4% paraformaldehyde in PBS for 15 min.
3. Rinse three times with PBS.
4. Permeabilize cells with 0.1% Triton X-100 in PBS for 10–12 min.
5. Rinse three times with PBS.
6. Block the cells with 1% BSA solution (0.05% Tween) for 2 h at room temperature.
7. Rinse three times with PBS.
8. Dilute primary antibodies in an appropriate concentration in blocking solution (see Note 7).
9. Add primary antibody in blocking solution at 150 μL/well concentration. Incubate for 2 h at room temperature or overnight at 4°C.
10. Rinse three times with PBS.
11. Add secondary antibody in blocking solution at 150 μL/well concentration. Incubate for 1 h at room temperature. Keep the samples away from light.
12. Rinse three times with PBS.
13. To stain nucleus, add To-PRO in PBS (1:1,000 dilution) at 150 μL/well concentration and incubate for 15 min.
14. Rinse three times with PBS.
15. Mount on slides by using mounting media.

3.4.2 Chondrogenic Differentiation

In the presence of TGFβ1 and small molecules such as insulin, dexamethasone MSCs undergo chondrogenic differentiation gaining chondrogenic morphology and producing cartilage-specific extracellular matrix molecules.

For this purpose, seed MSCs in growth medium (DMEM-Glutamax/10% FBS/1% penicillin) to the peptide nanofiber-coated wells at 5×10^4 cells/cm^2 density. After 24 h of incubation,

exchange the growth medium with chondrogenic medium as described in Section 2.

Safranin-O Staining

Safranin-O staining is used to show spatial organization of sulfated glycosaminoglycans. It is a cationic dye that binds to tissue glycosaminoglycans. The intensity of staining is proportional to the amount of glycosaminoglycans.

1. Remove the medium and wash cells with pre-warmed PBS.
2. Fix the cells with 4% PFA for 15 min followed by washing three times with PBS at room temperature.
3. In order to eliminate nonspecific binding, treat cells on scaffolds with 2% BSA/PBS for 30 min.
4. Treat cells with 0.1% (w/v) Safranin-O in 0.1% (v/v) in acetic acid for 5 min at room temperature.
5. Wash cells with 0.1% acetic acid extensively.

Glycosaminoglycan Assay

DMMB assay is one of the accepted protocols for rapid quantification of sulfated glycosaminoglycans in tissue.

1. Remove the medium and wash cells with pre-warmed PBS.
2. Add 500 μL papain solution on cells and wait for 5 min at RT.
3. Then collect cells after extensive pipetting into 1.5 mL Eppendorf tubes. Seal caps of tubes with parafilm.
4. Place the tubes on a hot plate at 65°C for 16–18 h. The digests are used for both sulfated glycosaminoglycan quantitation by DMMB assay and DNA quantitation. Total DNA content will be used to normalize sulfated glycosaminoglycan content.
5. Quick spin Eppendorf tubes after 16–18 h. You can store papain digests in a −20°C freezer. In order to increase the total DNA extracted from tissue, apply repeated freeze–thaw cycle.
6. Determine total DNA per experimental group by using Qubit dsDNA-HS quantitation kit according to manufacturer's instructions.
7. For DMMB assay, prepare serial dilutions of chondroitin sulfate standards in papain to generate a standard curve (Table 1).
8. Then aliquot 50 μL of sample, controls, and standards in a clear bottom 96-well plate.
9. Add 100 μL of DMMB dye solution on each well using a multichannel pipette.
10. Read optical density of samples at 590 and 530 nm values. Subtract the absorbance values of cell-free control groups from the absorbance values of experimental groups.

Table 1
Volumes for chondroitin sulfate standard curve

Concentration of CS (μg mL^{-1})	Volume of 1 mg mL^{-1} CS (μL)	Volume of papain (μL)
0	0	1,000
5	5	995
10	10	990
15	15	985
20	20	980
25	25	975
30	30	970
35	35	965

4 Notes

1. In our laboratory, we utilize MSCs from different origins like human, rat, and mouse. We have observed that using stem cells between passage 4 and 10 gives the most reliable and reproducible results.
2. Papain enzyme and cysteine are highly unstable. For this reason prepare Cys/PBE buffer and papain solution fresh and sterile.
3. DMMB dye solution is stable for 3 months. Check optical density and discard if it decreases appreciably.
4. Mechanical properties and ligand density of peptide gels can be tuned by their concentration. For this reason, molarity and volume of peptide solutions can be changed depending on the purpose of the study.
5. Alternatively, PBS and HEPES buffer can also be used to dissolve peptides. Depending on the sequence of each peptide and p*K*a, adjust pH to 7.4 by using 1 M NaOH or 1 M HCl.
6. Because xylene dissolves plastics, transfer 3D constructs from TCP to glass dishes before xylene changes.
7. Carry out the immunocytochemistry protocol without adding primary antibody to detect nonspecific binding of secondary antibodies.

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