Bone Marrow Mesenchymal Stem Cells Adhesion Assay

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[Abstract] Mesenchymal stem cells (MSCs) are widespread in adult organisms and involved in tissue maintenance and repair as well as in the regulation of hematopoiesis and immunologic responses. As cell adhesion play important roles in cell interactions and signaling, to thoroughly evaluate the adhesion ability of MSCs is of vital importance to clarify the mechanism of self-renewal, proliferation, activation and migration of MSCs in different microenvironments. Based on the method by Siler *et al*., 2000, we revised the protocol in order to provide details on how to evaluate the adhesion ability of MSCs from bone marrow (BMSCs) on extracellular matrix (ECM) protein laminins. The current protocol can also be easily translated to MSCs with other treatments or ECMs such as collagens, fibronectin, *etc.*

Materials and Reagents

1. 96 well culture plate (Sigma-Aldrich, Corning® Costar®, catalog number: CLS3595)
2. Human bone marrow derived MSCs, isolated and cultured as previously described (Kern *et al*., 2006)
   Note: Cells in the third cell passage were used.
3. Laminin 511&521 (Biolamina, catalog number: LN511; LN521)
4. DMEM (Thermo Fisher Scientific, Invitrogen™, catalog number: 11965092)
5. Fetal bovine serum (FBS) (Thermo Fisher Scientific, Invitrogen™, catalog number: 10099141)
6. Trypsin (Thermo Fisher Scientific, Invitrogen™, catalog number: 25200-056)
7. Soybean trypsin inhibitor (Thermo Fisher Scientific, Invitrogen™, catalog number: 17075-029)
8. BSA (Sigma-Aldrich, catalog number: V900933)
9. Blocking buffer (0.5% BSA in PBS without Ca^{2+} and Mg^{2+})
10. Wash buffer (0.1% BSA in PBS without Ca^{2+} and Mg^{2+})
11. 2% SDS (Sigma-Aldrich, catalog number: 71729)
12. 4% paraformaldehyde (Sigma-Aldrich, catalog number: 158127)
13. NaCl (Sigma-Aldrich, catalog number: S7653)
14. KCl (Sigma-Aldrich, catalog number: 746436)
15. Na_2HPO_4 (Sigma-Aldrich, catalog number: 795410)
16. KH_2PO_4 (Sigma-Aldrich, catalog number: P0662)
17. $CaCl_2{\cdot}2H_2O$ (Sigma-Aldrich, catalog number: 223506)

18. $MgCl_2 \cdot 6H_2O$ (Sigma-Aldrich, catalog number: M9272)
19. HCl (Sinopharm chemical reagent Being Co.ltd, catalog number: 10011008)
20. Crystal violet powder (Sigma-Aldrich, catalog number: C6158)
21. Ethanol (Sinopharm chemical reagent Being Co.ltd, catalog number: 10009159)
22. PBS with/without Ca^{2+} and Mg^{2+} (see Recipes)
    Note: Commercial DPBS with/without calcium and magnesium can also be used.
23. 0.1% crystal violet staining solution (see Recipes)

Equipment

1. Small shaker for microtiter plate (IKA, model: MS3 Digital)
2. Scanner (UMAX, model: POWERLOOK 2100XL)
3. Series II 3110 Water-Jacketed CO_2 chamber (Thermo Fisher Scientific, Forma™, catalog number: 3111)
4. Microscope (Nikon, model: ECLIPSE TE2000-S)
5. Microplate reader (ThermoFisher Scientific, model: Multiscan MK3) or spectrometer with 550 nm wavelength available.

Procedure

1. Coat plate with laminins
   a. Slowly thaw recombinant laminins at 2 °C to 8 °C before use.
   b. Dilute the thawed laminin stock solution with 1x DPBS (Ca^{2+}/Mg^{2+}) to 10 μg/ml, Add 60 μl diluted laminin solution to each well of 96 well culture plate (The final coating concentration is 2 μg/cm^2). Make sure the laminin solution is spread evenly across the surface. Leave some wells uncoated as negative control.
   c. Incubate at 2 °C to 8 °C overnight.
   d. Wash with wash buffer (200 μl/well) for 2 times.
   e. Block plates with blocking buffer (200 μl/well) at 37 °C in CO_2 chamber for 60 min.
   f. Wash with wash buffer (200 μl/well) for 2 times.

   Note: All the wash procedures do not need some time incubation, the followings are the same.
2. Prepare and seed cells
   a. Taking a 10 cm culture dish as an example, wash the cells with 2 ml PBS.
   b. Detach the cells with 2 ml 0.25% Trypsin-EDTA, neutralize trypsin with 2 ml 0.25 mg/ml soybean trypsin Inhibitor.
   c. Mix the cells by pipetting up and down using a 1 ml pipette, then transfer them to a 15 ml sterile tube and centrifuge at 300 *x g* for 5 min.
   d. Resuspend the cells in 2 ml DMEM with 0.5% FBS.

e. Count the cells and dilute to a final concentration of 2 x 10^5/ml, add 100 µl (2 x 10^4 cells) to each well. Set up at least triplicate wells for each condition.
Note: Cell number varies depending on the coated ECM, for laminins, 2 x 10^4 is appropriate. However, 4 x 10^5 cells may be required for non-coated wells.

f. Incubate at 37 °C (in a CO_2 chamber) for 20 min.
Note: Adhesion time depends on the coated ECM. It is useful to observe the adherent condition under microscope at different time points, choosing between 5 min and 30 min (5, 10, 15, 20, 30), even do a time course first. In our system, cells adhere to the laminin-coated wells within at least 10 min, while for non-coated wells, it may take longer time.

3. Stop the assay
   a. Discard the seeding medium carefully without scratching the bottom of wells.
   b. Rinse cells once with 200 µl PBS per well.
   c. Shake the plate at 2,000 rpm for 15 sec.
   d. Discard the PBS and wash with 200 µl wash buffer per well for 3 times.
   e. Fix with 200 µl 4% paraformaldehyde per well. Incubate at room temperature for 10-15 min.
   f. Wash with 200 µl PBS per well.
4. Staining and analysis
   a. Add 100 µl 0.1% crystal violet to each well and incubate for 10 min at room temperature.
   b. Remove the crystal violet and wash with ddH_2O (200 µl/well) for 3 times.
   c. Turn the plates upside down on the absorbent papers. Let the plates dry up completely.
   d. The plates can be scanned by a scanner firstly.
   e. Add 100 µl 2% SDS to each well and incubate for 10 min with gently shaking at room temperature.
   f. Read the absorbance within 5 min on a microplate reader/spectrometer at a wavelength of 550 nm.

Representative data

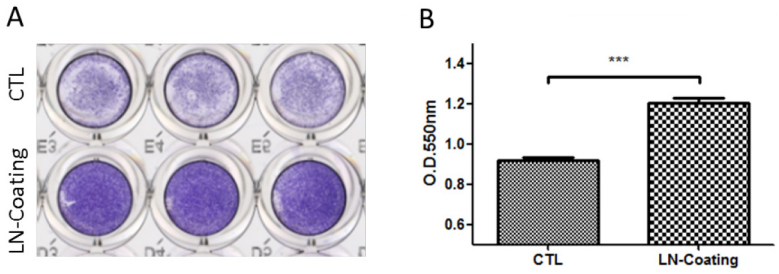

Figure 1. Laminin 511 promotes the adhesion of bone marrow mesenchymal stem cells (BMSCs). A. The scanned image of crystal violet staining of BMSCs adhered to laminin 511 and BSA coated wells; B. The O.D. values from the dissolved crystals are shown for (A), the data are presented as the mean ± SEM from three replicate wells; ***, $P < 0.001$.

Recipes

1. PBS with/without Ca^{2+} and Mg^{2+} (Reference 1)
   a. Dissolve the following in 800 ml distilled H_2O
      8 g NaCl
      0.2 g KCl
      1.44 g Na_2HPO_4
      0.24 g KH_2PO_4
      For PBS with Ca^{2+} and Mg^{2+}, supplement with the following
      0.133 g $CaCl_2 \cdot 2H_2O$
      0.10 g $MgCl_2 \cdot 6H_2O$
   b. Adjust pH to 7.4 with HCl
   c. Adjust volume to 1 L with additional distilled H_2O
   d. Sterilize by autoclaving
2. 0.1% crystal violet staining solution
   To make up 50 ml crystal violet solution, dissolve 50 mg crystal violet powder in (5 ml ethanol/45 ml water).

References

1. Phosphate-buffered saline (PBS). (2006) *Cold Spring Harb Protoc*: doi:10.1101/pdb.rec8247.
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3. Siler, U., Seiffert, M., Puch, S., Richards, A., Torok-Storb, B., Muller, C. A., Sorokin, L. and Klein, G. (2000). Characterization and functional analysis of laminin isoforms in human bone marrow. *Blood* 96(13): 4194-4203.

