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Cardiac stem cell niche, MMP9, and culture and differentiation of embryonic stem cells

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Summary

Embryonic stem cells (ESC) are totipotent, self-renewing and clonogenic having potential to differentiate into a wide variety of cell types. Due to regenerative capability, it has tremendous potential for treating myocardial infarction (death of myocardial tissue) and diabetes (death of pancreatic beta cells). Understanding the components regulating ESC differentiation is the key to unlock the regenerative potential of ESC-based therapies. Both the stiffness of extracellular matrix (ECM) and surrounding niche / microenvironment play pivotal roles in ESC differentiation. Matrix metalloproteinase-9 (MMP9) induces fibrosis that causes stiffness of the ECM and impairs differentiation of cardiac stem cells into cardiomyocytes. Here, we describe the method of ESC culture and differentiation and the expression of MMP9 and its inhibitor, tissue inhibitor of metalloproteinase-4 (TIMP4) in differentiating ESC.

Keywords

Stem cell; MMP9; TIMP-4; Differentiation; Extracellular matrix; Cardiomyocytes

1. Introduction

Stem cell therapy is an emerging area in cardiovascular diseases, where the dead myocardium after myocardial infarction (MI) can be replenished by stem cell transplantation (1-8). However, the mechanism of stem cell mediated regeneration of myocardium is still nebulous. Recent studies suggest that the surrounding niche plays pivotal role in stem cell differentiation (6,9-17). One of the key factors involved in survival and differentiation of stem cell is mechano-sensitivity of surrounding niche, which is provided by stiffness of

extracellular matrix (ECM) (18-21). The stiffness of ECM determines the contractility of cardiomyocytes and cardiac tissue repair (22,23). One of the important enzymes involved in induction of stiffness of ECM is matrix metalloproteinase-9 (MMP9) that induces cardiac fibrosis (24,25). Although clinical studies revealed that MMP9 plays crucial role in diastolic dysfunction (26), protects against ischemia-reperfusion (27) and acts as biomarker for heart failure (28), its role in stem cell differentiation was not clear. Recently, we have reported that MMP9 is involved in regulation of survival and differentiation of cardiac stem cells by inducing stiffness of ECM (25). Since ablation of MMP9 differentially regulate miRNAs (29) and miRNAs play key role in differentiation of stem cells (8,30-34), it is also expected that MMP9 may inhibit differentiation of stem cells by regulating miRNAs. However, role of MMP9 in embryonic stem cells (ESC) differentiation is unclear. Therefore, we determined the role of MMP9 and its inhibitor, tissue inhibitor of metalloproteinase-4 (TIMP4) in differentiating embryonic stem cells.

We cultured disaggregated mouse embryonic stem cells (ESC) without mouse leukemia inhibitory factor (LIF) and allowed it to differentiate. Since heart is the first organ formed during embryogenesis (35), we expected to obtain cardiomyocytes, the default pathway of differentiation. We determined the levels of MMP9 and TIMP4 in both differentiated and un-differentiated ESC.

2. Materials

Use all the materials in sterile conditions. The ESC and mouse embryonic fibroblast (MEF) cells should be stored in a liquid nitrogen tank whereas the culture medium should be stored at 4°C.

2.1. Mouse ESC culture medium components

1. Prepare Dulbecco's Modified Eagle Medium (DMEM) medium by dissolving DMEM powder with high glucose, L-glutamine, pyridoxine hydrochloride and 110mg/L sodium pyruvate but without NaHCO₃ into doubled distilled water at 15-30°C with gentle stirring. The amount of water should be 5% less than the total desired volume. Add 3.7g of NaHCO₃/ liter (Gibco) into the medium.
2. Adjust pH to 7.1-7.2, which is 0.2-0.3 below the desired pH 7.4. The container should be closed after pH adjustment. The pH rises 0.1-0.3 upon filtration.
3. To prepare complete medium, add 1% Non-essential amino acid (Gibco), 1% sodium pyruvate (Gibco), 20% fetal bovine serum (FBS) (Atlanta Biologicals), 1% Penicillin /Streptomycin antibiotics (Cellgro), and 0.1% β-Merceptoethanol (Gibco) into the DMEM medium. To prepare 609.2 ml of complete medium, add 6 ml of non-essential amino acid, 6 ml of sodium pyruvate, 120 ml of FBS, 1.2 ml of 50mm β-Merceptoethanol, 6ml of antibiotics into 470 ml of DMEM medium
4. Add mouse leukemia inhibitory factor (LIF) (Millipore) at the concentration of 125 U / ml to the ESC culture medium (*see* Note 1).

5. Immediately sterilize the above medium by passing it through a 0.22µm polyethersulfone sterilizing filter (Corning) inside the Biological safety cabinet (*see* Note 2).
6. Store the complete sterilized medium at 4°C.
7. Warm the medium to 37°C before using it for culturing.

2.2. ESC differentiation medium components

1. ESC culture medium without LIF.

2.3. MEF culture medium components

1. The MEF culture medium is similar to the ESC culture medium except the concentration of FBS is 10%.

2.4. MEF inactivation

1. MEFs are frozen in MEF culture medium + 10% DMSO @ 5×10^6 / cryovial and stored in liquid nitrogen. MEFs can be inactivated by γ -irradiation (5,000 rads) in a cell irradiator packed with dry ice to prevent thawing during irradiation. They are used as a feeder layer for ESCs (*see* Note 3).

2.5. Additional components

1. Ultra low attachment 6 well plate (Costar).
2. Sterile tissue culture dish (10cm)
3. Ultrapure water with 0.1% gelatin (Millipore).
4. Light sensitive and without phenol red, Tryple Express trypsin (Gibco).
5. Cell culture grade Dimethyl sulfoxide (DMSO) (Fisher Scientific).
6. Ultrapure distilled water (Gibco).
7. Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco).

3. Methods

3.1. MEF Culture

1. Thaw frozen MEFs quickly in a water bath maintained at 37°C for 90 sec and dilute vial contents with MEFs medium in a 15 ml conical tube and spin @ 270g for 5 min. (*see* Note 3).
2. One thawed cryovial of 5×10^6 MEFs is enough for 1 confluent 10 cm plate.

¹LIF inhibits differentiation of ESC. Dilute LIF in the ESC medium and aliquot into small tubes to avoid freeze-thaw cycle. We used PCR tubes to aliquot in the manner that one tube can be used in one time.

²The commercially available complete culture medium can be also used.

³During warming of frozen tubes, try to keep the cap region above the water level. Also, spray 70% alcohol and clean it with Kimwipes before taking it into biosafety cabinet. It will help to keep the cells in sterilized condition.

3. Culture γ -irradiated MEFs on a sterile tissue culture plate coated with 0.1 % gelatin in ultrapure water under a biosafety cabinet and place in a cell culture incubator maintained at 37°C with 5% CO₂ (*see* Note 4).
4. Remove gelatin at the time of ESC plating (*see* Note 5).
5. To plate MEFs, resuspend the pelleted MEFs in 10 ml MEF medium, distribute into the gelatin coated plate and place in incubator for at least 24 hrs to allow MEFs to become confluent before adding ESCs.

3.2. ESC Culture

1. 24 hrs after plating MEFs, remove the medium. Wash 1x with DPBS. Add 10 ml ESC medium with LIF. Place back into the incubator for 1 hr.
2. Thaw frozen ESCs quickly in a water bath maintained at 37°C for 90 sec and dilute cryovial contents with ESC medium into a 15 ml conical tube and spin @ 270g for 5 min. (*see* Note 3).
3. Spin the tube at 270g for 5min.
4. Remove the supernatant by vacuum using sterilized pipette.
5. Add 5 ml of fresh warm ESC medium with LIF and resuspend the pellet gently (*see* Note 6).
6. Distribute this 5ml of ESC suspension to the 10 cm plate containing the MEFs and place back into the incubator (*see* Note 7).
7. The plated ESCs exhibit a small round morphology. They attach to the MEFs and proliferate forming oval shaped colonies (Fig. 1A).
8. Change culture medium every day to allow proper growth of ESCs. LIF must be added each time during medium change.
 1. ESCs can be expanded as needed. Trypsinize the entire plate for 5 min. Collect trypsinized cells into a 15 mL conical tube and add an equal amount of ESC medium to counteract the trypsin. Now proceed from step 3 passaging the ESCs onto as many MEF feeder cell plates as needed (*see* Note 8).
9. The aggregate of ESC is called Embroid body (EB). Do not allow EB to come into contact with one another as this will induce differentiation. Undifferentiated EB colonies will have defined borders, which become less defined when overgrown.
10. When EB attains substantial size (Fig. 1B), passage them or proceed with ESC differentiation.

⁴The non-irradiated MEFs can be treated with Mitomycin C at the concentration of 10 μ g/ml for 3-4 hrs to mitotically inactivate MEFs. Mitomycin C inhibits DNA synthesis and nuclear division.

⁵The removal of gelatin from the plate immediately before ESC plating provides better coating than removal of gelatin beforehand.

⁶We found that adding first 100-200 μ L of medium to the pellet and disaggregating it by pipetting it up and down and then adding the rest volume of medium into the disaggregated 100-200 μ L of medium is better method to dissolve the pellet.

⁷To get homogenous cell suspension, it is recommended to dispense the thawed cells drop-wise in all parts of the 10 cm plates.

⁸The number of EB in the medium is important because EB has tendency to attach to each other in vicinity and form a single big EB. To maintain a moderate and size of EB, it is necessary to maintain them in a manner that they do not contact.

3.3. ESC differentiation

- 2 . Use ESC medium without LIF for differentiation of ESC (ESC differentiation medium).
- 3 . To remove feeder cells from ESC, coat a 10 cm plate with 0.1% gelatin for 30-45 min.
- 4 . Remove gelatin at the time of ESC plating (*see* Note 5).
- 5 . Trypsinized ESCs with MEFs with 5 ml of Tryp LE Express and disaggregate it with a fire polished pipette.
- 6 . Transfer the disaggregated ESC into a 15 ml tube.
- 7 . Add 8 ml of ESC medium to the 5ml of trypsinized cells and mix it by pipetting up and down.
- 8 . Centrifuge the cell containing medium at 270g for 5min.
- 9 . Remove the supernatant by vacuum using sterilized Pasteur pipette.
- 10 . Resuspend the pellet with 10ml of fresh medium.
- 11 . Transfer the medium into 0.1% gelatin coated plate and incubate it for 1 hr (*see* Note 9).
- 12 . After 1hr, transfer the medium from 10 cm gelatin coated plate to a 15 ml tube.
- 13 . Centrifuged the tube at 270g for 5min.
- 14 . Remove the supernatant and resuspend the pellet in 1ml of ESC medium.
- 15 . Count the ESC number (*see* Note 10).
- 16 . Dilute ESCs in a manner that 540,000 ESCs are suspended in 36 ml of ESC medium without LIF (15,000 cells /mL). This volume is good for two 6-well plates @ 3ml of cell suspension /well of the plate (*See* Note 11).
- 17 . The plated cells are not disturbed for 48hrs. Differentiating ESCs will begin to agglomerate into free-floating EBs.
- 18 . After 48 hrs, change the medium daily (*See* Note 12).
- 19 . EB attains considerable size by 72-96 hrs (Fig. 1C) and are ready for further differentiation. By default, each EB will differentiate into cardiomyocytes (Fig.

⁹MEF has more adherent capacity and they have higher binding affinity to gelatin than the ESCs. MEFs are also larger than ESCs and sink to the bottom of the plate faster than ESCs. Therefore, majority of MEFs attach to the surface in 1hr whereas ESCs remain in suspension.

¹⁰The cells number can be counted by hemocytometer. For that, take 10 μ L of cell suspension, and add 90 μ L of Trypan blue. Mix it well and spread 10 μ L of mixed solution onto hemocytometer. Score the number of cells in the four quadrants in the four corners. The number of cells will be calculated by the formula: Number = (total cells in four quadrants /4) $\times$ 10 $\times$ 10⁴.

¹¹To get 100% confluent MEFs, regular MEFs from two 10cm plates are stored in a single vial. These vials are irradiated with γ -rays and stored in liquid nitrogen. When used for plating even after cell death during the process of freezing and thawing, the number of MEFs is high enough for 100% confluence. The excess number of MEF does not affect the ESC binding and culture.

¹²To change the medium, collect EBs and media into a 15 mL conical tube and allow 2 min for EBs to sink to the bottom. Aspirate the supernatant without disturbing the settled EBs. Add 3ML of fresh ESC media without LIF. Triturate very gently to re-distribute the EBs without breaking them apart. The EBs can now be transferred back to the ultra-low attachment 6-well plates.

1D). The beating of cardiomyocytes can be observed under light microscope at 10X and 20X magnifications (Fig. 2A-B).

To determine the role of MMP9 and TIMP4 in differentiation of ESC, we stained the differentiated and undifferentiated ESC with MMP9. The results revealed that MMP9 is robust in undifferentiated (Fig. 3C) than differentiated (Fig. 3A-B) ESC. We also compared central versus remote regions of differentiating ESC for MMP9 expression because the central region is in active stage of differentiation and remote region has terminally differentiated cells. The results show that MMP9 is down regulated in central region ((Fig. 3A) but comparatively highly expressed in the remote region (Fig. 3B). Since TIMP4 inhibits MMP9, we stained the differentiating ESC with TIMP4. The comparison of MMP9 and TIMP4 in central region of ESC revealed that TIMP4 is highly expressed (Fig. 4) in differentiating ESC whereas MMP9 is attenuated in the same region (Fig. 3A). These findings indicate that MMP9 is inhibited and TIMP4 is induced during differentiation of ESC.

In diabetic condition, MMP9 is activated (24,25). To understand the effect of hyperglycemia on differentiation of ESC, we treated differentiating ESC with 5mM (physiological dose: CT) and 25mM (high dose) of D-glucose for 24 hrs. Both of them are stained with MMP9. The comparative results revealed that MMP9 is down regulated in CT (Fig. 5A) but it is robust in hyperglycemic ESC (Fig. 5 B (i) and (ii)). It suggests that hyperglycemia induces MMP9 in differentiating ESC that could have inhibitory effect on differentiation.

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Fig. 1. The different stages of embryoid body (EB). **A.** One day after plating of embryonic stem cell (ESC) on mouse embryonic fibroblasts (MEFs) shown in low magnification (10X). The round shape EB is shown by arrows. **B.** The 3 days EB shown in high magnification (20X). **C.** The MEF free undifferentiated floating EB. **D.** Differentiated and attached EB.

Fig. 2. Different regions of differentiated ESC. **A.** From central (black spot, arrow) to the remote (distant from the black spot) of the differentiated EB at low magnification (10X). **B.** The high magnification (20X) view of the area of differentiated ESC, where contractile cardiomyocytes are observed.

Fig. 3.

Expression of MMP9 in differentiating ESC. **A.** The expression of MMP9 (green color) in differentiating area, the central region of EB. The blue color is dapi, which stains nucleus. **B.** The expression of MMP9 in the terminally differentiated region (remote from the center of EB). **C.** The expression of MMP9 in undifferentiated EB. Scale bar is 50µm.

Fig. 4.

The expression of TIMP4 (green color) in differentiating region of EB. The left panel show dapi (blue) that stains nucleus, the middle panel show TIMP4 staining (green) and the right panel show merged imaged with blue and green. Scale bar is 50µm.

Fig. 5.

The differentiated EBs are treated with 5mM and 25mM of D-glucose for 24 hrs and stained with MMP9 (green) and dapi (blue). **A.** The left panel control (5mM) group show less expression of MMP9. **B.** The right two panels B (i), and B (ii) show robust expression of MMP9 (green). Scale bar is 50µm.