

Coculture between hMADS and Mouse Adult CM

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[Abstract] Heart failure occurring after acute myocardial infarction (MI) is among the main causes of death in Western countries. Cell therapies, particularly those based on mesenchymal stem cells (MSC), represent one of the most promising approaches to repair damaged heart tissues. Several reports have provided evidences that injection of mesenchymal stem cells improved heart function following myocardial infarction (Shake *et al.*, 2002; Zimmet and Hare, 2005; Zeng *et al.*, 2007). Nevertheless, the mechanism(s) by which MSC exert their therapeutic action is far from being understood, and further knowledges in this field are required especially to optimize efficiency of current cardiac cell therapies. To assess the regenerative mechanisms developed by MSC *in vitro*, we developed the method described above which is expected to mimic the micro-environment typical of an infarcted heart. This method consists in a species mismatch coculture between mouse terminally differentiated cardiomyocytes in a distressed state and human Multipotent Adipose Derived Stem cells (hMADS cells) used herein as an MSC model.

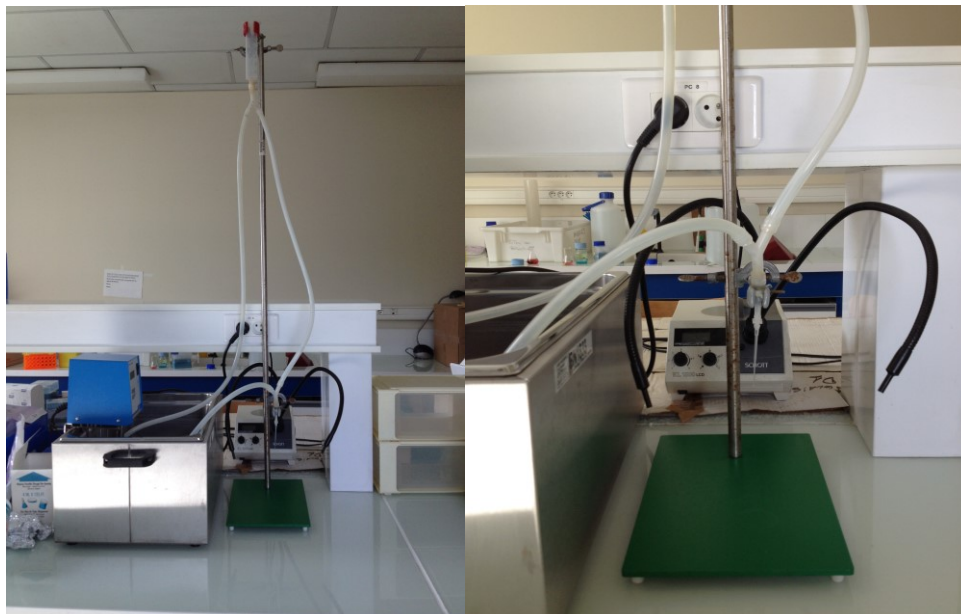
Materials and Reagents

1. hMADS cells were isolated as described previously (Rodriguez *et al.*, 2005)
2. Perfusion Buffer stock solution (sPB)
3. Liberase blendzyme TM (Roche Diagnostics, catalog number: 05401119001)
4. Trypsin (Sigma-Aldrich, catalog number: T4549)
5. Calcium chloride
6. Annexin V/7AAD staining (BD Biosciences, catalog number: 559763)
7. 18 α -glycyrrhetic acid (18 α -GA) (Sigma-Aldrich, catalog number: G-8503)
8. M Latrunculin A (Life Technologies, catalog number: L12370)
9. Nocodazole (Sigma-Aldrich, catalog number: M-1404)
10. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S9625)
11. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P4504)
12. Potassium phosphate monobasic (KH₂PO₄) (Sigma-Aldrich, catalog number: P5379)
13. Sodium phosphate dibasic (Na₂HPO₄) (Sigma-Aldrich, catalog number: S0876)
14. Magnesium sulfate heptahydrate (MgSO₄·7H₂O) (Sigma-Aldrich, catalog number: M9397)

15. Sodium bicarbonate (NaHCO_3) (Sigma-Aldrich, catalog number: S5761)
16. Potassium bicarbonate (KHCO_3) (Sigma-Aldrich, catalog number: P9144)
17. 1 M HEPES buffer solution (Sigma-Aldrich, catalog number: M3375)
18. Taurine (Sigma-Aldrich, catalog number: T0625)
19. Phenol Red (last) (Sigma-Aldrich, catalog number: P5530)
20. 5.5 mM glucose (Sigma-Aldrich, catalog number: G7528)
21. 0.25 mg/ml liberase blendzyme (Roche Diagnostics, catalog number: 1988417)
22. Trypsin (Sigma-Aldrich, catalog number: T4549)
23. 10% newborn calf serum (Life Technologies, Gibco[®], catalog number: 16010167)
24. 5% bovine calf serum (BCS) (HyClone, catalog number: SH30073)
25. Pentobarbital sodium (CEVA, CIP number: 6742145)
26. Heparin choay (sanofi-aventis, CIP number: 3048450)
27. 1x perfusion buffer stock solution (see Recipes)
28. 2,3-Butanedione monoxime (BDM) (Sigma-Aldrich, catalog number: B0753) (see Recipes)
29. Perfusion buffer (pH 7.46) (PB) (see Recipes)
30. Digestion buffer (see Recipes)
31. Stopping buffer 1 (see Recipes)
32. Stopping buffer 2 (see Recipes)

Equipment

1. Langendorff apparatus (see below)



2. Water bath at 37 °C

3. Surgical silk 4.0
4. Petri dishes (Dutscher Scientific, catalog number: 628103)
5. Surgical instruments
6. Disposable transfer pipets (VWR International, catalog number: 612-1681)
7. Falcon 15 ml propylene conical tubes (Dutscher Scientific, catalog number: 352095)

Procedure

A. Cardiomyocytes isolation

Ventricular cardiomyocytes were obtained from 2 to 5 months old male C57BL/6J mice (Janvier) according to the AfCS Procedure Protocols PP00000125 by O'Connell T, Ni Y.

1. Perfusion buffer, digestion buffer, stopping buffers (1 and 2) must be prepared daily.
2. Perfusion buffer and digestion buffer must be warmed to 37 °C prior to use.
3. Prepare the Langendorff perfusion apparatus. The circulating water bath is set so that the outflow from the tip of the cannula is 37 °C. The perfusion system is rinsed with 100 ml of purified water then with the perfusion buffer for at least 5 min and air bubbles are eliminated.
4. 2-5 months old male mice were anesthetized with a solution of pentobarbital (60 mg/kg) and received heparin (200 UI/kg).
5. Heart was excised and perfused on a Langendorff apparatus with calcium-free oxygenated (95% O₂, 5% CO₂) perfusion buffer at 37 °C.
6. Then, heart was perfused for few minutes with the digestion buffer, the same calcium-free perfusion buffer supplemented with 0.25 mg/ml of liberase blendzyme TM, 0.14 mg/ml of trypsin and 12.5 μM of calcium chloride.
7. Heart was removed, placed in a dish containing stop buffer 1 and further diced into small pieces using small scissors. Auricles and aorta were removed. Cellular dissociation was achieved by gentle mechanical agitation in stopping buffer 1.
8. Cell suspension was transferred to a 15 ml polypropylene conical tube. The plate was rinsed with 2.5 ml of room temperature stopping buffer 1. This washing solution was then added to the cell suspension to reach a final volume of 5 ml. Stop buffer solution 1 contains serum to inactivate proteases. The final calf serum concentration is 5%, and the final calcium concentration is 12.5 μM.
9. Cardiomyocytes were sedimented by gravity for 8-10 min in the 15 ml tubes at 37 °C. For each tube, the supernatant was eliminated after sedimentation and the pellet was ~~resolved~~ resuspended in 5 ml of stop buffer 2 added to increasing concentration of Ca²⁺.
 - 5 ml of SB2 + 50 μl 10 mM Ca²⁺ (112 μM)
 - 5 ml of SB2 + 100 μl 10 mM Ca²⁺ (212 μM)

- 5 ml of SB2 + 25 μ l 100 mM Ca^{2+} (500 μ M)
 5 ml of SB2 + 50 μ l 100 mM Ca^{2+} (1 mM)
10. The viability of freshly isolated CM was assessed by flow cytometry analysis following Annexin V/7AAD staining.

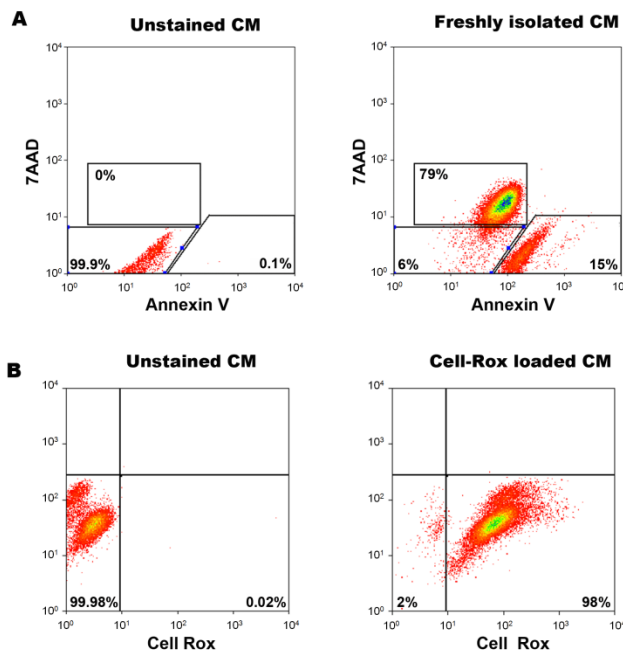


Figure 2. Suffering state of freshly isolated mouse CM (Figeac *et al.*, 2014)

B. Coculture

Notes:

- a. Freshly isolated mouse cardiomyocytes were cocultured with mesenchymal stem cells in different conditions depending on the parameter to assess.
 - b. Although most of our experiments were performed with hMADS cells, some of them were done with hMSC from bone marrow generous gift from EFS, Créteil (Acquistapace, *et al.*, 2011).
1. To assess cardiomyocyte reprogramming process, cocultures were placed in DMEM supplemented with 10% FBS, Gentamycine (1/500) and Fungizone (1/100) in a 1:1 ratio, each cell type seeded at 3,500 cells per square centimeter.
 2. To prevent physical contact between the two cell types, cell-culture inserts containing a polycarbonate membrane with different pore sizes (from 0.4 μ m up to 3 μ m pore size) were used.

Note: We use a 1 μ m pore size insert which allows the diffusion of soluble factors, exosomes and large microparticules as apoptotic bodies to demonstrate that these cell-to-

cell communication pathways were not involved in the paracrine alteration of mesenchymal stem cells through coculture. We also used 0.4 μm pore size which allows the diffusion of soluble factors and small exosomes but impedes the transfer of microvesicles and apoptotic bodies to confirm that cell-to cell communication through soluble soluble factors and exosomes did not affect stem cell phenotype and the generation of hybrid cardiac progenitor cells.

3. To measure soluble factors released by MSC during the coculture, each of the two cell types was seeded at 10^5 cells/ml (ratio 1:1) in a growth medium, containing low amount of fetal calf serum, namely, DMEM supplemented with 0.8% FBS, Gentamycine (1/500) and Fungizone (1/100).
4. As control for these experiments, hMADS and adult cardiomyocytes were seeded at 10^5 cells/ml of DMEM supplemented with 10% and 0.8% FBS, according the experiments considered. We used 10% FBS concentration to perform experiments on the characterization of cell fusion processes and the phenotype of resulting hybrid cells. We used 0.8% FBS concentration to study secretome changes induced by coculture in order to decrease the risk of contamination by the soluble factors contained in the serum.
5. To determine the communication pathways involved in the paracrine activation of hMADS cells, we performed coculture experiments in presence of several pharmacological compounds. Fresh cocultures were performed in 0.8% FBS DMEM medium during 24 h in presence of 100 mM 18α-glycyrrhetic acid to inhibit gap junction and 2.5×10^{-8} M Latrunculin A or 5×10^{-8} M Nocodazole, to inhibit tunneling nanotube formation.

Representative data

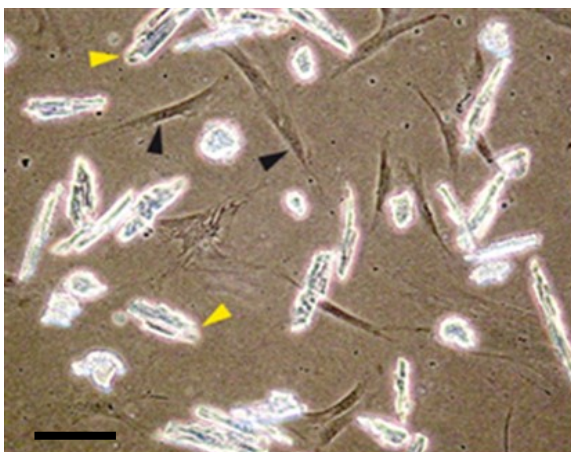


Figure 3. Coculture between hMADS cells (black arrowhead) and mouse adult cardiomyocytes (yellow arrowhead) at day 0. Scale bar, 30 μm (Acquistapace *et al.*, 2011)

Recipes

1. 1x perfusion buffer stock solution (AfCS Solution Protocols, protocol ID: PS00000452 from Kachura M)
 - 113 mM sodium chloride (NaCl)
 - 4.7 mM potassium chloride (KCl)
 - 0.6 mM potassium phosphate monobasic (KH_2PO_4)
 - 0.6 mM sodium phosphate dibasic (Na_2HPO_4)
 - 1.2 mM magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)
 - 12 mM sodium bicarbonate (NaHCO_3)
 - 10 mM potassium bicarbonate (KHCO_3)
 - 10 mM HEPES buffer solution 1 M
 - 30 mM taurine
 - 0.032 mM Phenol Red (last)

Add all components to 900 ml purified water in a 2 L beaker
 Stir until all components are thoroughly dissolved
 Transfer to a 1 L graduated cylinder and adjust final volume to 1 L with purified water
 Prewash a 0.22 μm filter unit with 10 ml of purified water
 Sterilize the solution
 Stored at 4 °C
2. 2,3-Butanedione monoxime (BDM) (AfCS Solution Protocols, protocol ID: PS00000438)
 - 500 mM 2,3-Butanedione monoxime

Weigh BDM and add to 30 ml purified water in a 100 ml beaker
 Stir until solution is thoroughly dissolved
 Transfer solution to a 50 ml graduated cylinder and adjust final volume to 50 ml with purified water
 Prewash a 0.2 μm filter with 10 ml purified water
 Sterilize the solution by filtering through the prewashed filter
 Stored at 4 °C
3. Perfusion buffer (PB) (pH 7.46) (AfCS Solution Protocols, protocol ID: PS00000451 by Kachura M)
 - 1x stock perfusion buffer; 0.98x (AfCS Solution Protocols, protocol ID: PS00000452)
 - 500 mM, 10 mM 2,3-Butanedione monoxime (BDM)
 - 5.5 mM glucose

Weigh glucose and add to 400 ml of stock perfusion buffer in a 1 L beaker
 Add 10 ml of BDM to the beaker and stir until all components are thoroughly dissolved

- Transfer solution to a 500 ml graduated cylinder and adjust final volume to 500 ml with stock perfusion buffer
- Cover with Parafilm and mix by inversion
- Prewash a 0.22 μm 500 ml filter unit with 10 ml purified water
- Sterilize the solution by filtering through the prewashed filter unit
- Final pH is 7.46 after filtration
4. Digestion buffer (AfCS Solution Protocols, protocol ID: PS00000447)
 - 1x perfusion buffer (pH 7.46)
 - 0.25 mg/ml liberase blendzyme
 - 0.14 mg/ml trypsin
 - 12.5 μM calcium chloride (CaCl_2 , 100 mM; AfCS Solution Protocols, protocol ID: PS00000441)

Weigh liberase blendzyme and add to 30 ml of perfusion buffer in a 100 ml beaker

Measure and add trypsin and 100 mM CaCl_2 to the beaker

Stir until all components are thoroughly dissolved

Adjust final volume to 50 ml with perfusion buffer in a graduated cylinder

Cover with parafilm and mix by inversion

Prewash a 0.2 μm filter attached to a 60 ml syringe with 10 ml purified water

Sterilize the solution by filtering through the prewashed filter
 5. Stopping buffer 1 (AfCS Solution Protocols, protocol ID: PS00000449)
 - 1x perfusion buffer (pH 7.46) (AfCS Solution Protocols, protocol ID: PS00000451)
 - 10% newborn calf serum
 - 12.5 μM calcium chloride (CaCl_2) 10 mM (AfCS Solution Protocols, protocol ID: PS00000440)

Pipette 1 ml of NCS and 12.5 μl of CaCl_2 into a 10 ml graduated cylinder

Adjust final volume to 10 ml with perfusion buffer

Cover with parafilm and mix by inversion

Prewash a 50 ml tube filter with 10 ml purified water

Sterilize the solution by filtering through the prewashed filter
 6. Stopping buffer 2 (AfCS Solution Protocols, protocol ID: PS00000450)
 - 1x perfusion buffer (pH 7.46) (AfCS Solution Protocols, protocol ID: PS00000451)
 - 5% bovine calf serum (BCS)
 - Calcium chloride (CaCl_2) 10 mM (AfCS Solution Protocols, protocol ID: PS00000440)
 - 12.5 μM

Pipette 1.5 ml of BCS and 37.5 μl of CaCl_2 into a 50 ml graduated cylinder

Adjust final volume to 30 ml with perfusion buffer

Cover with parafilm and mix by inversion

Prewash a 0.2 μ M filter attached to a 35 ml syringe with 10 ml purified water
Sterilize the solution by filtering through the prewashed filter

Acknowledgments

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