Transwell Co-culture of Bone Marrow Macrophages with Tumor Cells

Mackenzie K. Herroon and Izabela Podgorski*

Department of Pharmacology, Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, USA
*For correspondence: ipodgors@med.wayne.edu

[Abstract] Bone is a primary site of metastasis from prostate and breast cancers. Bone marrow macrophages (BMMs) are mediators of inflammatory processes and are thought to promote tumor growth in the skeletal sites. In order to elucidate how their interactions with tumor cells impact aggressiveness of metastatic tumors in bone *in vitro* methods are required. By employing a system in which BMMs and tumor cells are grown separately, yet share the media and exchange soluble factors, contribution of each cell type in the context of BMM-tumor cell relationship in the bone marrow can be investigated. Additional advantages of this system include the ability to study: 1) phenotypic changes in BMMs and tumor cells upon co-culture; 2) cell-specific modulation of protein and gene expression; and 3) effects on proliferation and cell survival. It is noteworthy, that this transwell co-culture system is not limited to BMMs and tumor cells and can be easily modified to include other components of bone marrow microenvironment (*e.g.*, adipocytes, stromal cells, osteoblasts).

Materials and Reagents

1. Mice (FvBN strain)
2. PC3 cells (ATCC, catalog number: CRL 7934)
3. L929 cells (ATCC, catalog number: VR-1404)
4. DMEM (Sigma-Aldrich, catalog number: D2902)
5. MEM-alpha (Sigma-Aldrich, catalog number: M0644)
6. Fetal bovine serum (Thermo Fisher Scientific, HyClone catalog number: SH3008803)
7. Pencillin-Streptomycin (Life Technologies, Gibco®, catalog number: 15140-122)
8. 0.25% Trypsin-EDTA (Life Technologies, Gibco®, catalog number: 25200-056)
9. 1x Sterile PBS (Sigma-Aldrich, catalog number: P3813)
10. 70% ethanol
11. RNeasy Plus Mini Kit (QIAGEN, catalog number: 74134)
12. QIAshredder (QIAGEN, catalog number: 79654)
13. RLT buffer (part of RNeasy Plus Mini Kit, shown above), includes 2-Mercaptoethanol (Sigma-Aldrich, catalog number: M7522)

14. Sucrose (Sigma-Aldrich, catalog number: S9378)
15. MES (Sigma-Aldrich, catalog number: M8250)
16. Complete DMEM (see Recipes)
17. L929 conditioned media (see Recipes)
18. BMM media (see Recipes)
19. SME lysis buffer (see Recipes)

Equipment

1. 6-well plates (Corning, Costar®, catalog number: 3516)
2. Transwell inserts (Corning, catalog number: 3412)
3. Petri dishes (Thermo Fisher Scientific, catalog number: 08-757-13)
4. 100 mm tissue culture dishes (Corning, catalog number: 430293)
5. T75 flasks (Corning, catalog number: 430725)
6. Cell scrapers (SARSTEDT AG, catalog number: 83.1830)
7. Sterile fine-tip transfer pipets (Thermo Fisher Scientific, catalog number: 232-1S)
8. 10 ml syringes (Luer-Lok) (BD, catalog number: 309604)
9. 5, 10, & 25 ml serological pipets
10. Needles (26 Gauge & 20 Gauge) (BD, catalog numbers: 305111 & 305176, respectively)
11. Dissecting forceps and scissors
12. Scalpel
13. 1.5 ml tubes (Thermo Fisher Scientific, catalog number: 3464)
14. 15 ml centrifuge tubes (Thermo Fisher Scientific, Nunc®, catalog number: 339650)
15. 50 ml centrifuge tubes (Thermo Fisher Scientific, Nunc®, catalog number: 339652)
16. 5 K Millipore centrifugal concentrator (Amicon Ultra-4) (Millipore, model: UFC800596)
17. Centrifuge
18. Incubator (5% CO_2, 37 °C) (Napco Series 8000 DH CO_2 Incubator)
19. Biosafety cabinet (The Baker Company, SterilGARD®)

Procedure

A. Obtaining BMMs from murine bone marrow

1. Sacrifice donor mouse (male, 6-8 weeks of age, in-house bred) using CO_2 inhalation and cervical dislocation.
2. In sterile hood, rinse hind legs with 70% ethanol and remove, making sure to cut above head of femur. Place skinned legs in a petri dish with 1x sterile PBS.

3. Remove muscle and connective tissue from bones (both femur and tibia), scraping them clean with scalpel and placing into fresh 1x sterile PBS.
4. Place fresh scalpel and dissecting forceps into beaker of 70% ethanol. Remove one bone from PBS onto dry petri dish, and cut both ends of bone, exposing marrow.
5. Using 26 gauge needle and 10 ml syringe, drill into bone marrow and slowly flush out marrow using BMM media into 50 ml centrifuge tube. Flush 2 ml from one side, flip bone, flush 2 ml from the other side - 4 ml total per bone.
6. Repeat step A5 for all 4 bones into 1 x 50 ml centrifuge tube.
7. Using 20 gauge needle and 10 ml syringe, gently mix the cell suspension 4x by aspiration into syringe to break up any clumps.
8. Bring volume of BMM media and cells up to 36 ml. Plate into 3x petri dishes with 12 ml per 100 mm petri dish (~9-10 x 10^6 cells/dish). Allow cells to grow undisturbed for ~84 h in cell culture incubator (at 37° C, 5% CO_2).

   Note: Petri dishes are used instead of treated cell culture plates to allow easier passaging of BMMs. These cells tend to tightly adhere to tissue culture-treated cells and flasks and are difficult to lift for subsequent experiments.

B. Transwell set up

1. Both BMMs and PC3 cells should be at 70-80% confluency and ready for use the same day.
2. BMMs:
   a. Remove media and lightly rinse BMM dish with 1x PBS. Add ~8 ml of fresh BMM media to dish and gently remove cells using a sterile fine-tip transfer pipet and cell scraper, collecting into a 15 ml centrifuge tube.
   b. Perform a cell count on a single-cell suspension, and plate 0.35 x 10^6 BMMs in 2 ml of BMM media directly in the 6-well plates.
3. Using sterile forceps, gently place transwell inserts in each well above freshly plated cells. By their design inserts are secured in place by the walls of the well. They will remain suspended and touch the media, but not the bottom of the plate allowing for free exchange of soluble factors between the cells on the bottom and top of the insert.
4. PC3s
   a. Allow PC3 cells to grow to ~70-80% confluency in T75 flask.
   b. Rinse with 1x sterile PBS and dissociate the cells using 2 ml of 0.25% Trypsin-EDTA.
   c. Use 8 ml complete DMEM to stop the activity of the trypsin and collect all 10 ml in a 15 ml centrifuge tube.
   d. Centrifuge the cells at 130 *x g* for 5 min at RT to pellet, and re-suspend in an appropriate amount of complete DMEM (~8 ml) to perform a cell count.

e. Plate 0.2 x 10^6 PC3s in 2 ml of complete DMEM on top of the transwell inserts.

5. Allow the cells to grow for 48 h undisturbed in cell culture incubator.
6. When plating for control conditions without the transwell, plate the same number of cells directly on the 6-well plate, in a mix of 2 ml complete DMEM and 2 ml BMM media.

C. Changing co-culture to serum-free conditions (if necessary)

1. For experiments requiring analysis of media conditioned by cells in co-culture and to avoid interference from serum in the media, transwell co-cultures may be changed to serum-free conditions 12-16 h prior to harvest.
2. Briefly, after 48 h, gently wash the cells 3x with 1x sterile PBS. To do so, remove media from the top of the transwell, lift the transwell, remove media from the bottom of the well, add 2 ml of PBS to the bottom of the well, replace the transwell, add 2 ml of PBS to the transwell, and gently agitate. Repeat at least 2 times.
3. Plate 2 ml of serum-free MEM-alpha to the bottom of the well, and 2 ml of serum-free DMEM to the transwell. Allow cells to sit for 12-16 h undisturbed in cell culture incubator.
4. If change to serum-free conditions is not necessary, 3x PBS washes should be performed immediately prior to sample harvest for analyses.

D. Harvesting experimental protein samples

1. Media:
   a. Collect media into 15 ml centrifuge tube. Transfer any transwells to new, unused 6-well dishes.
   b. Remove any floating cells by centrifuging collected media at 130 *x g* (to keep the cells intact) for 5 min at 4 °C.
   c. Transfer media to new 15 ml centrifuge tube.
   d. Remove any additional cell debris by centrifuging at 830 *x g* for 10 min at 4 °C. It is necessary that the two spins are performed separately and intact cells are removed before 830 x g spin is performed.
   e. Freeze media as collected at -80 °C for future use or concentrate if needed using 5 K Millipore Centrifugal concentrator.
   f. Transfer media to the concentrator, and spin at 3,000 *x g* at 4 °C until desired volume is achieved.
   g. Store concentrated media at -80 °C for future use.
2. Protein lysates - BMMs
   a. After media is collected, add required amount of SME (~200 µl) to cells and set plate on ice.
   b. Gently scrape the cells and collect into 1.5 ml tube.

c. Freeze at -80 °C.

3. Protein lysates - PC3s
   a. After media is collected, add 2 ml 0.25% Trypsin-EDTA to transwells, and 1 ml 0.25% Trypsin-EDTA to wells used as controls (*i.e.* directly on plastic).
   b. After ~5 min at 37 °C, cells should be dissociated. Stop the trysinization with 4 ml complete DMEM. This volume is sufficient for control wells.
   c. Using a sterile fine-tip transfer pipet, gently rinse and collect cells from the wells into a 15 ml centrifuge tube. For the transwells, add an additional 4 ml of complete DMEM to the transwell, and rinse again, adding the additional media to the original tube.
   d. Spin the cells at 130 *x g* for 5 min at RT.
   e. Remove the media, and gently wash the pellet by re-suspending in ~10 ml of 1x sterile PBS.
   f. Spin the cells at 130 *x g* for 5 min at RT, and remove all PBS.
   g. Re-suspend the pellet in SME (~200 µl) and transfer into 1.5 ml tube.
   h. Freeze at -80 °C.

E. Harvesting samples for RNA analyses
1. After 48 h, gently wash the cells 1x with PBS as described in step C1.
2. Collect the PC3 cells as indicated in steps D3a-d. Wash pellet an additional time with PBS (repeat steps D3e-f an additional time). Add RLT Buffer from RNeasy Plus Mini Kit to the pellet. Use ~250 µl per well collected. Transfer to fresh QIAshredder.
3. Add ~250 µl of RLT Buffer per well directly to washed BMM cells. Scrape cells and collect into fresh QIAshredder.
4. Proceed with RNA extraction according to directions supplied with RNeasy Plus Mini Kit.

Recipes

1. Complete DMEM
   445 ml DMEM, sterile filtered
   50 ml heat-inactivated fetal bovine serum
   5 ml Pencillin-Streptomycin 10,000 U each/ml
2. L929 conditioned media
   a. L929 cells grown to ~70-80% confluency in 10 ml using 100 mm tissue culture dish over 48 h in complete DMEM
   b. Media collected from cells into 15 ml centrifuge tube
   c. Cells removed from media by centrifuging at 130 *x g* for 5 min
   d. Media transferred to new 15 ml centrifuge tube

e. Cell debris removed from media by centrifuging at 830 *x g* for 10 min

f. Media transferred to new 15 ml centrifuge tube and used

3. BMM media (30% L929 CM; 20% FBS)
   12 ml L929 conditioned media
   5.3 ml heat-inactivated fetal bovine serum
   22.7 ml MEM-alpha media
4. SME lysis buffer
   250 mM sucrose
   25 mM MES
   1 mM EDTA
   In deionized water
   pH to 6.5, add 0.1% TritonX-100 before use

Acknowledgments

This protocol was adapted from our manuscript Herroon *et al.* (2013). Grant support was provided by DOD PC074031, DOD PC030325, Wayne State University start-up fund and MICR: P30 CA 22453.

References

1. Herroon, M. K., Rajagurubandara, E., Rudy, D. L., Chalasani, A., Hardaway, A. L. and Podgorski, I. (2013). Macrophage cathepsin K promotes prostate tumor progression in bone. *Oncogene* 32(12): 1580-1593.

