Video Article

Dual Effects of Melanoma Cell-derived Factors on Bone Marrow Adipocytes Differentiation

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Abstract

The crosstalk between bone marrow adipocytes and tumor cells may play a critical role in the process of bone metastasis. A variety of methods are available for studying the significant crosstalk; however, a two-dimensional transwell system for coculture remains a classic, reliable, and easy way for this crosstalk study. Here, we present a detailed protocol that shows the coculture of bone marrow adipocytes and melanoma cells. Nevertheless, such a coculture system could not only contribute to the study of cell signal transductions of cancer cells induced by bone marrow adipocytes, but also to the future mechanistic study of bone metastasis which may reveal new therapeutic targets for bone metastasis.

Video Link

The video component of this article can be found at https://www.jove.com/video/57329/

Introduction

Bone metastases are widespread among advanced cancer patients, but a curative treatment is still unavailable. Beyond specializing in storing energy as fat, adipocytes can support tumor growth and metastasis in bone marrow and other organs[1,2,3,4,5,6]. Moreover, adipocytes play an essential role in regulating cancer cell biology[7,8,9,10] and metabolism[4,11,12,13,14,15,16], as well as in bone metastasis[1,4,12]. In the bone marrow niche, adipocytes can also affect the biological behavior of cancer cells[4,6,17]. The interplay between bone marrow adipocytes and cancer cells with osteotropism is significant for an understanding of bone metastasis. However, little is known.

Based on the current studies, various methods are applied to adipocytes, including two- or three-dimensional (2/3D) and *ex vivo* cultures[17,18,19,20,21]. Recently, Herroon *et al.* designed a new 3D-culture approach to study interactions of bone marrow adipocytes with cancer cells[22]. Although the 3D coculture is optimal for mimicking physiological interactions between adipocytes and cancer cells *in vivo*, it suffers from poor reproducibility[22,23]. In comparison to a 2D coculture system, a 3D coculture system may provide different cellular phenotypes, such as cell morphology[21,22,24,25,26]. Moreover, the *ex vivo* culture of isolated cancellous bone tissue fragments can lead to a robust outgrowth of adipocytes from cultured bone marrow cells[17].

In contrast to these previous models, however, the 2D cell culture model remains a classic, reliable, and easy technique for quickly scanning candidate molecules and the phenotypes changed in either adipocytes or cancer cells *in vitro*[1,4,6,12,15,27]. To better understand the crosstalk between bone marrow adipocytes and melanoma cells, we provide a detailed protocol for a 2D coculture system of bone marrow adipocytes with melanoma cells.

Protocol

NOTE: All cells used in this protocol should be grown for at least three generations after thawing from frozen stock cells.

1. Harvest Melanoma Cell-derived Factors

1. **Preparations**
   1. Obtain B16F10 cells and a mouse melanoma cell line.
      NOTE: For this protocol, a mouse melanoma cell line was obtained from the Stem Cell Bank of the Chinese Academy of Sciences.
   2. Make a complete medium for the B16F10 cell culture (100 mL). Use Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/mL of penicillin, and 50 µg/mL of streptomycin.

3. Make a serum-free medium for the starvation of the B16F10 cells (50 mL). Use DMEM medium without FBS, 50 U/mL of penicillin, and 50 µg/mL of streptomycin.

2. **Prepare melanoma-conditioned medium (CM)**
   1. Seed 2.0 x 10^5 B16F10 cells in a 100 mm dish with 10 mL of 10% FBS DMEM. Then incubate the cells in a 37 °C incubator in an atmosphere of 5% CO_2 for 24 h.
   2. When the cells reach about 80% of confluence, remove the culture medium, wash the cells twice with 5 mL of phosphate-buffered saline (PBS) (1x, pH 7.4), and replace the medium with a 10 mL serum-free medium for the starvation.
   3. Return the cells to the incubator for 18–24 h.
   4. After the starvation, collect the conditioned medium (CM) with a pipette and transfer it to a fresh 50 mL centrifuge tube.
      NOTE: The conditioned medium is the supernatant of the B16F10 cell cultures. Make sure the B16F10 cells are in good condition before collecting the CM.
   5. Centrifuge the CM for 5 min at 1,000 x *g* at 4 °C.
   6. Pipette the supernatant and transfer it to a fresh sterile tube or bottle.
      NOTE: Avoid pipetting off the pellet at the bottom of the tube.
   7. To remove the cell debris, filter the CM by passing it through a syringe-connected 0.45 µm filter on top of a sterile bottle or tube.
      NOTE: 0.22 µm filters are optimal for filtering the CM.
   8. Store the filtered supernatant at -20 °C until use.
      NOTE: It is best not to store the supernatant at -20 °C for more than one month. Alternatively, store the CM at -80 °C for more extended periods of time for the differentiation of adipocyte.

2. Induction of Bone Marrow Adipocytes by Melanoma Cell-derived Factors

1. **Preparations**
   1. Make 100 mL of adipogenic induction medium[28]. Use a complete DMEM supplemented with 5 µg/mL of insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 1 µM dexamethasone. Store it up to two weeks at 4 °C.
   2. Prepare 100 mL of adipogenic maintenance medium. Use a complete DMEM or mixed with 50% melanoma CM supplemented with 5 µg/mL of insulin.
   3. Make an Oil Red O stock solution. Dissolve 30 mg of Oil Red O powder in 10 mL of isopropanol (≥ 99.5%) and mix them well by vortex.
      NOTE: Store it up to 1 year at 4 °C.
   4. Make an Oil Red O working solution. In the fume hood, dilute the Oil Red O stock solution (from step 2.1.3) with deionized water in a ratio of 3:2. Incubate the mixture for 15 min at room temperature and filter it through a 0.22 µm filter before use.
      NOTE: Only use the fresh working solution within 2 h.

2. **Adipocyte differentiation**
   1. Culture a stromal cell line 14F1.1 from mouse bone marrow[29] in a 60 mm dish with a complete DMEM at 80% confluency in a 37 °C incubator in an atmosphere of 5% CO_2.
      NOTE: Alternatively, replace 14F1.1 with primary bone marrow-derived mesenchymal cells[30].
   2. Trypsinize the cells with 1.5 mL of a 0.25% trypsin solution in an incubator for 1–2 min.
   3. Add 5 mL of 10% FBS medium to the cells and count the cell numbers with a glass hemocytometer; then adjust the cell density to about 1–2 x 10^5/mL.
   4. Seed 5 x 10^4 of 14F1.1 cells per well in a 24-well plate with 0.5 mL of adipogenic induction medium. After two days of incubation, the cells reach about 90% of confluence.
      NOTE: This step is very critical. Bone marrow stromal cells cannot be induced to adipocytes without an adipogenic induction medium.
   5. Remove the induction medium and wash the cells twice with 1 mL of PBS each time.
   6. Change to 1 mL of adipogenic maintenance medium and continue to culture the cells until adipocyte maturation.
      NOTE: Under a microscope, adipocytes look like small or large soap bubbles and are very easy to recognize. Usually, mature adipocytes are present on the 6^{th} or 8^{th} day.
   7. Stain the mature adipocytes with Oil Red O or collect the adipocytes for a quantitative polymerase chain reaction (qPCR) analysis.

3. **Adipocyte Staining**
   1. Wash the differentiated 14F1.1 cells 2x with PBS and fix them in 3.7% formaldehyde for 1 h.
   2. Rinse the cells in 60% isopropanol and allow them to dry completely.
   3. Add 0.2 mL of the Oil Red O working solution to the wells and incubate it at room temperature for 0.5 to 2 h.
   4. Wash the cells with water to remove the unbound dyes.
   5. Photo the dyed adipocytes under a light microscope.

3. Coculture of Bone Marrow Adipocytes with Melanoma Cells

1. **Preparations**
   1. Prepare mature bone marrow adipocytes: induce the 14F1.1 cells into mature BM adipocytes in 24-well plates with the adipogenic induction medium for 2 days, and then maintain them in the adipogenic maintenance medium for an additional 6–8 days.
   2. Prepare the B16F10 cells: culture the B16F10 cells in 60 mm dishes with 5 mL of DMEM medium.
   3. Moisten the membrane insert (*e.g.* transwell): add 150 µL of FBS-free DMEM medium to the 0.4 µm pore-size membrane inserts and immerse them upon the wells of 24-well plates filled with 500 µL of FBS-free DMEM medium.

2. **Coculture setting**

1. Slowly pipette off the medium of each insert.
   NOTE: Do not destroy the membrane when removing the medium.
2. Seed the B16F10 cells ($0/10^3/10^5/10^6$) in the 0.4 µm pore inserts from step 3.2.1.
3. Replace the adipogenic maintenance medium in the wells with the mature bone marrow adipocytes from step 3.1.1. with 600 µL of complete DMEM medium.
4. Transfer the inserts filled with the B16F10 cells (from step 3.2.2) upon the wells with the mature bone marrow adipocytes (from step 3.2.3) for the coculture experiments.
5. Incubate the cocultures at 37 °C and 5% CO_2 for 2 days.
6. After 48 h of incubation, remove the inserts of the coculture and then wash the adipocytes 2x with 500 µL of 1x PBS.
   NOTE: Optionally, collect the B16F10 cells in the inserts for analysis if needed.
7. Stain the adipocytes with the Oil Red O working solution or collect the adipocytes for qPCR analysis.
   NOTE: See the staining procedure in step 2.3.
8. Photo the dyed adipocytes under a light microscope.

4. qPCR Analysis of the Adipocyte-specific Gene Signature

1. Add 0.5 mL of ready-to-use RNA isolation reagent per well in a 24-well plate in which mature adipocyte is ready to detect.
2. Follow a standard protocol for the RNA extraction and the cDNA synthesis[31].
3. Use the primer for 18S and GAPDH as the control, and CEBPα/β, PPARγ, FABP4, leptin, Pref-1, or adiponectin for the adipocyte-specific gene signature (**Table 1**).
4. Run the following reaction in a thermal cycler: 95 °C for 20 s, followed by 40 cycles of 95 °C for 1 s, and 60 °C for 20 s.
5. Use the $2^{-\Delta\Delta Ct}$ method to calculate the fold changes[32].

Representative Results

In the bone marrow, adipocytes can appear in the tumor microenvironment[1,13,33,34,35] at an early stage for supporting tumor progression through soluble factors or activating osteoclastogenesis[6,12,36], especially in the context of obesity[6,12] and/or aging[37]. Our previous studies[1] have noted that the number of bone marrow adipocyte changes dramatically during the early stage of melanoma metastases to bone[1]. Indeed, the amounts of adipocytes accumulate in the tumor area and support the tumor cells' growth in the bone marrow when the mice fed with a high-fat diet[6], suggesting that bone marrow adipocytes might play a significant role in the metastatic bone marrow niche[37]. However, the dynamic crosstalk of tumor cells and bone marrow adipocyte remains unclear. Therefore, we were dedicated to addressing this issue by the 2D coculture system of bone marrow adipocyte and melanoma cells.

Tumor-derived factors promote the differentiation of bone marrow adipocytes:
Figure 1a represents a typical workflow for inducing bone marrow adipocytes with tumor-conditioned medium (TCM). A representative experiment indicates 14F1.1 cells, plated as single cells at a density of 5×10^4 cells per well in the presence of an adipogenic induction medium in the first 2 days, and then maintained within the adipogenic maintenance medium (with or without TCM) for 6–8 days. Mature adipocyte contains lipid droplets; therefore, the differentiated adipocytes can be easily identified by Oil Red O staining (**Figure 1b**) and quantified by qPCR with an adipocyte-specific gene signature (**Figure 1c**).

Overload of melanoma cells induces de-differentiation in bone marrow adipocytes:
Tumor cells can modify the environment to survive, especially under hypoxic conditions[1,4,6]. To mimic a tumor-burden secondary effect on a bone marrow adipocyte, we performed the 2D coculture system with a continued increase of melanoma cell number in the upper inserts (**Figure 2a**). **Figure 2b** shows that the lipid storage in the adipocytes is deprived to a greater extent at the highest density of melanoma cells (10^6 cells/well) compared with the deprivation at the lowest frequency of melanoma cells (10^3 cells/well). **Figure 2c** presents a gene profiling of these adipocytes in the adipocytes in the coculture. Compared with the coculture with the lowest density of melanoma cells, pref-1 level increased obviously, and the expression of Leptin, adiponectin, FABP4, CEBPβ, and PPARγ was decreased, which suggests that the tumor-burden secondary effect can be attributed to the de-differentiation process in the adipocytes. Besides, an overload of tumor cells could induce necrosis in adipocytes[38].

In summary, we observed an opposite melanoma cell-driven effect on bone marrow adipocytes by soluble factors.The opposite effect on bone marrow adipocytes is of high interest, suggesting that bone marrow adipocytes are dynamic regulators of the tumor cell metastasize to bone marrow.

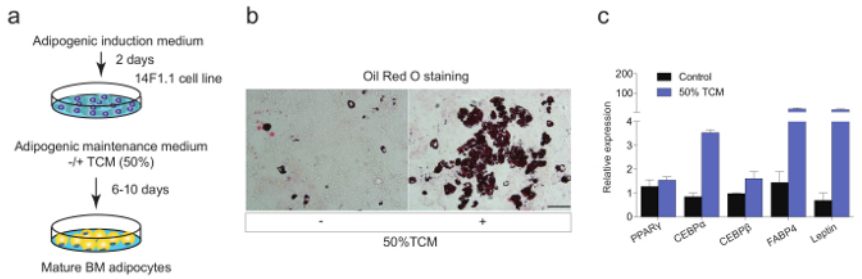

Figure 1: Tumor-derived factors promote an adipocyte differentiation. (**a**) This panel shows a schematic view of the experimental setting which uses 14F1.1 cell lines and a melanoma-derived conditioned medium. (**b**) This panel shows the imaging of a differentiated bone marrow adipocyte. (**c**) This panel shows the analysis of an adipocyte-specific gene signature by qPCR. TCM = tumor-conditioned medium. qPCR: quantitative polymerase chain reaction. Scale bar = 50 µm. This figure has been modified from Wang *et al.*[1]. Please click here to view a larger version of this figure.

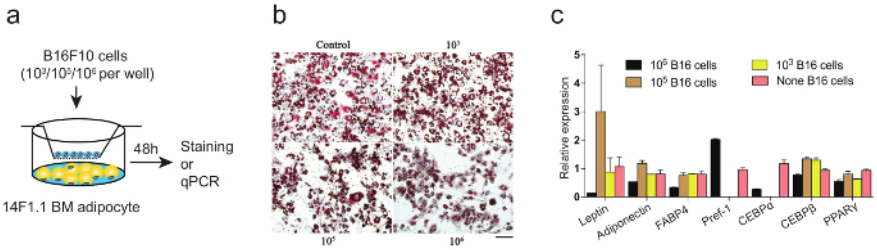

Figure 2: Tumor-burden effects on adipocyte. (**a**) This panel shows a schematic view of the experimental setting: a coculture of 14F1.1 cell lines and melanoma cells. (**b**) This panel shows the imaging of a bone marrow adipocyte in the coculture system. (**c**) This panel shows a gene profiling of adipocytes by qPCR. qPCR: quantitative polymerase chain reaction. Scale bar = 50 µm. This figure has been modified from Wang *et al.*[1]. Please click here to view a larger version of this figure.

Primers		
Gene name	**Forward sequence (5' to 3')**	**Reverse sequence (5' to 3')**
PPARγ	CTGATGCACTGCCTATGAGC	GGGTCAGCTCTTGTGAATGG
CEBPα	AAG AGC CGC GAC AAG GC	GTC AGC TCC AGC ACC TTG TG
CEBPβ	CAACCTGGAGACGCAGCACAAG	GCTTGAACAAGTTCCGCAGGGT
FABP4	TGAAATCACCGCAGACGACAGG	GCTTGTCACCATCTCGTTTTCTC
Leptin	ATC TGA AGC AAG CCA TCA GC	CCA GTC ACC AGA GGT CAA GC
Pref-1	GACACTCGAAGCTCACCTGG	GGAAGGCTGGGACGGGAAAT
Adiponectin	GCG ATA CAT ATA AGC GGC TTC T	GCA GGC ATC CCA GGA CAT C

Table 1: Table of primers used for qPCR.

Discussion

Cocultures with inserts have been widely used to study cell-to-cell interactions. The 2D coculture system is an effective way to observe how the two parts crosstalk *in vitro*, by which we here showed two different cancer cell-driven effects on bone marrow adipocytes. Many labs have utilized this method to investigate the crosstalk between adipocytes and cancer cells[6,12,27,39].

In general, therefore, a 2D coculture is the best straightforward approach to the cell-to-cell crosstalk study.However, when it comes to preparing an undifferentiated cell line for further research, there are some unique challenges. In the protocol described here, the health and functional status of the bone marrow stromal cell is very critical for the adipocytes differentiation. The protocol could be modified to replace the 14F1.1 cells with primary bone marrow mesenchymal stem cells. Furthermore, the pre-incubation of the 14F1.1 cells in the adipogenic induction medium for several days is another critical step. Without this pre-incubation, the bone marrow stromal cells will fail to differentiate into adipocytes, which has been detected in our and others' studies[40]. These phenomena also explain why some similar protocols may result in different or even opposite effects and highlight the importance of such an easy, repeatable, and detailed protocol for the crosstalk study of tumor cells and bone marrow adipocytes.

Although the 2D coculture may lose some critical information in comparison to 3D or *ex vivo* cultures, it remains easy to be standardized, monitored, and eventually manipulated for further use[20,21,41]. A future application of the technique may decide how an osteoblast changes when it is cocultured with cancer cells.

Disclosures

The authors have nothing to declare.

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