Published in final edited form as:

Methods Mol Biol. 2017 ; 1509: 115–122. doi:10.1007/978-1-4939-6524-3_11.

Profiling the MicroRNA Payload of Exosomes Derived from Ex Vivo Primary Colorectal Fibroblasts

Rahul Bhome^{1,2,*}, Rebecca Goh¹, Karen Pickard¹, Massimiliano Mellone¹, A. Emre Sayan¹, and Alex Mirnezami^{1,2}

¹Cancer Sciences, University of Southampton, Southampton, UK

²University Surgical Unit, Southampton General Hospital, Southampton, UK

Abstract

The tumor microenvironment is a heterogeneous and dynamic network that exists between cancer and stroma, playing a critical role in cancer progression. Certain tumorigenic signals such as microRNAs are derived from the stroma and conveyed to cancer cells (and vice versa) in nanoparticles called exosomes. Their identification and characterization is an important step in better understanding cellular cross talk and its consequences. To this end we describe how to culture primary ex vivo derived fibroblasts from colorectal tissue, isolate their exosomes, extract exosomal RNA and perform microRNA profiling.

Keywords

Tumor microenvironment; Stroma; Fibroblast; Exosome; Colorectal cancer

1 Introduction

It is important to appreciate the tumor microenvironment (TME) as a functional ecosystem that exists between cancer cells and the surrounding stromal milieu. In recent times, there has been increased recognition of the key part played by the microenvironment in the control of both normal physiological processes and development of the malignant phenotype [1]. For example, activated stroma is strongly associated with the acquisition of increased proliferation, invasion and chemoresistance of cancer cells [2]. Consequently, the study of TME methods to delineate the cellular cross talk between different cell types and compartments is of great importance. This will not only enhance our understanding of fundamental biological processes but may pave the way towards more novel therapeutic strategies. Using techniques such as laser capture microdissection and modern single cell isolation methodologies, the stromal and epithelial compartments of a tumor can be separated and subsequently analyzed to determine the origin of deregulated signals [3]. For example, functionally relevant microRNA-21 originates from stromal myofibroblasts rather than cancer cells in the colorectal setting [4, 5].

*Corresponding author: Rahul Bhome r.bhome@soton.ac.uk.

MicroRNAs (miRNAs) are small noncoding RNA sequences, which are approximately 20 nucleotides long. Pri-miRNAs are transcribed by RNA polymerase II and undergo enzymatic cleavage within the nucleus (drosha) to form pre-miRNAs and within the cytoplasm (dicer) to form mature miRNAs. The resulting miRNA sequence then binds the 3' untranslated region of coding mRNAs repressing translation [6]. In this way miRNAs can regulate a number of cellular processes including proliferation, differentiation and apoptosis [7], all of which are relevant to cancer progression.

MiRNAs can be transferred between cells in the TME, allowing one cell to alter protein expression in another [8]. This paracrine signaling is facilitated by nanoparticles called exosomes. Exosomes are extracellular vesicles between 40 and 100 nm in diameter with a lipid bilayer. Unlike shedding vesicles and microparticles, their biosynthesis and secretion is complex and enzyme dependent [9]. Exosomes are secreted by all cellular components of the TME, including cancer cells [10], fibroblasts [11], innate and acquired immune cells [12], and vascular endothelial cells [13]. Moreover, the impact of exosomal transfer on target cells is not momentary but lasts for at least several days [14].

In order to identify stromal microRNA signals in the colorectal cancer setting, we describe how to: (1) culture primary fibroblasts from human colorectal tissue, (2) isolate fibroblast exosomes, (3) label and transfer exosomes from one cell type to another, (4) extract total RNA from exosomes, and (5) perform exosomal miRNA profiling.

2 Materials

2.1 Culture of Primary Fibroblasts

1. 10 cm diameter dish.
2. Scalpel.
3. Forceps (non-toothed).
4. 12-well plate.
5. Phosphate buffered saline (PBS) supplemented with 2 % (double-strength) penicillin–streptomycin (Penicillin (200 U/mL)–streptomycin (200 µg/mL; Sigma-Aldrich), and 0.1 % (0.25 µg/mL) amphotericin B (Fungizone; Thermo Fisher Scientific) (*see* Note 1).
6. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20 % fetal calf serum (FCS), 2 % penicillin–streptomycin and 0.1 % amphotericin B.
7. Trypsin–EDTA solution (0.25 %; Sigma-Aldrich).
8. Ciprofloxacin (BIOMYC-3 100×; Biological Industries).

2.2 Isolation of Exosomes from Primary Fibroblasts

1. Exosome deplete FCS (produced by collecting the supernatant from FCS which has been centrifuged at $100,000 \times g$ for 16 h).
2. 50 mL polypropylene (Falcon) tubes.

3. 0.22 μm filter with polyethersulfone (PES) membrane (Merck Millipore).
4. 50 mL (Luer lock) syringe.
5. Polycarbonate ultracentrifuge tubes 26.3 mL (Beckman Coulter).
6. Ultracentrifuge with rotor capable of $100,000 \times g$ (e.g., Sorvall Discovery 100 s with titanium rotor TFT 50.38).
7. PBS.
8. Appropriate solvent for further processing of exosomes e.g., Laemmli buffer 2x, Lysis solution (RNAqueous®-Micro Total RNA Isolation Kit, Ambion), sterile water.

2.3 Labeling and Transfer of Exosomes

1. Vybrant Cell Labeling Solution DiO: absorption 484 nm emission 501 nm (Molecular Probes).
2. Laemmli Buffer 2x.
3. Pierce BCA Protein Assay Kit (Thermo Fisher).

2.4 Isolation of Exosomal RNA

1. RNAqueous®-Micro Total RNA Isolation Kit (Ambion).
2. Nuclease-free 1.5–2 mL tubes.
3. 100 % ethanol.
4. Nuclease-free water.

2.5 MicroRNA Array

1. Cancer MicroRNA qPCR Array with Quantimir™ (System Biosciences).
2. Primer plate - dried primers for 95 cancer-associated microRNAs (plus U6 for normalization).
3. Power SYBR Green PCR Mastermix (Applied Biosystems).
4. 96-well qPCR reaction plate.
5. Optical adhesive cover (Applied Biosystems).
6. Nuclease-free water.
7. Thermocycler.
8. qPCR instrument e.g., ABI7500 (Applied Biosystems).

3 Methods

3.1 Culture of Primary Colorectal Fibroblasts

1. Collect fresh biopsy (1-2 cm) of malignant and normal colon/rectum (ensuring ethical approval for study and patient consent) in 10 mL PBS supplemented with antimicrobials.
2. Transfer to a 10 cm dish and wash biopsy three times with 10 mL PBS. Do not aspirate PBS after last wash.
3. Divide biopsy into 2–5 mm fragments using sterile scalpel and forceps.
4. Score the bottom of each well in a 12-well plate with a cross ('X').
5. Place the biopsy fragments individually on to the center of each cross allowing them to attach.
6. Add 750 μ L medium (DMEM supplemented with 20 % FCS and antimicrobials) to each well ensuring that the biopsy fragment is not dislodged.
7. Incubate at 37 °C/5 % CO₂.
8. Change medium at 24 h and every 72 h thereafter, checking for outgrowth of fibroblasts and microbial infection at each interval.
9. When fibroblasts are greater than 70 % confluent (usually at 4–6 weeks), expand each well into one T25 flask.
10. For expansion: aspirate medium from well, wash twice with PBS, add 500 μ L warm trypsin, incubate at 37 °C for 5 min.
11. Then add 1 mL fresh medium, aspirate entire contents of well and transfer to a 15 mL Falcon tube.
12. Centrifuge at 400 $\times g$ for 5 min to pellet cells. Discard supernatant.
13. Resuspend cell pellet in 5 mL PBS, centrifuge again at 400 $\times g$ for 5 min. Discard supernatant.
14. Resuspend washed cell pellet in 6 mL fresh medium and transfer to a T25 flask.
15. Add 60 μ L BIOMYC-3.
16. Incubate at 37 °C/5 % CO₂.
17. Expand to T75 and then T175 flasks when cells are 100 % confluent using appropriate volumes of trypsin and medium. It is not necessary to use BIOMYC-3 for these expansions.

3.2 Isolation of Exosomes from Primary Colorectal Fibroblasts

1. Primary fibroblasts should be cultured in FCS-deplete medium for 72 h prior to exosome isolation (*see* Notes 2–4).
2. Pre-chill ultracentrifuge rotor to 4 °C.

3. Harvest conditioned medium from all flasks and transfer to 50 mL Falcon tubes.
4. Centrifuge at $400 \times g$ for 5 min to pellet out floating cells. It is not necessary to decant supernatant at this step (*see Note 5*).
5. Centrifuge at $2000 \times g$ for 10 min to pellet out debris.
6. Attach 0.22 μm filter to 50 mL syringe and remove plunger. Transfer supernatant into the chamber of the syringe. Reattach the plunger and drive supernatant through filter into a fresh 50 mL Falcon tube.
7. Transfer equal volumes of filtered supernatant into ultracentrifuge tubes. Ensure that filled tubes are balanced (to within the manufacturer's recommended limit).
8. Place tubes into ultracentrifuge rotor. Ultracentrifuge at $100,000 \times g$ for 75 min at 4 °C (*see Note 6*).
9. The exosome pellet should be visible on the radial aspect of ultracentrifuge tube. Carefully aspirate supernatant without disturbing pellet. (If the pellet is not visible, leave 1 mL at the bottom of the tube and use this when pooling exosomes together - *see next step*.)
10. Resuspend the exosome pellet in 200 μL PBS. Pool exosomes together into one ultracentrifuge tube. Fill tube to capacity with PBS.
11. Ultracentrifuge again at $100,000 \times g$ for 75 min at 4 °C.
12. Aspirate supernatant as above.
13. Resuspend washed exosome pellet in 200 μL PBS (for cell transfer), 200 μL 2 $\times$ Laemmli buffer (western blotting), 100 μL Lysis solution (RNA extraction) or 50 μL sterile water (unfixed imaging by transmission electron microscopy) (*see Note 7*).
14. Store samples at 4 °C for short term (<1 week) or -80 °C for longer term. Samples for electron microscopy should only be stored at 4 °C (*see Note 8*).

3.3 Labeling and Transferring Exosomes

1. Isolate exosomes as above (up to and including 3.2.9).
2. Resuspend exosome pellet in 200 μL PBS and add 1 μL DiO.
3. Incubate for 20 min at 37 °C.
4. Fill tube to capacity with PBS and ultracentrifuge again at $100,000 \times g$ for 75 min at 4 °C.
5. The washed exosome pellet will be colored with dye. Aspirate supernatant. Resuspend pellet in 200 μL in PBS (*see Note 9*).
6. Measure exosomal protein concentration by BCA (or similar) assay as an index of exosome number (*see Note 10*).

7. Add the appropriate amount of solubilized exosomes to achieve a concentration of 20-30 $\mu\text{g/mL}$ in the target cell medium. This can be titrated in subsequent experiments.
8. Incubate for 24 h at 37 °C.
9. Visualize using fluorescence microscope with appropriate filter to confirm the presence of fluorescent exosomes within target cells.

3.4 Extraction of Exosomal RNA

1. Resuspend washed exosome pellet in 100 μL Lysis Solution and transfer to a 2 mL nuclease-free tube.
2. Pre-wet the Micro Filter with 30 μL of Lysis Solution.
3. To recover both large and small RNA species add 1.25 volumes of 100 % ethanol to the lysate and vortex gently.
4. Load the lysate/ethanol mixture on to the Micro Filter and centrifuge for 1 min at maximum speed to bind the RNA.
5. Add 180 μL of Wash Solution 1 to the filter and centrifuge for 10 s at $16,000 \times g$.
6. Add 180 μL of Wash Solution 2/3 to the filter and centrifuge for 10 s at $16,000 \times g$. Repeat this step once.
7. Remove the Micro Filter cartridge from the collection tube and discard the flow-through. Replace the Micro Filter cartridge into the same collection tube.
8. Centrifuge the Micro Filter cartridge for 1 min at maximum speed to dry the filter.
9. Transfer the Micro Filter cartridge to a clean nuclease-free tube.
10. Elute the RNA in 25 μL of nuclease-free water by centrifuging for 30 s at $16,000 \times g$.
11. Measure the concentration of RNA using a NanoDrop spectrophotometer.
12. Store exosomal RNA at -80°C .

3.5 Cancer MicroRNA qPCR Array with Quantimir™

1. A minimum amount of 125 ng RNA is required.
2. Set up the Quantimir™ reverse transcription reaction according to the manufacturer's instructions. A total volume of 20.5 μL cDNA is produced.
3. On ice, resuspend forward primers (in the primer plate) with 12.5 μL nuclease-free water per well (*see* Note 11).
4. Load 1 μL primer into each well of the 96-well qPCR reaction plate and cover with adhesive film to prevent evaporation.

5. Combine 2× Power SYBR Green PCR Mastermix, nuclease-free dH₂O and universal reverse primer with 20 µL synthesized cDNA according to the manufacturer's instructions.
6. Load equal volumes of cDNA/Mastermix to each primer in the 96-well qPCR reaction plate.
7. Cover the plate with an optical adhesive cover.
8. Centrifuge plate briefly to bring contents of each well to the bottom.
9. Run real-time qPCR using the following parameters; 50 °C for 2 min, 95 °C for 10 min and 40× cycles of 95 °C for 15 s/60 °C for 1 min.
10. Perform a melt curve after qPCR run to verify specificity of the reaction.
11. Normalize miRNA expression with U6 snRNA CT values (*see* Note 12).
12. Calculate fold changes in 95 cancer-associated microRNAs between paired samples, e.g., normal versus cancer-associated exosomes.

4 Notes

1. Penicillin–streptomycin comes as penicillin (10000 U/mL) and streptomycin (10 mg/mL). Fungizone comes as amphotericin B 250 µg/mL. Working concentrations are given in the text as % v/v.
2. Each confluent T175 flask should contain 18 mL medium to concentrate exosomes without depriving cells.
3. 12× T175 flasks of fibroblasts will produce exosomes from which approximately 125–250 ng RNA can be extracted. This is adequate for most downstream analyses.
4. Carcinomatous cells produce approximately three to four times more exosomes than mesenchymal cells as evidenced by total protein and RNA content. A high amount of starting material is therefore required for exosomal protein/RNA analysis from fibroblasts.
5. It is not advised to commence the initial spin at 2000 × *g* as this may lyse cells releasing organelles into the supernatant.
6. We have found that using an ultracentrifuge with a fixed angle rotor produces a visible exosome pellet (on the radial aspect of the tube) whereas swinging bucket rotors do not.
7. The final exosome pellet is resuspended in whichever solvent is most appropriate for downstream analysis.
8. Samples for electron microscopy should not be frozen as this leads to artifact.
9. Exosome labeling can be incorporated into the routine isolation technique to improve visibility of the pellet.

10. Alternative methods of exosome quantification include lipid assay [15] and NanoSight technology [16].
11. Manufacturer instructions suggest 10 μL of cDNA but 12.5 μL will ensure 10 complete reactions.
12. U6 snRNA was not differentially regulated in our stromal exosome samples, so we use this as an endogenous control. U6 snRNA is also a commonly used endogenous control for normalization of cellular microRNA levels.

References

1. Bhome R, Bullock M, Al Saihati H, et al. A top down view of the tumor microenvironment: structure, cells and signaling. *Front Cell Dev Biol.* 2015; 3:33. [PubMed: 26075202]
2. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011; 144:646–674. [PubMed: 21376230]
3. Emmert-Buck M, Bonner R, Smith P, et al. Laser capture microdissection. *Science.* 1996; 274:998–1001. [PubMed: 8875945]
4. Bullock MD, Pickard K, Mitter R, et al. Stratifying risk of recurrence in stage II colorectal cancer using deregulated stromal and epithelial microRNAs. *Oncotarget.* 2015; 6:7262–7279. [PubMed: 25788261]
5. Bullock MD, Pickard KM, Nielsen BS, et al. Pleiotropic actions of miR-21 highlight the critical role of deregulated stromal microRNAs during colorectal cancer progression. *Cell Death Dis.* 2013; 4:e684. [PubMed: 23788041]
6. Mirnezami A, Pickard K, Zhang L, et al. MicroRNAs: key players in carcinogenesis and novel therapeutic targets. *Eur J Surg Oncol.* 2009; 35:339–347. [PubMed: 18644693]
7. Esquela-Kerscher A, Slack F. Oncomirs—microRNAs with a role in cancer. *Nat Rev Cancer.* 2006; 6:259–269. [PubMed: 16557279]
8. Valadi H, Ekström K, Bossios A, et al. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol.* 2007; 9:654–659. [PubMed: 17486113]
9. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol.* 2013; 200:373–383. [PubMed: 23420871]
10. O'Brien K, Lowry M, Corcoran C, et al. miR-134 in extracellular vesicles reduces triple-negative breast cancer aggression and increases drug sensitivity. *Oncotarget.* 2015; 6:32774–32789. [PubMed: 26416415]
11. Hu Y, Yan C, Mu L, et al. Fibroblast-derived exosomes contribute to chemoresistance through priming cancer stem cells in colorectal cancer. *PLoS One.* 2015; 10:e0125625. [PubMed: 25938772]
12. Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol.* 2009; 9:581–593. [PubMed: 19498381]
13. van Balkom BW, de Jong OG, Smits M, et al. Endothelial cells require miR-214 to secrete exosomes that suppress senescence and induce angiogenesis in human and mouse endothelial cells. *Blood.* 2013; 121:3997–4006. [PubMed: 23532734]
14. Lee C, Mitsialis SA, Aslam M, et al. Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension. *Circulation.* 2012; 126:2601–2611. [PubMed: 23114789]
15. Osteikoetxea X, Balogh A, Szabó-Taylor K, et al. Improved characterization of EV preparations based on protein to lipid ratio and lipid properties. *PLoS One.* 2015; 10:e0121184. [PubMed: 25798862]
16. Mehdiani A, Maier A, Pinto A, et al. An innovative method for exosome quantification and size measurement. *J Vis Exp.* 2015; 95:e50974. [PubMed: 25650897]