Chapter 2

Isolation of Extracellular Nanovesicle MicroRNA from Liver Cancer Cells in Culture

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Abstract

MicroRNA can be transferred across cells within extracellular vesicles such as exosomes. In order to analyze the biological effects of extracellular vesicle microRNA, it is necessary to isolate these vesicles and to extract their miRNA content.

Here, we describe an approach to the isolation of cellular nanovesicles from liver cancer cell lines that can be used for the isolation of RNA and microRNA.

Key words Exosomes, Nanovesicles, MicroRNA, Centrifugation, Hepatocellular cancer, RNA isolation

1 Introduction

The release from cells of small vesicles, termed extracellular vesicles, is now being recognized to play an important role in intracellular communications [1]. These extracellular nanovesicles, such as exosomes, can be found in biological fluids such as blood, urine, and ascites [2–4]. A variety of normal cells or tumor cells in culture have been shown to secrete these nanovesicles into the cell culture medium [5–8]. Exosomes have been shown to contain membranous and cytoplasmic proteins. Moreover, exosomes can be taken up by other cells and thereby can transfer these proteins with the potential ability to modulate cellular activities. Recent studies have revealed that exosomes carry not only proteins but also RNAs such as microRNAs (miRNAs) [7, 8]. Moreover, transfer of microRNA across cells by exosomes can contribute to modulation of cell signaling in target cells.

MicroRNAs are a class of small noncoding RNAs, 19–24 nucleotides in length, that can modulate gene expression by inhibiting the translation of messenger RNAs (mRNAs) or directing the degradation of mRNAs [9]. MicroRNAs found in blood may be present within exosomes thereby allowing for stability from degradation by RNases. Deregulated expression of microRNAs has been

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reported in many different physiological and pathophysiological processes, such as cancers [10]. Recently, we have shown that microRNAs found in hepatocellular carcinoma (HCC) cells derived nanovesicles could target TGF-β-activated kinase 1 signaling in recipient cells contributing to hepatocarcinogenesis [11]. Moreover, circulating microRNAs in blood show distinct expression profiles in cancer patients [12]. Thus, the study of exosomal miRNA content can be valuable in understanding cellular processes involved in disease pathogenesis as well as providing insights into potential targets or markers of disease.

Here we describe the methods of isolating nanovesicles from culture medium of HCC cell lines, and a method of extracting total RNAs from these that is suitable for the analysis of miRNA expression. The isolation method involves differential ultracentrifugation, and the nanovesicles isolated have characteristic size, morphology, and protein expression consistent with exosomes. Since the approach is not biased towards selective isolation of exosomes, we will refer to these as nanovesicles. The methods were optimized in two human hepatocellular cancer cell lines—Hep3B, and PLC/PRF/5—and have been successfully used for several other cell lines such as KMCH, KMBC, and MzChA-1 human cholangiocarcinoma cell lines, as well as for normal human hepatocytes and cholangiocytes. For other cell lines, the procedure may need further optimization.

2 Materials

2.1 Nanovesicle Isolation from Cell Culture Medium of HCC Cell Lines

1. Refrigerated centrifuge with swinging bucket rotors for 15 and 50 ml tubes.
2. Ultracentrifuge: Beckman Optima L60 Ultracentrifuge (Beckman Coulter, Fullerston, CA).
3. Ultracentrifuge rotor: Type 50.2 Ti (Beckman Coulter).
4. Polycarbonate tubes for ultracentrifuge: Beckman Polycarbonate Aluminum Bottle with Cap Assembly, PC 26.3 ml (Beckman Coulter).
5. 50 ml polypropylene centrifuge tubes.
6. Growth medium (*see* **Note 1**): A commonly used growth medium for many human HCC cell lines is Dulbecco's modified Eagle's medium (DMEM), containing 10 % fetal bovine serum (FBS). To prepare vesicle-depleted medium, first make DMEM containing a double volume of FBS (*see* **Note 3**), e.g., by adding 112 ml of FBS to 500 ml of DMEM.
7. Cell culture dishes: 10-cm dishes.
8. 0.22-μm pore filter: Bottle top filters.

2.2 RNA Extraction from Tumor Cell-Derived Nanovesicles

1. Trizol (*see* **Note 4**).
2. 1.5 ml nuclease-free tube.
3. Nuclease-free water.
4. Chloroform.
5. Isopropanol.
6. 75 % ethanol: Add 2.5 ml of nuclease-free water to 7.5 ml of molecular biology grade pure ethanol.
7. Refrigerated benchtop microcentrifuge.

2.3 Quality of RNA Extraction

1. Spectrophotometer.
2. Bioanalyzer (Agilent 2100 Bioanalyzer, Agilent Technologies, Inc., Santa Clara, CA).

3 Methods

The procedure of isolation of extracellular nanovesicles from cell culture medium of HCC cells is based on differential sedimentation properties. A schematic of the isolation by sequential ultracentrifugation is shown in Fig. 1. Perform the centrifugation at 4 °C unless otherwise specified.

3.1 Preparation of Vesicle-Depleted Medium (See Note 2)

1. Transfer the growth medium into polycarbonate tubes, and centrifuge at 100,000 × *g* for overnight.
2. Transfer the supernatant to a new bottle. Transfer should be done carefully to avoid contact or disruption of the pellet of vesicles or other debris from the FBS.
3. Add 500 ml of DMEM without FBS to the supernatant from **step 3** to make vesicle-depleted medium containing a final FBS concentration of 10 %.
4. Sterilize the medium using 0.22-μm pore filter and store at 4 °C for up to 2 weeks.

3.2 Isolation of Nanovesicles from Tumor Cell Culture Medium

1. Prepare thirty 100-mm dishes of cell culture by plating HCC cells at 1×10^6 cells per dish with 11 ml of vesicle-depleted medium (*see* **Note 5**).
2. After 3 or 4 days collect the cell culture medium from each dish using a pipette and aliquot into 50 ml polypropylene tubes (*see* **Note 6**).
3. Centrifuge the medium in 50 ml tubes at 300 × *g* for 10 min to spin down any detached cells in the medium.
4. Carefully collect the supernatant using a pipette and transfer to new 50 ml tubes without disruption or contamination from the pellets of detached cells. Centrifuge at 2,000 × *g* for 20 min to remove detached cells or cell debris.

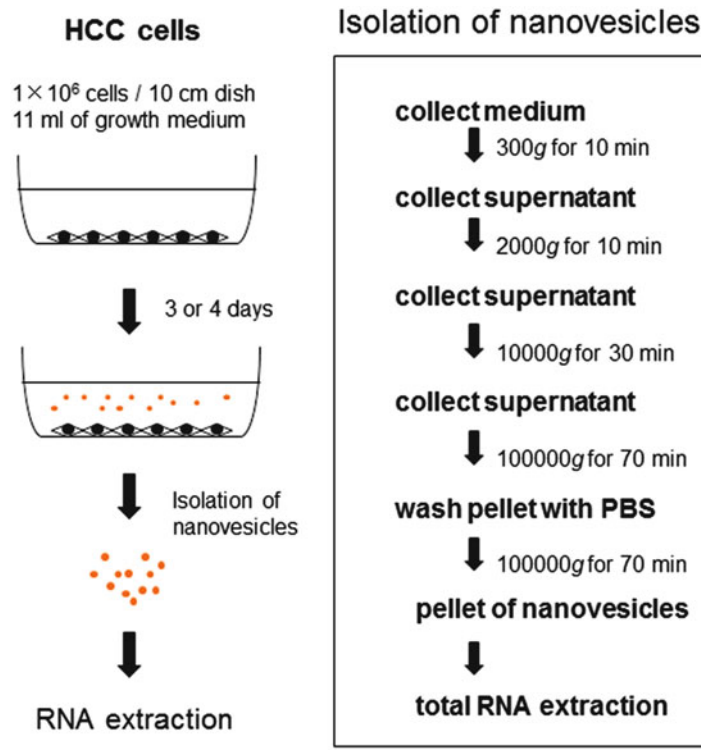

Fig. 1 Schematic representation of the procedure of nanovesicle isolation from HCC cells in culture

5. Collect the supernatant carefully without carrying over any of the pellets of cell debris using a pipette and transfer it to polycarbonate ultracentrifuge tubes, about 25 ml/tube.
6. Put a mark on the surface of each ultracentrifuge tube. The mark allows locating the pellet of cell debris on the bottom of tubes after the centrifugation. Balance the tubes, put the tubes in the ultracentrifuge rotor orienting with the mark side up, and centrifuge at 10,000 × *g* for 30 min to remove cell debris.
7. Collect the supernatant using a pipette and transfer it to new polycarbonate ultracentrifuge tubes (*see* **Note 7**).
8. Put a mark on the surface of each ultracentrifuge tube to assist with localization of the nanovesicle containing pellet at the bottom of tubes after centrifugation. Balance the tubes, and place in the rotor orienting with the mark side up, and centrifuge at 100,000 × *g* for 70 min to obtain the nanovesicle containing pellet.
9. Remove the supernatant carefully by pouring it off. Resuspend the pellet by adding 12 ml of phosphate buffered saline (PBS) to each tube and washing the pellet off from the inside wall of

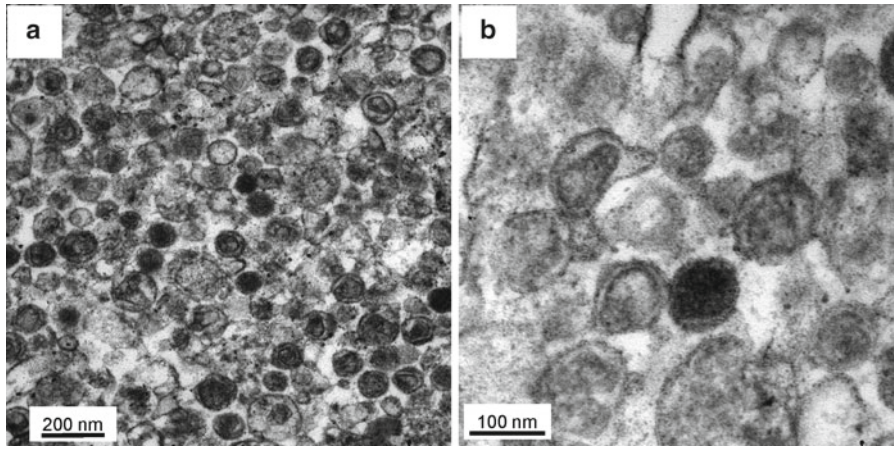

Fig. 2 Morphology of nanovesicles isolated from the culture medium. Electron microscopy was performed on a whole mount isolated from PLC/PRF/5 cells after differential centrifugation. The size and morphology of the isolated nanovesicles are consistent with those of exosomes. (**a**) Low magnification. (**b**) High magnification

the ultracentrifuge tubes (*see* **Note 8**). Use the same PBS to collect pellets from all other ultracentrifuge tubes to minimize dilution. After collecting all of the pellets, transfer the 12 ml of PBS containing resuspended nanovesicle pellets to a new ultracentrifuge tube.

10. Take fresh 12 ml of PBS using a pipette and wash the wall of ultracentrifuge tubes trying to collect any residual nonvisible nanovesicles that remain attached to the wall. Combine the PBS containing nanovesicles in the ultracentrifuge tube. This will give a total volume of ~24 ml of PBS containing nanovesicles in the ultracentrifuge tube.
11. Centrifuge the PBS containing nanovesicles at 100,000 × *g* for 70 min.
12. Remove the supernatant carefully. Add 1 ml of Trizol reagent onto the pellet, lyse it completely by repetitive pipetting, and proceed to RNA extraction.
13. The pellet can be resuspended in 50–200 μl of PBS and stored at −80 °C or used for other downstream experiments such as examination with electron microcopy (EM). Representative EM pictures of nanovesicles that are released into cell culture media by PLC/PRF/5 cells are shown in Fig. 2.

3.3 RNA Extraction from Tumor Cell-Derived Nanovesicles

1. Transfer the nanovesicles lysed in 1 ml of Trizol reagent to a 1.5 ml RNase-free tube and incubate for 5 min at room temperature to allow the complete dissociation of nucleoprotein complex.

2. Add 200 µl of chloroform, cap the tube tightly, and mix well by shaking vigorously for 15–30 s. Incubate the tube for 5 min at room temperature.
3. Centrifuge the tube at 12,000 × *g* for 15 min at 4 °C.
4. Transfer the upper aqueous phase carefully to a new 1.5 ml tube without drawing the intermediate phase or bottom organic phase.
5. Add 500 µl of 100 % isopropanol (Fisher Scientific), close the cap tightly, and incubate at −20 °C for overnight (*see* **Note 9**).
6. Centrifuge at 12,000 × *g* speed for 60 min at 4 °C (*see* **Note 10**).
7. Remove the supernatant carefully using a pipette trying not to move the pellet of RNA. Sometimes the pellet may not be visible.
8. Add 600 ml of 75 % ethanol carefully trying not to move the pellet of RNA and centrifuge at 12,000 × *g* for 5 min.
9. Remove the ethanol carefully using a pipette, add 600 ml of 75 % ethanol, and centrifuge at 12,000 × *g* for 5 min.
10. Repeat the wash step.
11. Remove the ethanol carefully using a pipette and air-dry the pellet.
12. Resupend the pellet of total RNA from exosomes with 10 µl of RNase-free water by pipeting (*see* **Note 11**).
13. Examine the quality and quantity of RNA using a spectrophotometer or by capillary electrophoresis using a Bioanalyzer (*see* **Note 12**). Representative images of capillary electrophoresis are shown in Fig. 3.

4 Notes

1. Use appropriate growth medium depending on each cell line.
2. The serum used for cell culture medium contains exosomes [13, 14]. To selectively analyze nanovesicles that are secreted from cultured cells, any preexisting exosomes present in serum must be depleted.
3. Using medium with a double volume of FBS allows us to deplete vesicles from a larger amount of FBS using a single centrifugation. Centrifugation of FBS itself to deplete vesicles is not preferable due to the viscosity of FBS.
4. Other non-filter-based approaches for RNA isolation could also be used.
5. The number of cells to be plated in a dish needs will need to be optimized for each cell line, as the yield of nanovesicles varies between cell lines. The amount of cell culture medium used will also depend on the capacity of the centrifuge and rotors.

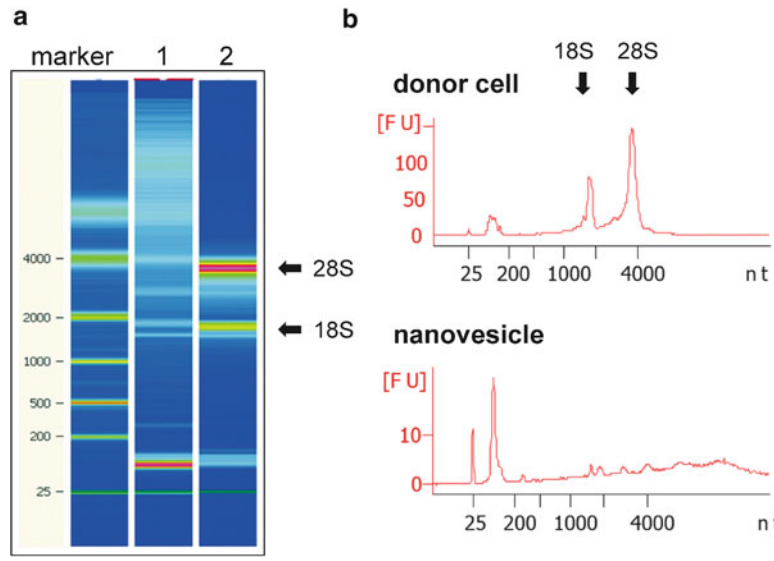

Fig. 3 (**a**) Gel image and (**b**) electropherogram of total RNA extracted from nanovesicles in cell culture. RNA was extracted from PLC/PRF/5-derived nanovesicles (*lane 2*) or from their corresponding donor cells (*lane 1*) and analyzed by capillary electrophoresis (Bioanalyzer; Agilent Technologies). The RNA content of nanovesicles is different from the RNA from the cells of origin, with the majority of RNAs below 2 kb in size and with a very low fraction of 18S ribosomal RNA and 28S ribosomal RNA

We try and obtain the maximum volume of vesicle-depleted medium possible. The amounts mentioned in the protocol are based on the use of a 50.2Ti rotor that will accommodate 12 centrifugation tubes allowing for a total 300 ml of medium to be centrifuged in a single run.

6. The duration of the culture will depend on the growth rate for each individual cell line. We maintain cultures in vesicle-depleted medium by collecting the cells by trypsinization, and seeding them at 1×10^6 cells in 11 ml of vesicle-depleted medium per dish for future isolations.
7. Collection of the supernatant should be carried out carefully to minimize contamination from cell debris. The pellet of cell debris may not always be readily visible.
8. The pellet of exosomes may not be visible. In that case, the location of the invisible pellet could be inferred from a mark made on the surface of the ultracentrifuge tubes.
9. Overnight precipitation of RNA in isopropanol at −20 °C may increase the yield of small RNAs [15].
10. A longer centrifugation time may improve the yield of small RNA.

11. It may be necessary to use a smaller amount of RNAse-free water to dissolve the RNA pellet in order to increase the RNA concentration of the suspension. In this case, the wash step could be repeated to reduce any potential effect of carryover of reagents or ethanol on either spectrophotometry or downstream experiments.
12. Different methods of isolation of RNA may yield different yields for microRNA, and the method used should be consistent to avoid any variations in yield or content of RNA isolated from nanovesicles [16].

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