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The Colorectal Cancer Microenvironment: Strategies for Studying the Role of Cancer-Associated Fibroblasts

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Abstract

Colorectal cancer (CRC) is a key public health concern and the second highest cause of cancer related death in Western society. A dynamic interaction exists between CRC cells and the surrounding tumor microenvironment, which can stimulate not only the development of CRC, but its progression and metastasis, as well as the development of resistance to therapy. In this chapter, we focus on the role of fibroblasts within the CRC tumor microenvironment and describe some of the key methods for their study, as well as the evaluation of dynamic interactions within this biological ecosystem.

Keywords

Colorectal cancer; Tumor microenvironment; Cancer-associated fibroblasts; Extracellular vesicles

1 Introduction

Colorectal cancer (CRC) is of significant public health concern, and represents 9% of all cancer incidence, and the third highest cause of cancer-related death in Western civilizations [1, 2]. CRC is a multipart cellular entity composed of malignant cancer cells, surrounded by a variety of non-transformed cell types, bound together by an extracellular matrix. These components assemble together to form the tumor microenvironment (TME), and interact with one another by a variety of means to create a functional and dynamic ecosystem, able to promote and enhance tumor initiation, growth, invasion, metastasis, and resistance to therapy [3, 4].

The seminal observations of Paget more than a century ago in his seed (cancer cells) and soil (the microenvironment) hypothesis demonstrate that the importance of the TME has long been appreciated [5]. Nevertheless, the underlying mechanisms for the importance of the TME and its constituents in promoting the hallmarks of cancer remained opaque for many years, as the enabling methods and techniques for dissecting the different inter-cellular and cell-matrix signalling systems were not available. Over the last few decades however, several innovative methods for the study of the different components of the TME, and more importantly their dynamic interactions with one another have been identified, and have dramatically enhanced our understanding of these complex interactions.

These techniques are helping decipher the processes taking place within the TME, and provide significant promise also for highlighting opportunities to manipulate the TME for

therapeutic gain in the future. Many current treatment strategies directly targeting CRC cells remain disadvantaged by the high genetic and chromosomal instability in CRC and the resistance to therapy that develops from the outgrowth of impervious clones under selective pressure [6, 7]. In stark contrast, the genetically stable stromal cells are therefore a more attractive alternative for therapeutic targeting and modulation of the TME [8].

In the present manuscript, we describe some of the key methods for the study of colorectal cancer-associated fibroblasts, and their interactions with cancer cells. Our focus is on methods for their isolation and in vitro culture; direct isolation and molecular profiling from human tumor tissue; examination of their signaling output via extracellular vesicles (EVs); their utility in 3-dimensional in vitro coculture assays; and finally evaluation of their role in in vivo models of CRC.

Deciphering the complex cross talk and interplay that exists between components of the TME with these methods will advance our understanding of tumor behavior, and help pinpoint novel opportunities for therapeutic manipulation, facilitating continued progression toward more personalized treatment approaches.

2 Materials

2.1 Isolation and Culture of Fibroblasts

1. 10 cm diameter dish.
2. Forceps and scalpel.
3. 12-well plate.
4. Phosphate buffered saline (PBS) supplemented with 2% (double-strength) penicillin–streptomycin (penicillin, 200 units/mL—streptomycin, 200 µg/mL) and 0.1% amphotericin B (Fungizone) (*see Note 1*).
5. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal calf serum (FCS), 2% penicillin–streptomycin, and 0.1% amphotericin B.
6. Ciprofloxacin (BIOMYC-3 100×).
7. Trypsin–EDTA solution (0.25%).

2.2 Molecular Profiling by Laser Capture Microdissection

1. Cryostat.
2. Mounted slides.
3. Microtome.
4. Xylene.
5. Ethanol (75%, 100%).
6. Cresyl Violet.
7. Laser microdissection (LMD) platform (e.g., Leica AS, Leica Microsystems).

8. Lysis buffer.
9. RNAqueous®-Micro Kit.
10. RecoverAll™ kit.
11. NanoDrop™ 1000 spectrophotometer.
12. Agilent 2100 Bioanalyzer with RNA 6000 PicoChip.

2.3 CRC Fibroblast EVs

2.3.1 Isolation of EVs from CRC Fibroblasts

1. EV-depleted FCS (FCS ultracentrifuged at $100,000 \times g$ for 16 h).
2. 50 mL falcon tubes.
3. Polycarbonate ultracentrifuge tubes.
4. Ultracentrifuge/rotor (e.g., Sorvall Discovery 100 s/TYP TFT 50.38 rotor).
5. Appropriate solvent for downstream analysis: PBS/protein lysis buffer/RNA lysis buffer/sterile water.

2.3.2 Labelling and Transfer of EVs

1. Protein Lysis Buffer
2. Vybrant Cell-Labeling Solution (Molecular Probes).
3. BCA Protein Assay Kit.

2.3.3 Extraction of RNA from EVs

1. RNase-free water.
2. 100% ethanol.
3. RNase-free 1.5–2 mL tubes.
4. miRNeasy Micro Kit (Qiagen).

2.4 Organotypic Coculture Systems with CRC Fibroblasts

1. Culture medium components: Collagen type I (rat-tail), Matrigel, FCS, 10× DMEM, 1× DMEM.
2. Other components: Fibroblasts/stromal cells of choice, 25% glutaraldehyde, 24- and 6-well plates, steel grids, nylon mesh, full keratinocyte growth medium (KGM) of choice (without cholera toxin).

2.5 Cancer-Associated Fibroblasts In Vivo

1. 6–8-week-old immuno-incompetent mice, e.g., SCID or nude mice.
2. Sterile hood with laminar flow and oxygen, and inhalational anesthetic agent, e.g., Isoflurane.

3. Operating surgical loupes with 2.5–3.5× magnification.
4. Sterile small animal surgery equipment: sterile drapes and skin prep, e.g., 10% Povidine–iodine, small animal clipper, warming pad.
5. 1 mL Micro-Fine 29–30 Gauge syringe (or Hamilton syringe), sterile saline, sterile 4/0 absorbable suture material (e.g., polyglactin 910 suture).

3 Methods

3.1 Isolation and Culture of Colorectal Fibroblasts

1. Collect fresh tumor and normal colorectal mucosa (ensure institutional ethical approval and patient consent) in PBS supplemented with antimicrobials (*see* Note 2).
2. Wash three times with PBS and do not aspirate after final wash.
3. Divide sample into 2 mm sections using sterile scalpel and forceps.
4. Score the bottom of a 12-well plate with a cross (“X”).
5. Place tissue sections on to the center of each cross.
6. Add 750 μ L medium (DMEM supplemented with 20% FCS and antimicrobials) to each plate, while ensuring the specimen 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 (Fig. 1a, b).
9. When 70% confluent, expand into a T25 flask (usually 4–6 weeks). Add BIOMYC-3 (1/100) to the growth medium for the first expansion.

3.2 Laser Microdissection of CRC Stroma

1. Fresh frozen specimens: use cryostat to divide specimen into 8–10 μ m thickness sections on to membrane mounted slides.
2. FFPE specimens: use microtome to section specimen and deparaffinize in xylene for 1 min.
3. Fix tissue sections in 75% ethanol for 30 s.
4. Stain with Cresyl Violet for 1 min.
5. Dehydrate in 100% ethanol for 1 min.
6. Leave sections to air dry.
7. Use LMD platform according to manufacturer’s instructions to separate epithelial from stromal layers, producing a $1 \times 10^6 \mu\text{m}^2$ cut from each layer.
8. Collect microdissected specimen in 50 μ L lysis buffer (Fig. 1c–e).

9. Proceed immediately to total RNA extraction using RNAqueous®-Micro Kit for fresh-frozen tissue or Recover-All™ kit for FFPE in accordance with the manufacturer's instructions.
10. Assess yield and purity of RNA using spectrophotometer and/or bioanalyzer.

3.3 Isolation of EVs from Primary Ex Vivo CRC Fibroblasts

1. Primary fibroblasts should be cultured in FCS-depleted medium for 48–72 h prior to isolation of exosomes (*see* Notes 3–5).
2. Harvest the conditioned media from cells being studied, and transfer to a 50 mL falcon tube.
3. Centrifuge at $500 \times g$ for 5 min at room temperature to pellet out floating cells. It is not necessary to decant supernatant at this step (*see* Note 6).
4. Centrifuge again at $2000 \times g$ for 10 min at room temperature to pellet out debris.
5. Decant supernatant into fresh 50 mL falcon tubes and centrifuge at $10,000 \times g$ for 30 min at 4 °C to pellet out microparticles/apoptotic bodies.
6. Transfer equal volumes of supernatant into ultracentrifuge tubes. Ensure tubes balance each other to within limits stated by the manufacturer.
7. Place tubes into ultracentrifuge rotor. Centrifuge at $100,000 \times g$ for 70 min at 4 °C (*see* Note 7).
8. The 1–2 mm diameter EV pellet will be visible on the radial aspect of ultracentrifuge tube. Carefully aspirate supernatant without disturbing pellet.
9. Resuspend the EV pellet in 200 μ L PBS and pool samples together in one ultracentrifuge tube.
10. In the event that you cannot see a pellet, aspirate all but 1 mL of supernatant, mix well, and add this to the pool.
11. Fill tube to capacity with PBS.
12. Ultracentrifuge again at $100,000 \times g$ for 70 min at 4 °C and aspirate supernatant as above.
13. Resuspend washed EV pellet in 200 μ L PBS (for cell transfer), 200 μ L protein lysis buffer (western blotting), 700 μ L QIAzol Lysis Reagent (RNA extraction), or 50 μ L sterile water (for imaging by transmission electron microscopy) (*see* Note 8).
14. Store samples at 4 °C for short term (<1 week) or –80 °C for longer term. Samples for electron microscopy should not be frozen (*see* Note 9).
15. EV samples should be validated as follows: transmission electron microscopy with negative staining (e.g., 5% ammonium molybdate/1% trehalose) for size and morphology (Fig. 2a); western blotting for presence of transmembrane (e.g., CD63 and CD81) and endosomal (e.g., ALIX and TSG101) markers, and

absence of other organelle markers (e.g., GM130 and cytochrome C); as well as nanoparticle tracking analysis (Nanosight™; Fig 2b).

3.4 Organotypic Coculture with CRC Fibroblasts

3.4.1 Day 1—Gels

1. Prepare a cell suspension of fibroblasts at 250,000 cells/gel in 1× DMEM.
2. Make up gels in the following ratio:

(a) Collagen: matrigel (1:1)	7 volumes
(b) 10× DMEM	1 volume
(c) FCS	1 volume
(d) CRC fibroblasts	1 volume

3. Add 1 mL of the above mix into each well of a 24-well plate.
4. Incubate at 37 °C for 1 h.
5. Add 1 mL of 1× DMEM on top and incubate overnight at 37 °C.

3.4.2 Day 2—Plating out CRC Cells

1. Aspirate medium from the top of the gels.
2. Plate out desired number of tumor cells (usually 500,000 per gel in 1× DMEM).
3. Incubate overnight at 37 °C.
4. Place required number of sterile nylon sheets in a 10 cm dish.
5. Make up gel coat for nylon sheets (250 µL of mix per sheet):

(a) Collagen	7 volumes
(b) 10× DMEM	1 volume
(c) FCS	1 volume
(d) 1× DMEM	1 volume
NB If the solution is yellow, neutralize with 0.1 M NaOH until it turns pink	

6. Add 250 µL gel coat to each nylon sheet and incubate at 37 °C for 15–30 min.
7. Make up 1% glutaraldehyde (in PBS) and add 10 mL to each dish. Incubate at 4 °C for 1 h.
8. Wash three times with PBS and once with 1× DMEM and leave in 1× DMEM overnight at 4 °C.

3.4.3 Day 3—Raising Gels on to Grids

1. Place a steel grid into each well of a 6-well plate and place a gel-coated nylon sheet on it (with sterile forceps).
2. Remove gel from 24-well plate using a sterile spatula and place it on the nylon sheet (*see* Note 10).
3. Fill the well with KGM such that it reaches the undersurface of the grid (4.8 mL KGM).
4. Replace medium every two days.
5. Harvest after 14 days of culture (or less if RNAi or treatments are added).
6. Remove whole organotypic from well and cut in half with disposable scalpel on cling film.
7. Fix both halves in a universal container filled with 10% formol saline for 24 h.
8. Replace formol saline with 70% ethanol after 24 h and leave overnight.
9. Section and mount as required (Fig. 3).

3.5 Coinjection of Colorectal Fibroblasts in Subcutaneous Location

1. Mix CRC cells and fibroblasts at desired ratio for experimental protocol.
2. Add Matrigel to the cell mixture (1:1 volume)
3. Transfer to container of choice and keep on ice until ready for injection.
4. Clip the hair on the intended surgical site on 6–8-week-old SCID mice (*see* Note 11).
5. Anesthetize under sterile conditions in a laminar flow hood.
6. Position on a warming pad, perform skin preparation over the operative field with 10% povidine–iodine, and drape the skin to expose the surgical site.
7. Inject 0.5 mL of sterile saline subcutaneously at desired site to raise a skin bleb.
8. Inject cell-Matrigel suspension into bleb.
9. Compress for 1 min and recover animal in recovery chamber.
10. Observe until fully recovered from anesthesia.
11. Monitor for tumor growth or metastasis by physical (size of tumor, weight loss, general health, etc.) or radiological parameters (e.g., small animal imaging units).

3.6 Coinjection of Colorectal Fibroblasts in Orthotopic Location

1. Mix CRC cells and fibroblasts at desired ratio for experimental protocol.
2. Add Matrigel to the cell mixture (1:1 volume).
3. Transfer to container of choice and keep on ice until ready for injection.

4. Clip the hair on the intended surgical site on 6–8-week-old SCID mice (*see Note 11*).
5. Anesthetize under sterile conditions in a laminar flow hood.
6. Position on a warming pad, perform wide abdominal skin preparation with 10% povidine–iodine, and drape the skin to expose the surgical site.
7. Lift the skin in the midline with a pair of toothed forceps and perform a careful incision longitudinally with a sharp pair of dissecting scissors.
8. Lift the incised area and extend the incision cephalad and caudally to desired length for adequate exposure (Fig. 4a).
9. With a pair of small atraumatic bowel grasping forceps (e.g., DeBakey or Tuttles forceps) gently grasp the cecum which is located typically in the left iliac fossa in mice and deliver into the wound area where it can be visualized (*see Note 12*).
10. Gently grasp the cecum to steady it and inject the cell suspension prepared earlier just under the surface of the colon to raise a bleb in the submucosal plane (*see Note 13*; Fig. 4b).
11. Press with a sterile cotton bud on the injection site for 1 min.
12. Return the bowel to the abdominal cavity and close the midline incision in layers with a continuous 4/0 suture.
13. Recover animal in recovery chamber and observe until fully recovered from anesthesia.
14. Monitor for tumor growth or metastasis by physical (weight loss, general health, etc.) or radiological parameters (e.g., small animal imaging units; Fig. 4c), or by evaluate metastatic pattern by organ collection and histological analysis at the end of the experiment (Fig. 4d).

4 Notes

1. Penicillin–streptomycin usually comes as penicillin (10,000 units/mL) and streptomycin (10 mg/mL). Fungizone comes as amphotericin B 250 µg/mL. Working concentrations are given in the “Materials” section.
2. Ensure rapid transfer from theater to laboratory to diminish cold ischemia time.
3. Each “T175” flask should contain 16–18 mL medium to concentrate exosomes without depriving cells.
4. 12× “T175” flasks of confluent fibroblasts ($3\text{--}4 \times 10^6$ cells/flask) will produce EVs from which approximately 300–400 ng RNA can be extracted. This is adequate for most downstream analyses.
5. Cancer cells produce approximately 3–4 times more EVs than fibroblasts.
6. Serial centrifugation is carried out to avoid lysing cells and releasing organelles into the supernatant.

7. We use a fixed angle rotor because the EV pellet is seen clearly on the radial aspect of the tube. A swinging bucket rotor can also be used.
8. The final EV pellet is resuspended in whichever solvent is most appropriate for downstream analysis.
9. Samples for electron microscopy should not be frozen as this leads to artifact from ice crystals.
10. This is a critical step—be gentle with the gel and make sure it is orientated the right way up.
11. To avoid cells staying on ice too long, shaving of the operative site and other set up aspects should be done prior to splitting/mixing of the cells.
12. The bowel wall in mice is thin and can be damaged if grasped too firmly leading to a postoperative perforation. Where possible push and manipulate without actual grasping of the bowel and never use toothed forceps.
13. It is important to avoid intra-luminal injection of the cell suspension as this will not lead to tumor development. Using operating loupes for magnification, and ideally a head light and careful injection to raise a bleb are critical.
14. Isolation of primary colorectal fibroblasts and EVs has been updated from our previous description [9].

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Fig. 1.

Normal and cancer associated ex vivo fibroblasts and laser microdissection. **(a)** Low power magnification showing dissected human CRC tumor tissue in a 12-well plate on the “X” made in the bottom of the plate; **(b)** 10x magnification of ex vivo cancer associated fibroblasts in culture next to the “X” **(c–e)** Laser capture microdissection of the stromal component of fresh frozen human colorectal cancer tumor tissue. The tumor is stained with Cresyl Violet to highlight epithelial tumor and stromal compartments and visualized **(c)**, marked for microdissection **(d)**, and captured **(e)**. Tumor after microdissection **(f)**

Fig. 2. Characterisation of EVs. Transmission electron micrograph (120,000 $\times$) of a single EV demonstrating bilayered morphology and size in the order of 100 nm (a); Size distribution of EVs by Nanoparticle Tracking Analysis: modal size 99 nm (b)

Fig. 3. Haematoxylin and Eosin staining of typical three-dimensional organotypic cocultures of CRC cells and ex vivo CRC fibroblasts (normal and cancer-associated fibroblasts) in a 1:1 Rat-Tail Collagen:Matrigel matrix (Fibroblast-Matrigel). Cancer-associated fibroblasts can enhance invasion of CRC into the simulated stromal model

Fig. 4.

Orthotopic in vivo model to study the role of colonic fibroblasts in tumor formation and metastasis. Mice are anesthetized by inhalational anesthesia and skin preparation and draping conducted. In a sterile laminar flow cabinet with full aseptic measures, a midline laparotomy is performed under magnified vision with operating loupes and a headlight (**a**). The cecum and terminal ileum are identified and exteriorized, and the cell suspension injected under direct vision to raise a clearly demarcated bleb on the surface of the cecum (**b**). After a variable length of time, mice are humanely culled and evaluated for distant metastases by imaging (**c**) and/or gross (**d–f**) or microscopic pathological analysis. Typical organs harvested include the lungs (**d**); the liver and spleen (**e**) (containing CRC metastases in the former but not latter); and the primary site of tumor injection in the cecum (**f**)