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SOP –ADSI-2.0	Author: **R. Gstir; P. Filipek; D. Lesiak** Approved: **Lukas Huber**	Issued: **25/03/2022** Revised:

ISOLATION AND MAINTENANCE OF CANCER ASSOCIATED FIBROBLASTS

Purpose

The SOP-ADSI-2.0 was issued to describe how to isolate, maintain, expand, and cryopreserve cancer-associated fibroblasts (CAFs) from colon cancer and how to generate CAFs conditioned media, for application in drug screening in co-culture with tumor organoids.

Scope

SOP 2.0 is intended to cover all resources, personnel and equipment needed for CAFs isolation, maintenance, expansion, and cryopreservation aswell as generation of CAFs conditioned media.

Introduction

Colonic cancer tissues were obtained upon surgical resection from the Medical University of Innsbruck. Patients were diagnosed with chemo-resistant colorectal cancer. Normal and cancer intestinal tissue have been passed to the Austrian Drug Screening Institute GmbH for the cultivation of organoids, isolation of CAFs and generation of CAFs conditioned media.

1. Reagents, solutions, and cell culture media

1.1 Reagents

- DPBS (1X) without Ca2+, Mg2+ (REF14190-144 Gibco)
- Penicillin-Streptomycin (P/S) (#15070063 Life Technologies)
- DMEM (1X) + GlutaMAX (REF31966-021 Gibco)
- Primocin 500 mg (Cat.# ant-pm-1 InvivoGen)
- Liberase DH (REF05401054001 Roche)
- Y-27632 (#120129 Abcam) dissolve powder in sterile H2O

- Sterile water
- RBC Lysis Buffer 10X (#420310 BioLegend) dilute in sterile H2O
- 0.25 % Trypsin-EDTA (#T4049-100ML Sigma-Aldrich)
- DMSO Hybri-Max (#D2650 Sigma-Aldrich)
- Advanced DMEM/F-12(Gibco-Thermo Fisher #12634028)
- Glutamax 100 x (GIBCO#35050038)
- HEPES (1 M) (GIBCO#15630080)
- RPMI Medium 1640 (1X) (REF52400-025 Gibco)
- Fetal Bovine Serum (#S1810-500 Biowest)

1.2 Compositions

- **DPBS + Pen/Strep**

500 mL DPBS (1x)

5 mL P/S 100x

- **GF-**

500 mL Advanced DMEM/F12

5 mL P/S (100X)

5 mL of HEPES (1M = 100X)

5 mL of Glutamax (100X)

Store at 4 °C

- **CAFs cultivation medium**

500 mL RPMI-1640

50 mL Fetal bovine serum 100%

5 mL P/S 100X

- **RBC Lysis Buffer 1X**

300 µl of RBC Lysis Buffer

2.7mL of sterile H2O

2. Equipment

- Incubator Binder APT. lineTM (150CE2)
- Laminar Air-Flow Labculture Plus ESCO, Class II BSC
- Centrifuge (4°C) Hettich Zentrifugen ROTIXA A50 RS
- Brightfield microscope Motic AE31 and camera ProgRes CF cool JENOPTIKA
- Table centrifuge VWR Mini Star
- Analog Vortex Mixer (VWR)
- Waterbath
- Sterile scalpel + blades
- Set of pipettes 10 µL, 200 µL, 1000 µL and pipette tips
- Pipette Aid and pipettes (10 mL, 25 mL, 50 mL)
- Box with crashed ice
- Dissection tweezers (#11150-10, #11254-20, #11274-20 Fine Science Tools)
- Scissors (#14058-09, #14059-09, #14110-15 Fine Science Tools)
- Mr. Frosty freezing container (#5100-0001 Thermo Fisher Scientific)

3. Plastics

- 100 cm petri dish #83.3902 SARSTEDT))
- 50 mL conical tubes (#ET5050B Euroclone)
- 15 mL conical tubes (#ET5015B Euroclone)
- Cryovials (#E.3110-6122 STAR LAB)
- pluriStrainer 100µm (#43-50100-03 pluriSelect)
- pluri-Connector Ring (#41-50000-03 pluriSelect)
- Cell culture flask, T-75 surface: standard, filter cap (83.3911.002 Sarstedt)
- Cell culture flask, T-25 surface: standard, filter cap (83.3910.002 Sarstedt)
- Sterile syringes, 10 mL (#4606108V Braun)
- Sterile Syringe Filters 0.45 µm PES

4. Procedure for the collection of normal and tumor tissue

Collect the dissected healthy tissue and tumor tissue directly from the operation th eatre and put it on ice! The tumor is immediately transferred to the pathology. Contact the pathologist to do the routine inspection of the tissue. Collect tissue from the tumor and healthy intestinal tissue independently in DPBS + Pens/Strep on ice (take care to prevent cross contamination of the tumor and the healthy tissue). Transfer tissue and patient agreement as fast as possible to the lab (not longer than 1-2 hours). The isolation of the CAFs as well as tumor cells and intestinal crypts should be performed in parallel by independent laboratory staff to minimize the time of ischemia. Perform all procedures on ice unless indicated otherwise.

5. Isolation of cancer-associated fibroblasts (CAFs) from tumor tissue

- Transfer tumor tissue with sterile forceps in a sterile 50 mL conical tube with 10 mL ice-cold DPBS + Pen/Strep (4°C). Shake 10-15 times, remove the PBS + Pen/Strep and replace with 10 mL ice-cold PBS + Pen/Strep. Repeat this process 2 more times until the supernatant no longer contains any visible debris.
- Collect tumor tissue then in a 10 cm petri dish. Dissect tumor tissue with sterile scissor into approximately 2-5 mm cubes. A portion of two to three tissue fragments is used for isolation of CAFs. The remaining ones are used for generation of intestinal organoids (SOP-ADSI-1.0).
- For isolation of CAFs, homogenize tumor tissue with a pair of sterile scissors and place in a 50 mL conical tube with DMEM + Pen/Strep + Primocin 100 µg/mL + Liberase 50 µg/mL(0.26 units/mL) + Y-27632 10 µM for 1 hour at 37°C at 250 rpm (1 g of tissue in 10 mL). shake vigorously every 15 min.
- Pass this fraction through a 100 µm cell strainer to remove debris. Add 2.5 – 5 mL of PBS + Pen/Strep to wash the cell strainer. Collect filtered solution in a 50 mL conical tube.
- Centrifuge single cells at 1200 rpm for 5 minutes at 4 °C. Discard supernatant.
- Resuspend the pellet with 3 mL of RBC Lysis buffer 1x (dilute in sterile H20). Incubate for 10 min at room temperature in the dark. Neutralize lysis adding 10 mL of CAFs cultivation medium.
- Centrifuge at 1200 rpm for 5 minutes at 4 °C. Discard supernatant.

- Resuspend the pellet with 5 mL of CAFs cultivation medium and pass this fraction through a 100 µm cell strainer to remove debris (optional). Collect the filtered solution in a 50 mL tube.
- Centrifuge at 1200 rpm for 5 minutes at 4 °C. Discard supernatant.
- Resuspend the pellet with 5 mL of CAFs cultivation medium.
- Centrifuge at 1200 rpm for 5 minutes at 4 °C. Discard supernatant.
- Resuspend the pellet with 1 mL of CAFs cultivation medium. Plate resuspended pellet in a T25 flask with 10 mL of CAFs cultivation medium for three days (37 °C, 5 % CO2, 95 % r.H.).

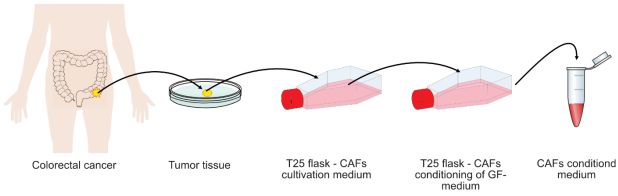

Figure 1. Schematic procedure - isolation of CAFs and generation of CAFs conditioned GF- media in T25 flask

6. Cultivation and expansion of CAFs

- After isolation cultivate CAFs in T25 flask for 1-2 weeks prior to first passage and exchange medium every 2-3 days. Therefore, aspirate old medium and apply 5 mL of new pre-warmed CAFs cultivation medium.
- For passaging CAFs into new flask, aspirate old medium and wash cells gently with pre-warmed DPBS 1x (3 mL for T25 flask, and 5 mL for T75 flask). Aspirate DPBS 1x and incubate CAFs with Trypsin-EDTA (1 mL for T25 flask, and 3 mL for T75 flask) for 7 min (37 °C, 5 % CO2, 95 % r.H.). Detach CAFs from bottom of flask, add pre-warmed CAFs cultivation medium (4 mL for T25 flask, and 7 mL for T75 flask) to inactivate Trypsin-EDTA. Resuspend CAFs and transfer into a new 50 mL conical tube. Centrifuge at 125 rpm for 5 min at room temperature. Aspirate supernatant and resuspend cell pellet carefully in 3 mL of pre-warmed CAFs cultivation medium. Add cell suspension into a new flask containing CAFs cultivation medium (5 mL total for T25 flask, and 15 mL total for T75 flask). In general passage CAFs 1:3 every 1-2 weeks, sufficient density assumed (approximately 80 % confluency).

7. Cryopreservation of CAFs

- Cryopreserve approximately 0.5 x 106 cells at -80 °C. Collect cells according to 6.2. with freezing medium (CAFs cultivation medium + 10 % DMSO) into cryovial.
- Snap freeze CAFs in cryovials with liquid nitrogen and place cryovials as fast as possible into freezing container placed at -80°C until final storage.
- For final storage move cryovials to liquid nitrogen tank.

8. Generation of CAFs conditioned medium

- Grow CAFs to confluency (see *Figure 2*) in T25 flasks with CAFs cultivation medium.
- Rinse confluent CAFs in T25 flask gently with 5 mL of DPBS 1x. Add 5 mL of pre-warmed GF- media and return flasks to 37 °C with 5 % CO2 and 95 % r.H. Collect as required after 24, 48 or 72 hours the conditioned medium. Double sterile filter the conditioned media with an eccentric tip syringe and sterile nonpyrogenic 0.45 µm filter.
- The conditioned medium can be stored at -20 °C or used directly.

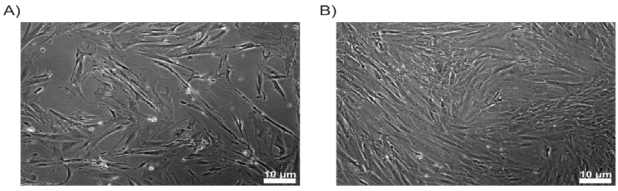

Figure 2. Cultivation of CAFs for generation of CAFs conditioned GF- media in T25 flask. Representative brightfield images of CAFs cultivated in CAFs cultivation medium. Size bar ≙ 10 µm A) Sub-confluency of CAFs B) CAFs grown to confluency.

9. Applicable to ADSI SOPs:

- SOP-ADSI-1.0
- SOP-ADSI-3.0

10. References

Naruse M, Ochiai M, Sekine S, Taniguchi H, Yoshida T, Ichikawa H, et al. Re-expression of REG family and DUOXs genes in CRC organoids by co-culturing with CAFs. Sci Rep-uk. 2021;11(1):2077.

Lebret SC, Newgreen DF, Thompson EW, Ackland ML. Induction of epithelial to mesenchymal transition in PMC42-LA human breast carcinoma cells by carcinoma-associated fibroblast secreted factors. Breast Cancer Res. 2007;9(1):R19–R19.

NOTES