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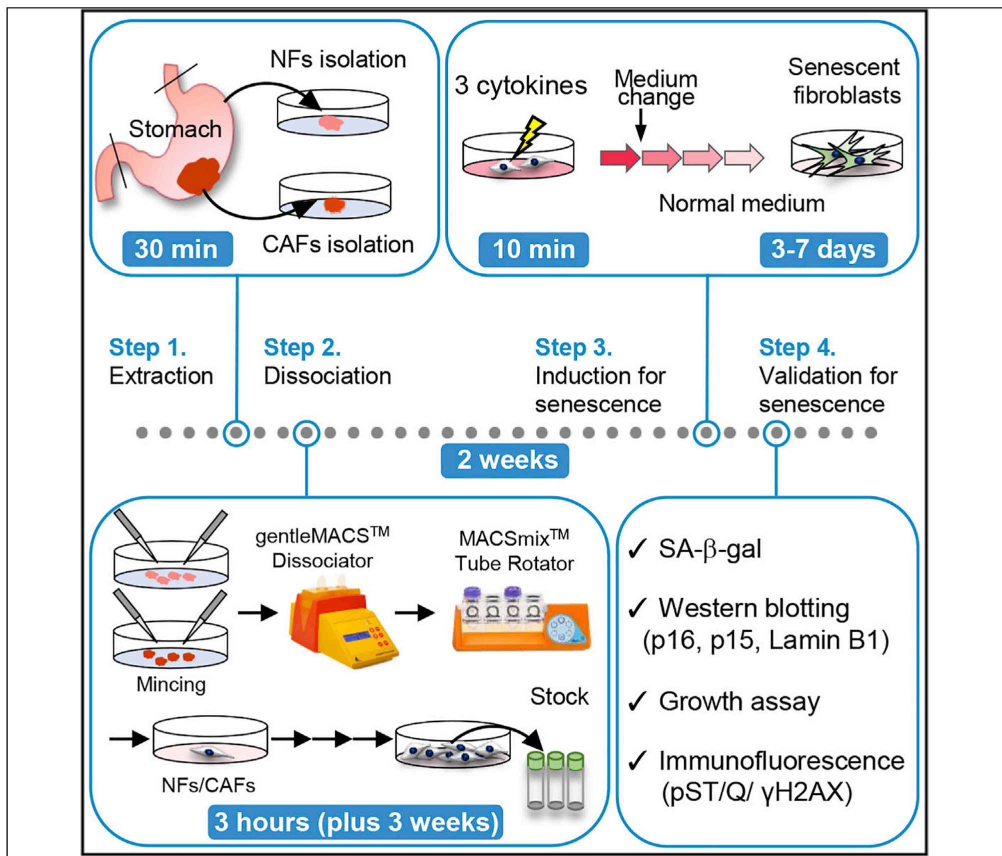
Protocol to establish cancer-associated fibroblasts from surgically resected tissues and generate senescent fibroblasts



Accumulating evidence suggests that the malignant behavior of cancer is influenced by stromal activity in the tumor microenvironment. Cancer-associated fibroblasts (CAFs), which are the main component of the cancerous stroma, play an important role in cancer development. Here, we describe a protocol to establish CAFs from surgically resected tissues. CAFs could be a vital tool for understanding the microenvironment and its impact on tumor progression and metastasis. Moreover, we generated inflammation-induced senescent fibroblasts that more closely mimic the tumor microenvironment.

Tadahito Yasuda, Mayu Koiwa, Atsuko Yonemura, Takahiko Akiyama, Hideo Baba, Takatsugu Ishimoto

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Highlights

Detailed protocol for extracting primary fibroblasts from human gastric cancer

High isolation rate of fibroblasts can be achieved

Protocol to induce senescent fibroblasts by inflammatory cytokines

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Protocol to establish cancer-associated fibroblasts from surgically resected tissues and generate senescent fibroblasts

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SUMMARY

Accumulating evidence suggests that the malignant behavior of cancer is influenced by stromal activity in the tumor microenvironment. Cancer-associated fibroblasts (CAFs), which are the main component of the cancerous stroma, play an important role in cancer development. Here, we describe a protocol to establish CAFs from surgically resected tissues. CAFs could be a vital tool for understanding the microenvironment and its impact on tumor progression and metastasis. Moreover, we generated inflammation-induced senescent fibroblasts that more closely mimic the tumor microenvironment.
For complete details on the use and execution of this protocol, please refer to Yasuda et al. (2021).

BEFORE YOU BEGIN

Preparation of reagents for primary fibroblast isolation

Timing: 20 min

1. Prepare culture medium and wash buffer (detailed recipe is available in the materials and equipment section)
   a. Culture medium: Prepare RPMI media containing 10% FBS by adding 50 mL of FBS to 450 mL of RPMI 1640.
   b. Wash buffer: Prepare 1× PBS containing 100 units/mL penicillin and 100 μg/mL streptomycin. Add 1 mL of 100× penicillin/streptomycin (Cat# 168-23191) to 99 mL of 1× PBS.

2. Prepare enzymes (H, R, and A provided in the Miltenyi Biotec Tumor Dissociation Kit using the manufacturer's protocol)/reagents.

   Note: Tumor Dissociation Kit: https://www.miltenyibiotec.com/US-n/products/tumor-dissociation-kit-human.html
   a. Prepare Enzyme H by reconstituting the lyophilized powder in each vial with 3 mL of RPMI 1640.
   b. Prepare Enzyme R by reconstituting the lyophilized powder in each vial with 2.7 mL of RPMI 1640.

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c. Prepare Enzyme A by reconstituting the lyophilized powder in each vial with 1 mL of Buffer A.

These reagents are recommended by Miltenyi Biotec for gentleMACS Dissociator. The gentleMACS Dissociator and these reagents are recommended for tissue digestion to keep the isolation rate of fibroblasts.

Preparation of reagents for the induction of senescent fibroblast sections

Timing: 10 min

3. Prepare 3 cytokines containing medium for the induction of senescent fibroblast.
   a. 3 cytokines containing medium: Prepare culture medium containing 3 cytokines (10 ng/mL recombinant human IL-1α + 10 ng/mL recombinant human IL-1β + 10 ng/mL recombinant human TNF-α)

   Note: We used the combination of three inflammatory cytokines (IL-1α, IL-1β, and TNF-α; 3 cytokines) in the current study based on our previous finding that the expression level of each cytokine was not associated with the prognosis of patients with gastric cancer and the simultaneous high expression of IL-1α, IL-1β and TNF-α was significantly associated with a shorter 5-year overall survival, suggesting that simultaneous stimulation with 3 cytokines causes gastric cancer progression and leads to a poor prognosis (Ishimoto et al., 2017).

   Note: 3 cytokines containing medium should be made each time to prevent inactivation of cytokines.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Alexa Fluor 488 goat anti-rabbit IgG	Molecular Probes	Cat# A11008
Alexa Fluor 594 goat anti-mouse IgG	Molecular Probes	Cat# A11005
Goat anti-mouse IgG-HRP	Santa Cruz	Cat# sc2005
Goat anti-rabbit IgG-HRP	Cell Signaling Technology	Cat# 7074
Mouse monoclonal to gH2AX	Millipore	Cat# 05-636
Mouse monoclonal to p15 INK4B	Santa Cruz	Cat# sc271791
Rabbit pSTQ	Cell Signaling Technology	Cat# 2851
Rabbit Lamin B1	Abcam	Cat# ab16048
Rabbit monoclonal to p16 INK4A	Abcam	Cat# ab108349
Rabbit polyclonal to anti-b-Actin	Cell Signaling Technology	Cat# 4967
Biological samples		
Human patient materials	Kumamoto University Hospital	N/A
Chemicals, peptides, and recombinant proteins		
Accutase cell detachment solution	Innovative Cell Technologies	Cat# AT-104
Albumin, from Bovine Serum (BSA), Crystallized	Wako	Cat# 012-15093
Bambanker	NIPPON Genetics	Cat# CS-02-001
Can Get Signal® Immunoreaction Enhancer Solution 1, 2	Toyobo	Cat# NKB-101
Cellstain DAPI solution	Dojindo	Cat# 340-07971
Fetal bovine serum (FBS)	Capricorn Scientific	Cat# FBS-12A
Incucyte® NucLight Rapid Red Reagent for nuclear labeling	Essen BioScience	Cat# 4717
Penicillin/streptomycin (×100)	FUJIFILM Wako Pure Chemical Corporation	Cat# 168-23191
RPMI 1640	FUJIFILM Wako Pure Chemical Corporation	Cat# 189-02145

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Senescence Detection Kit	BioVision	Cat# K320
Tumor Dissociation Kit, human	Miltenyi Biotec	Cat# 130-095-929
Recombinant DNA		
Recombinant Human IL-1α	Bay Biosciences	Cat# 8019
Recombinant Human IL-1β	Bay Biosciences	Cat# 8018
Recombinant Human TNF-α	Bay Biosciences	Cat# 8329
Software and algorithms		
BZ-X Analyzer	Keyence	https://www.keyence.co.jp/
Incucyte® S3 Live-Cell Analysis System	Essen BioScience	https://www.essenbioscience.com/ja/products/incucyte/incucyte-s3/
Other		
100 mm Collagen-coated dishes	IWAKI	Cat# 4020-010
Disposable Scalpel No. 11	FEATHER Safety Razor	Cat# no. 11
gentleMACS C Tubes	Miltenyi Biotec	Cat# 130-093-237
gentleMACS Dissociator	Miltenyi Biotec	Cat# 130-093-235
MACSmix Tube Rotator	Miltenyi Biotec	Cat# 130-090-753
MACS SmartStrainers (70 μm)	Miltenyi Biotec	Cat# 130-098-462

Alternatives: Any microscope that has brightfield and fluorescence functions can be used instead of the BZ-X Analyzer.

Alternatives: Primaria dishes (Cat# 353802, Corning) or tissue culture-treated dishes (Cat# 430166, Corning) coated with collagen (Cat# 354236, Corning) could be used instead of collagen-coated dishes (IWAKI).

MATERIALS AND EQUIPMENT

Culture medium		
Reagent	Final concentration	Amount
RPMI-1640	NA	450 mL
FBS	10%	50 mL
Total	NA	500 mL
Store at 4°C for up to 1 month		

Wash buffer		
Reagent	Final concentration	Amount
PBS	NA	99 mL
Penicillin/streptomycin	1% Pen-100 units/mL Strep-100 μg/mL	1 mL
Total	NA	100 mL
Store at 4°C for up to 2 weeks		

STEP-BY-STEP METHOD DETAILS

Extraction of fibroblasts from surgical resections

Timing: 30 min

This step describes how to extract cancer tissues and paired noncancerous tissues from surgical resections (Figure 1).

Note: The collection of human material must conform to all relevant institutional/governmental regulations. Informed consent was obtained from all subjects prior to collecting the primary human tissue materials.

1. Patient-derived material was obtained from stomach resections (total gastrectomy or distal gastrectomy) of primary cancers (Figure 1A).
2. CAFs were extracted from gastric cancer tissues and paired NFs were extracted from noncancerous tissues using a scalpel. NFs should be collected at least 5 cm away from cancer. (Figure 1B)

Note: In this protocol, a trained surgeon should be involved in the separation of gastric cancer (GC) and adjacent normal tissue as soon as possible after surgery. CAFs are isolated from advanced GC. Thus, the borderline between cancer and normal tissue is obvious. However, the borderline of type 4 GC would not be clear. In such a case, the tissue stiffness using palpable sensation is useful for determining the borderline.

Note: Extraction included the submucosa. All layers are not needed for extraction.

3. The dissected tissues were prepped by washing at least 3 times using wash buffer.

Note: Wash tissues using only gentle inversion.

4. The tissues were transferred to culture medium and placed on ice.

Dissociation of tissues for the generation of a single-cell suspension

Timing: 3 h (plus 3 weeks for culture expansion)

This step describes how to dissociate fibroblasts from collected tissues. The procedure follows the instrument manufacturer's protocols with a few modifications.

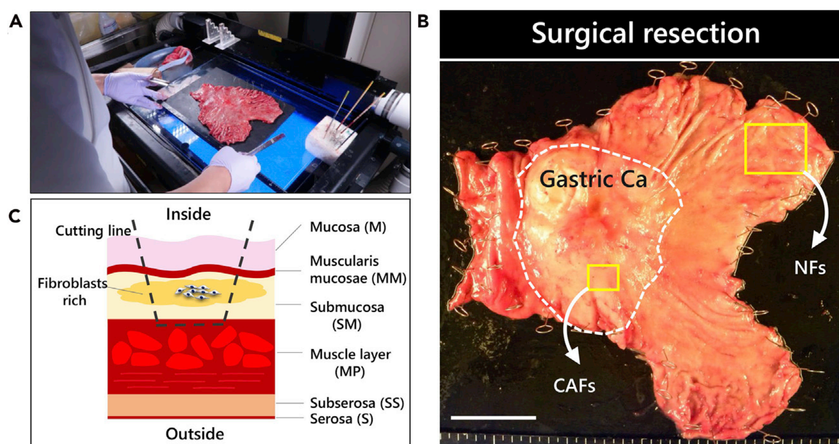

Figure 1. Extraction from surgically resected tissues
(A) Patient-derived materials were obtained from stomach resections.
(B) CAFs extracted from gastric cancer tissues (white dotted line) and paired NFs extracted from noncancerous tissues. Scale bar, 5 cm.
(C) Schema of the layer structure of the stomach.

Tumor Dissociation Kit: https://www.miltenyibiotec.com/US-n/products/tumor-dissociation-kit-human.html#gref

5. Prepare the enzyme mix by gently adding 4.7 mL of RPMI 1640, 200 μL of Enzyme H, 100 μL of Enzyme R, and 25 μL of Enzyme A into a gentleMACS C tube.
6. Using scalpels, mince each material into small pieces that are approximately 2–4 mm in size (Figure 2A).
7. After mincing, transfer the tissue pieces into a gentleMACS C tube containing the enzyme mix.
8. Tightly close the gentleMACS C Tube and attach it upside down onto the sleeve of the gentleMACS Dissociator (Figure 2B).

   Note: Ensure that the sample material is located in the area of the rotor.

9. Run the gentleMACS program h_tumor_01. For a visual guide, refer to Methods video S1 (Running the gentleMACS program).
10. After termination of the program, detach the gentleMACS C Tube from gentleMACS Dissociator (Figure 2C).
11. Incubate each sample for 30 min at 37°C under continuous rotation using the MACSmix Tube Rotator (Figure 2D).
12. Attach the gentleMACS C Tube upside down onto the sleeve of the gentleMACS Dissociator.
13. Run the gentleMACS program h_tumor_02.

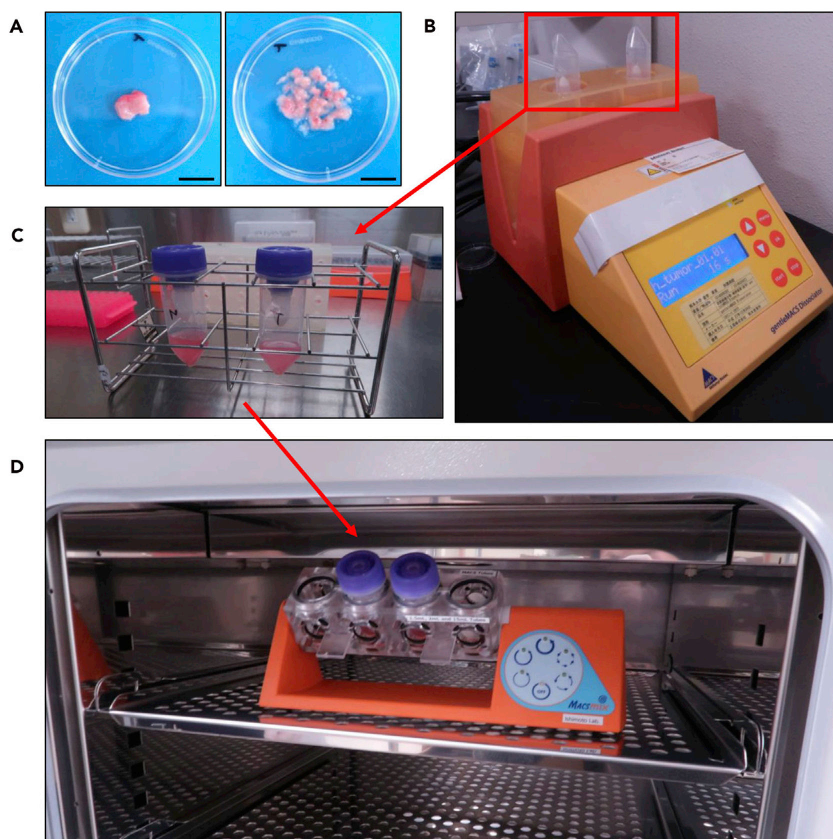

Figure 2. Dissociation of the extracted materials
(A) Minced samples of each material. Before mincing (left) and after mincing (right). Scale bar, 1 cm.
(B) Running the gentleMACS Dissociator program.
(C) Detaching the gentleMACS C Tube from the gentleMACS Dissociator.
(D) Incubating the materials at 37°C under continuous rotation using the MACSmix Tube Rotator.

14. After termination of the program, detach the gentleMACS C Tube from the gentleMACS Dissociator.
15. Incubate each sample for 30 min at 37°C under continuous rotation using the MACSmix Tube Rotator.
16. Attach the gentleMACS C Tube upside down onto the sleeve of the gentleMACS Dissociator.
17. Run the gentleMACS program h_tumor_03.

Note: The duration of the gentleMACS program is approximately 40 sec. The user manual is found at the following link: https://www.miltenyibiotec.com/upload/assets/IM0002089. PDF

18. Perform a short centrifugation at 300×*g* for 30 sec at room temperature (22°C–25°C) to collect the sample materials at the bottom of the tube.
19. Resuspend the sample in the culture media and apply the cell suspension to a MACS Smart-Strainer (70 μm) placed on a 50 mL tube.

Alternatives: Any 70 μm strainer could be used.

20. Wash the MACS SmartStrainer (70 μm) with 20 mL of RPMI 1640 to collect any remaining cells lodged in the cell strainer.
21. Centrifuge the cell suspension at 300×*g* at 4°C for 7 min. Aspirate the supernatant completely.
22. Culture the cells in a 10 cm collagen-coated dish in 10 mL of culture media in a 37°C incubator with 5% CO2 and 95% humidity.
23. Expand the cells until they reach a confluency of 90% in a 15 cm dish (Figure 3).

Note: The morphologies of primary NFs and CAFs are similar. A total of 3×10^6 cells are collected from a confluency of 90% in a 15 cm dish.

24. Store the cells in five tubes with 500 μL of Bambanker (Cat# CS-02-001), with approximately 6×10^5 cells in each tube.

Note: CD90 expression by flow cytometry is useful for validating the enrichment of primary fibroblasts before storing the cells. However, the purity of CD90-positive cells following this protocol ranges from 97% to 100% according to previous reports (Izumi et al., 2016), (Kisselbach et al., 2009). Therefore, the validation of CD90 by FACS staining is not essential.

Pause point: Sample can be stored at −80°C for up to 6 months at this point or processed as described below.

Induction of senescence using inflammatory cytokines

Timing: 20 min (plus 3–5 days for culture expansion)

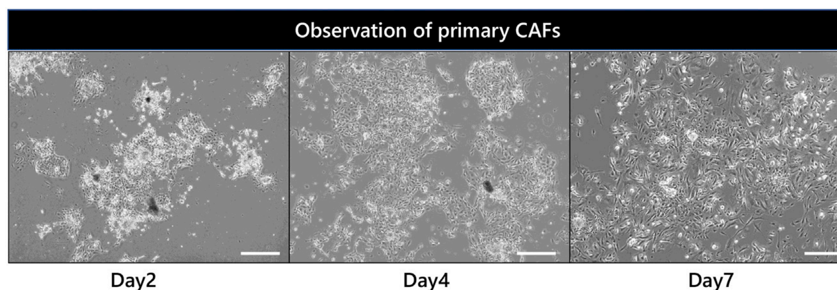

Figure 3. Observation of the primary CAFs at days 2, 4 and 7
Scale bar, 500 μm.

This step describes how to induce senescent fibroblasts using inflammatory cytokines.

25. Thaw and spin down at 210×*g* for 3 min to remove the freezing media, and culture primary fibroblasts in 10 mL of culture medium in a 10 cm plate.

Note: Cancer-associated fibroblasts (CAFs) are likely to shift to a senescent state under exposure to 3 cytokines (IL-1α, IL-1β, and TNF-α) compared to noncancerous fibroblasts (NFs) (Yasuda et al., 2021).

26. Incubate the cells in a 37°C incubator with 5% CO_2 and 95% humidity for 3–5 days until the cells are 90% confluent.

Timing: 20 min (plus 1 week for passaging)

27. Aspirate culture medium and wash cells once with 5 mL of 1×PBS.
28. Aspirate PBS and add 2 mL Accutase. Incubate at 37°C for 3 min.
29. Lightly tap the plate to lift the cells. If the cells are not all detached, incubate the plate at 37°C in increments of 2 min until all cells detach.

Note: Total time in Accutase should not exceed 7 min. Accutase is a gentler alternative to trypsin and is used for primary fibroblast cells to decrease cell damage and increase cell viability.

30. Dilute Accutase 1:5 using the culture medium. Collect the cells into a 15 mL tube.
31. Spin down at 210×*g* for 3 min and culture primary fibroblasts in 10 mL of culture medium in a 10 cm plate.

Note: For the induction of senescence, the cells should be 20%–30% confluent after two times of these steps (27–31).

Timing: 10 min

32. Change the culture medium to 3 cytokines containing medium for 24 h.
33. After 24 h, change the medium to culture medium without cytokines.

Timing: 3–7 days

34. Culture the fibroblasts for 3–7 days without passaging.

Note: To validate senescence, check the changes in the morphology (Figure 4A) and perform the following assays: senescence-associated β-galactosidase (SA-β-gal) assay (Figure 4B), western blot analysis with $p16^{INK4a}$, $p15^{INK4b}$ and Lamin B1, growth assay over a long period, and immunofluorescence staining with γH2A histone family member X (γH2AX) and phosphorylation of the consensus target sequences (pST/Q) (Figure 4).

For the SA-β-gal assay, we recommend using the Senescence Detection Kit (BioVision: K320) according to the manufacturer's protocol. https://www.biovision.com/documentation/datasheets/K320.pdf

For western blot analysis with $p16^{INK4a}$, $p15^{INK4b}$ and Lamin B1, we suggest using Cell Signaling Technology's standard protocol, which is available at https://en.cellsignal.jp/learn-and-support/protocols/protocol-western, and using the primary antibodies $p16^{INK4a}$ (Abcam: ab108349, 1:5000 dilution), $p15^{INK4b}$ (Santa Cruz: sc271791, 1:1000 dilution), Lamin B1 (Abcam: ab16048,

1:1000 dilution) and β-actin (Cell Signaling Technology: 4967, 1:1000 dilution) and the secondary antibodies anti-rabbit IgG-HRP (Cell Signaling Technology: 7074, 1:5000 dilution) and anti-mouse IgG-HRP (Santa Cruz: sc2005, 1:5000 dilution). Dilution buffer is Can Get Signal® Immunoreaction Enhancer Solution 1, 2 (TOYOBO); 1 for primary antibodies and 2 for secondary antibodies.

For the growth assay over a long period, we recommend waiting at least 10 days until the growth curve reaches a plateau. We suggest using the Incucyte S3 Live-cell rpmseeded onto 96-well plates and stained with NucLight Rapid Red (Essen BioScience: 4717).

For immunofluorescence staining for γH2AX and pST/Q, we suggest using Cell Signaling Technology's standard protocol, which is available at https://www.cellsignal.com/learn-and-support/protocols/protocol-if, and the primary antibodies γH2AX (Millipore: 05-636, 1:1000 dilution) and pST/Q (Cell Signaling Technology: 2851, 1:400 dilution) and the secondary antibodies Alexa Fluor 488 (Molecular Probes: A11008, 1:200 dilution) and Alexa Fluor 594 (Molecular Probes: A11005, 1:200 dilution with the DAPI solution (Dojindo: 340-07971) and 1:1000 for nuclear staining). Dilution buffer is 0.1% BSA/ PBS.

EXPECTED OUTCOMES

If this protocol is followed, then primary fibroblasts can be established from surgically resected tissue with efficiency higher than 70%. It is highly recommended to freeze and store these cells as soon as the culture is expanded. Primary fibroblasts from a 90% confluent 15 cm dish are stored in five tubes that each has approximately 6×10^5 cells. Primary fibroblasts can be thawed and cultured in 10 mL of culture medium in a 10 cm plate, which does not need to be collagen coated. Primary fibroblasts can be maintained and expanded in culture, although the expansion rates vary between cells derived from different patients. On average, CAFs that are 90% confluent can be passaged at a one to three ratio every 4–5 days. NFs can usually be passaged every three days at a one to three ratio.

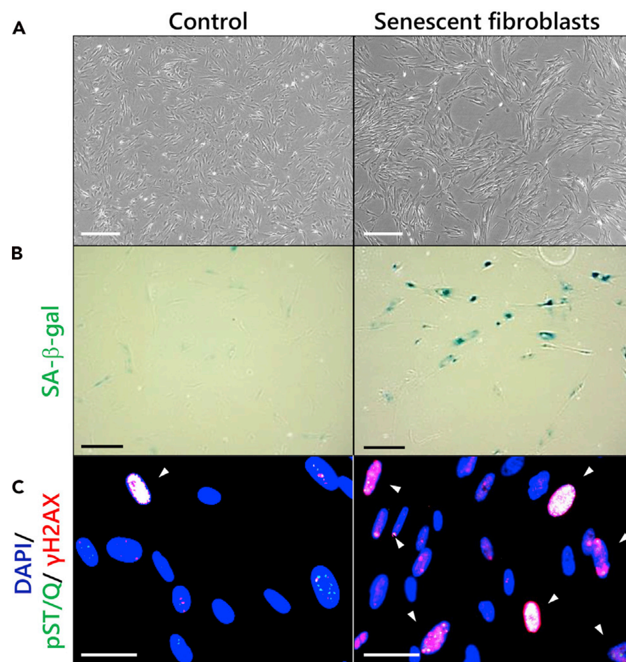

Figure 4. Characterization of the senescent fibroblasts

(A) Senescent fibroblasts had larger and more spindle-shaped cells than the controls. Scale bar, 500 μm.

(B) Senescence-associated β-galactosidase assay for senescent fibroblasts. Scale bar, 300 μm.

(C) Immunofluorescence staining for gH2AX and pST/Q. Scale bar, 50 μm.

LIMITATIONS

A CAF-specific marker has not been established thus far, although CD90 is a definitive marker of human primary fibroblasts (NFs and CAFs) (Izumi et al., 2016), (Kisselbach et al., 2009).

Biological heterogeneity and versatility of CAFs in various cancer tissues have been found (Bu et al., 2019), (Bu et al., 2020). However, heterogeneity is lost in isolated CAFs in vitro. Therefore, using isolated CAFs is inadequate to address the heterogeneity in vivo.

Primary fibroblasts can be expanded a limited number of times (usually up to 20–25 passages). Moreover, these cells generally show senescence over the course of serial passaging; thus, cells with fewer than 10 passages should be used in all experiments (Uchihara et al., 2020).

For the generation of senescent fibroblasts, we did not examine whether individual cytokine treatments (IL-1α, IL-1β, and TNF-α) can induce senescence in primary fibroblasts.

TROUBLESHOOTING

Problem 1

(Linked to 'step-by-step method details 2')

It is difficult to establish NFs from tissues due to their small size.

Potential solution

To solve this problem, at least 5 mm^2 of tissue for CAFs and 1 cm^2 of tissue for NFs are needed. Cancer tissues contain abundant stroma; thus, compared to NFs, CAFs can be easily extracted with a small amount of material.

Problem 2

(Linked to 'step-by-step method details 3')

Bacterial contamination often occurs during primary fibroblast isolation.

Potential solution

To reduce the risk of contamination, wash the extracted tissues with wash buffer at least 3 times by gentle inversion.

Problem 3

(Linked to 'step-by-step method details 22')

The yield of fibroblasts was low after culture.

Potential solution

To solve this problem, the extracted tissues should include the submucosa because the mucosal tissues lack fibroblasts.

Problem 4

(Linked to 'step-by-step method details 22')

Compared to CAFs, NFs are not as likely to adhere to the dish.

Potential solution

To solve this problem, collagen-coated dishes are required to expand primary fibroblasts, especially NFs.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Takatsugu Ishimoto (taka1516@kumamoto-u.ac.jp).

Materials availability

This study did not generate new or unique reagents.

Data and code availability

This study did not generate a dataset or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100553.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

T.Y. drafted and wrote the manuscript. T.Y., M.K., A.Y., and T.A. performed the experiments. H.B. and T.I. reviewed and proofread the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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