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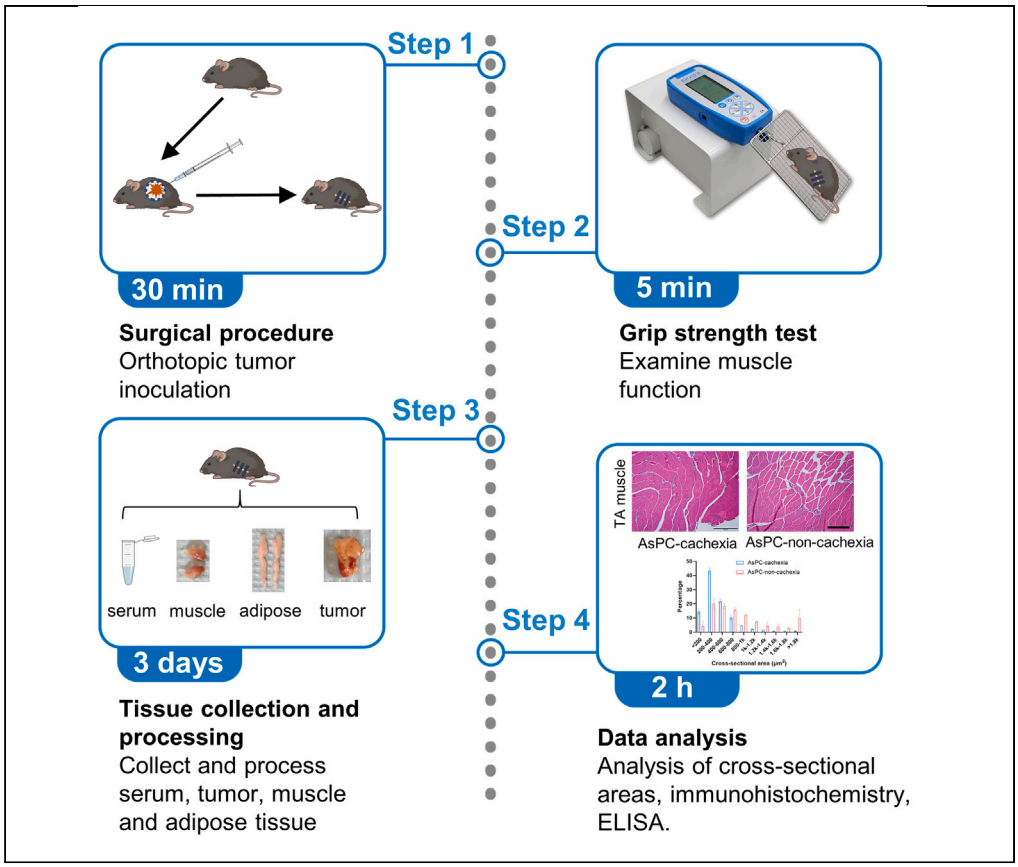
Protocol for establishing and evaluating a cancer cachexia mouse model



Cancer cachexia mouse models are needed to recapitulate the clinical features of patients with cachexia. Here, we present a protocol for the establishment and evaluation of cancer cachexia mouse models. We delineate the steps in preparing tumor cells for inoculation and surgical procedures. After the establishment of these mouse models, we describe essential techniques to assess cancer cachexia, including grip strength evaluation, tissue collection, and the calculation of cross-sectional areas of muscle tissue.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Zhijun Zhou, Jingxuan Yang, Mingyang Liu, ..., Yi-Ping Li, Yuqing Zhang, Min Li

yuqing-zhang@ouhsc.edu (Y.Z.)
min-li@ouhsc.edu (M.L.)

Highlights

Protocol for establishing a cancer cachexia mouse model

Details for assessing features of cancer cachexia, such as muscle wasting

Procedure for analyzing the cross-sectional areas of muscle fibers

Can be adapted to study cachexia in other cancer types besides pancreatic cancer

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Protocol for establishing and evaluating a cancer cachexia mouse model

Zhijun Zhou,[1,2,5] Jingxuan Yang,[1,2,5,6] Mingyang Liu,[1,2] Yu Ren,[1,2] Xiuhui Shi,[1,2] Yang Cai,[1,2] Alex X. Arreola,[1,2,3] Yi-Ping Li,[4] Yuqing Zhang,[1,2,*] and Min Li[1,2,7,*]

[1]Department of Medicine, The University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA

[2]Department of Surgery, The University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA

[3]Department of Pathology, The University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA

[4]Department of Integrative Biology & Pharmacology, The University of Texas Health Science Center at Houston, Houston, TX 77030, USA

[5]These authors contributed equally

[6]Technical contact

[7]Lead contact

*Correspondence: yuqing-zhang@ouhsc.edu (Y.Z.), min-li@ouhsc.edu (M.L.)
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SUMMARY

Cancer cachexia mouse models are needed to recapitulate the clinical features of patients with cachexia. Here, we present a protocol for the establishment and evaluation of cancer cachexia mouse models. We delineate the steps in preparing tumor cells for inoculation and surgical procedures. After the establishment of these mouse models, we describe essential techniques to assess cancer cachexia, including grip strength evaluation, tissue collection, and the calculation of cross-sectional areas of muscle tissue.

For complete details on the use and execution of this protocol, please refer to Liu et al.,[1] Yang et al.,[2] Shi et al.,[3] and Zhou et al.[4]

BEFORE YOU BEGIN

Cancer cachexia is systemic disorder involving a variety of organs that cannot be recapitulated by *in vitro* models, deeming mouse models essential. This protocol describes a step-by-step procedure to establish a cancer cachexia mouse model and highlights techniques to evaluate cancer cachexia features, including muscle wasting and adipose loss.

Sterilization of surgical instruments and materials

Timing: 1 h 30 min

1. The day before inoculation, all surgical instruments and related materials should be placed in an autoclavable box wrapped with aluminum foil, followed by autoclave sterilization.

Surgical instruments requiring sterilization: Surgical scissors, forceps and needle holders.

Related materials that need sterilization: Kimwipes.

Labeling the mice and recording body weight

Timing: 30 min to 1 h

2. Label each mouse with an ear tag and measure the body weight.
   a. Label and weigh each mouse before surgery.
   b. Evaluate the body weight of all mice. Ensure there is no outlier (>20% variation of median body weight) in terms of initial body weight.
   c. Randomly distribute the mice into different groups.

Preparing the instruments and materials

Timing: 30 min

3. Prepare the following instruments and materials: 1 mL syringe (with needle), disinfectant pad, sterile surgical drape, tape, desk lamps, electric shaver, vortex mixer, scale, anesthetic gas ventilator, Inotech Steri 250 Sterilizer with heated glass beads, heat pad, surgical instruments (knives, scissors, needle holders, tweezers, sutures).

Cell preparation

Timing: 20 min for AsPC-1 cells and 15 min for KPC cells

4. Monitor the cell lines for animal study.
   a. Culture the cells in RPMI-1640 medium supplemented with 1% Penicillin-Streptomycin, 10% Fetal Bovine Serum. For KPC cells, add 1% Non-Essential Amino Acids Solution into the basic culture medium mentioned above.
   b. Rinse the cells with 5 mL Phosphate-buffered saline twice. Add 1 mL Trypsin-EDTA (0.25%) into T75 flask to detach the cells.
   c. After 3–5 min, add fresh medium to the flask and collect the medium with cells into a 15 mL centrifuge tube.
   d. Centrifuge at 1000 rpm for 3 min.
   e. Remove the supernatant and resuspend the cells with fresh medium.
   f. Calculate cell numbers before seeding the cells. Seed around 3×10^6 AsPC-1 cells or 1×10^6 KPC cells in the T75 flasks.
   g. After 2 days, on the day of collecting the cells for *in vivo* study, the cells reach around 80% confluence, indicating that these cell lines are in a period of exponential growth on the day of inoculation.
5. Calculate the cells and make the desirable concentration.

Note: For AsPC-1 cells, we anticipate to inoculate 3×10^6 cells/mouse, therefore, we resuspend the AsPC-1 cells in RMPI-1640 medium in a concentration of 6×10^7 cells/mL. For KPC cells, we anticipate to inoculate 3×10^5 cells/mouse, therefore, we resuspend the KPC cells in RMPI-1640 medium in a concentration of 6×10^6 cells/mL.

⚠ CRITICAL: Repeat 3 times for the calculation of cell numbers to ensure that the cell numbers for inoculation are accurate.

Setting up equipment

Timing: 30 min

6. Prepare the anesthetic gas ventilator; prefill the ventilator with isoflurane and connect the ventilator to an oxygen source.
7. Prepare the cassettes. Label all the cassettes before tissue collection.
8. Turn on the Inotech Steri 250 Sterilizer with heated glass beads for instant sterilization.
9. Preheat the heat pad before the surgery.

Setting up the surgery platform

Timing: 30 min

10. Set up the equipment for anesthesia.
    a. Connect the anesthetic gas ventilator with the anesthetic induction chamber and hoses of anesthesia for individual mouse.
    b. Connect the anesthetic gas ventilator to oxygen supply.
    c. Fill the anesthetic gas ventilator with appropriate 5 mL isoflurane.
    d. Use the sterile surgical drape to prevent contamination to the sterile surgery area.
11. Turn on the heating pad to maintain the temperature of mice at 37°C during surgery.
12. Preheat sterilization beads to 240°C. These hot glass beads are used for instant sterilization of surgical instruments during the surgery.

Institutional permissions

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at The University of Oklahoma Health Sciences Center (OUHSC) and were conducted in compliance with the National Institutes of Health (NIH) Guide for the animal studies. Permissions should be obtained from relevant institutions before beginning animal work.

These cancer cachexia mouse models were developed using male nude mice and C57BL/6J mice aged 6–8 weeks. All mice were housed in a specific pathogen-free facility at OUHSC. A 12 h/12 h dark/light schedule was applied. The temperature of the room was set to 22°C and the humidity was 50%. Food and water were available at all times and were provided by the facility. Facility staff monitored the condition of the mice daily and notified the principal investigator of any necessary actions.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Isoflurane	Covetrus, USA	11695067772
RNAlater	Invitrogen, USA	AM7021
Decon's pure ethanol 200 proof	Decon Labs	2701
10% Phosphate-buffered formalin	Thermo Fisher Scientific, USA	SF100-20
Experimental models: Cell lines		
KPC	Liu et al.[1]	N/A
AsPC-1	ATCC	CRL-1682
Experimental models: Organisms/strains		
C57BL/6J, strain: 000664, sex: M, genotype: N/A, age: 6–8 weeks, range: 6–8 weeks	The Jackson Laboratory, USA	Strain #:000664 RRID:IMSR_JAX:000664
NU/J, strain: 002019, sex: M, genotype: homozygous for Foxn1<nu>, age: 6-8 weeks, range: 6–8 weeks	The Jackson Laboratory, USA	Strain #:002019 RRID:IMSR_JAX:002019
Software and algorithms		
ImageJ v1.53t	NIH, USA	https://imagej.net/ij/
Graphpad Prism 10	GraphPad, USA	https://www.graphpad.com/
Other		
Cryogenic vials	Wheaton, USA	W985863
Sterile alcohol prep pads	Fisherbrand, USA	22-363-750
Absorbent towels	Cardinal Health, USA	Cardinal Health 5550
Kimwipes	Kimtech, USA	Kimberly-Clark Professional 34256/EMD
Graefe forceps, 10 cm, straight, serrated	Fine Science Tools, USA	11050-10

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fine scissors – tungsten carbide, straight	Fine Science Tools, USA	14568-12
Ring forceps, 3 × 0.75 mm	Fine Science Tools, USA	11103-09
Ring Forceps, 6 × 0.9 mm	Fine Science Tools, USA	11106-09
Dumont #4 forceps, standard, Dumostar	Fine Science Tools, USA	11294-00
Halsey micro needle holder	Fine Science Tools, USA	12500-12
Ethicon suture: VCP494H, suture, size 4-0, 18 in. length, undyed color, triclosan, polyglactin 910, braided, 3/8 circle	Ethicon, USA	VCP494H
Scalpels	McKesson, USA	16-63110
Ball head tissue pins, AIMS	VWR, USA	10060-186
Blue Wax Large Tissue Process Disc PK10	VWR, USA	10060-180
Heat pad	Conduct Science, USA	RWD-69023
Cassette	Epredia, USA	B851090WH
Camera Nikon D5500	Nikon	D5500
1 mL TB syringe	BD, USA	309659
Phosphate-buffered saline	VWR, USA	45000-446
Corning RPMI 1640	VWR, USA	45000-398
Fetal bovine serum (FBS)	Thermo Fisher Scientific, USA	16000044
Trypsin-EDTA (0.05%), phenol red	Thermo Fisher Scientific, USA	25300054
Penicillin-Streptomycin-Glutamine (100X)	Thermo Fisher Scientific, USA	10378016
Sodium pyruvate	Thermo Fisher Scientific, USA	11360070
Non-essential amino acids solution (100X)	Thermo Fisher Scientific, USA	11140050
Grip strength meter	Bioseb, France	BIO-GS4
Anesthetic gas ventilator	Supera Scientific, USA	M3000
Vortex-Genie 2	Scientific Industries, Inc., USA	SKU: SI-0236
Inotech Steri 250 sterilizer	Simon Keller Ltd., Switzerland	KE-250
Electric shaver, BravMini+ purple	Wahl, USA	41590-0438
Scale for body weight test	Mettler Toledo	ME4002E
Scale for tissue weight test	Mettler Toledo	MS104S

STEP-BY-STEP METHOD DETAILS

Surgery preparation

Timing: 3 min/mouse

This section delineates the pre-operation procedure for the mice.

1. Measure the body weight of each mouse on the day of surgery.
2. Clean the surgical area.
   a. For C57BL/6J mouse, use the electric shaver to remove the hair from the left flank to create an area of around 3 × 3 cm.
   b. Use a disinfectant pad to remove residual hair and clean the area.

Mouse anesthesia

Timing: 3–5 min

This section delineates the steps to anesthetize the mice.

3. Anesthetize mice in the induction chamber.
   a. Place the mice in the anesthetic induction chamber supplied with 2–3% isoflurane, pure oxygen at a flow of 2–2.5 L/min.

Note: Ensure one end of the anesthetic induction chamber is connected to the ventilation system while the other is connected to an anesthetic gas scavenging system to prevent overdose.

b. Monitor the anesthesia depth of the mice. The mice will be fully anesthetized in about 2–3 min.

Note: Cut off the supply of anesthetizing gas and maintain the oxygen supply (2–2.5 L/min) for around 10 s before retrieving the mice from the induction chamber to prevent the leakage of anesthetizing gas.

4. Continue anesthesia through the breathing circuits.
   a. Place the fully anesthetized mice on the surgical desk. Maintain the anesthesia status through a breathing circuit.
   b. Check the anesthesia depth by tapping the feet of mouse.
   c. Apply ointment to the eyes of the mouse.

Surgery

Timing: 3–5 min/mouse

This section delineates the details of surgical procedure, including making the incision, tumor inoculation and closing the incision.

5. Clean the surgical area with disinfectant alcohol pad, circling outwards.
6. Cover the mouse with a fenestrated surgical drape.
7. Inject Bupivacaine (1 mg/kg) subcutaneously prior to the surgical incision.
8. Make a 1 cm incision on the skin at the left flank of the mouse.
9. Carefully make a 1 cm incision on the peritoneum.

Note: To avoid accidental damage to the organs in peritoneal cavity, use forceps to pull up the peritoneum when making an incision.

10. Use ring forceps to carefully pull the spleen out, exposing the pancreas which is attached to the spleen (Figure 1A).

Note: Avoid the use of tweezers to drag the pancreas out directly as this may induce injury and postoperative complications, such as pancreatitis.

11. Vortex the cells (we use a strength level of 6 [out of 10]) and draw the cells from the Eppendorf tube, using a 1 mL syringe. Remove any air bubbles and attach a needle (27G) to the syringe.
12. Inoculate 50 μL medium with AsPC-1 cells (6×10^7 cells/mL) or KPC cells (6×10^6 cells/mL).

Note: When doing the inoculation, avoid the vessels on the pancreas to prevent hemorrhaging (Figure 1A).

13. Return the pancreas and spleen and close the incision with a 4/0 absorbable suture.

Note: Close the peritoneum incision first and then close the incision on the skin with a suture.

⚠ CRITICAL: Surgical instrument sterilization between mice is required. Sterilize the surgical instruments with heated glass beads. This is critical, especially when there is leakage during tumor cells inoculation or when you are inoculating different tumor cells.

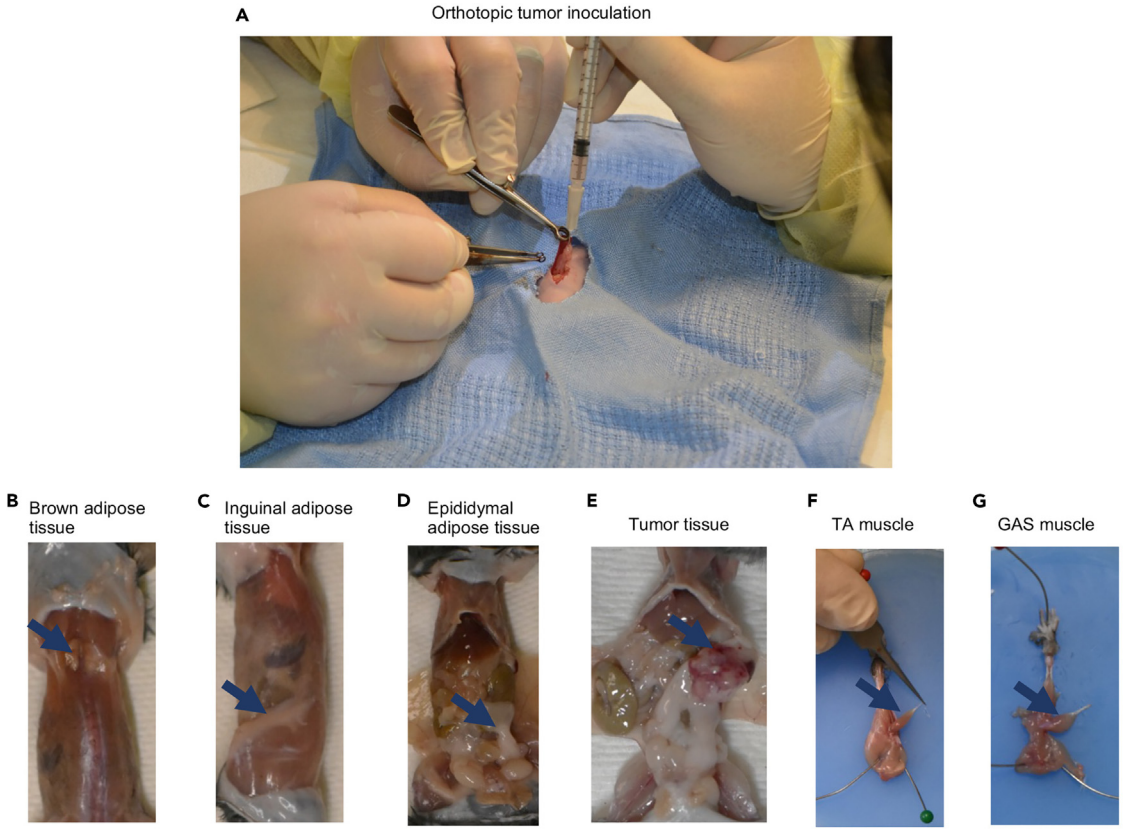

Figure 1. Orthotopic tumor inoculation and the location of adipose tissue and muscle tissue

(A) Orthotopic inoculation of pancreatic cancer cells.
(B) Location of brown adipose tissue.
(C) Location of inguinal white adipose tissue.
(D) Location of epididymal white adipose tissue.
(E) Location of tumor tissue.
(F) Location of TA muscle tissue.
(G) Location of GAS muscle tissue. The arrow indicates the location of the tissue.

Recovery after surgery

Timing: 5–10 min/mouse

This section delineates the steps to help the mice to recover from the operation.

14. Return the mouse to a cage which is placed on top of a heating pad.
15. Monitor the status of the mouse. When it awakes and moves freely, put it back into the home cage.
16. Monitor the mice closely post-surgery. Analgesics should be given daily within 48 h post-surgery and whenever needed after 48 h.
17. Inject Ketoprofen (2 mg/kg) subcutaneously post-surgery with daily injection up to 4 days.

Grip strength measurement post-surgery

Timing: 10–15 min/mouse

This section delineates the details of measuring the grip strength of the mice.

18. Measure mouse body weight 4 days after the surgery. Measure the body weight 3 times a week. When average body weight loss reaches 5%, monitor the body weight every day.

Note: Closely monitor body weight. The exact time which mice begin to lose weight varies depending on the cell lines used and can vary between mice.

19. The grip strength of the mice is measured by a grip strength meter (BIOSEB, France), which records the maximum grip strength of the mouse. Briefly, the mouse is placed on a metal grid and then pulled backwards by holding the tail. Measure 5 times. The maximum grip strength will be displayed on the screen.

Note: The measurement unit can be set as gram, newton, or lb.

Note: Measure grip strength starting from Day 7.

Note: Make sure to reset the meter between each mouse.

Note: Avoid pulling the metal grid directly by hands, as it may impair the accuracy of the meter.

Note: The data can be exported through the flash drive or recorded manually.

Tissue collection

Timing: 30–45 min/mouse

This section delineates the procedure of collecting serum, tumor tissue, adipose tissue and muscle tissue of the mice at the end of the study.

20. Begin tissue collection when some mice reach moribund.
21. Set up the platform for tissue collection.
    a. Set up the camera for optimal resolution and color.
    b. Prepare and label the tubes for tissue collection.
22. Measure the body weight of the mouse on the day of tissue collection.
23. Anesthetize the mouse, draw blood, and then perform cervical dislocation.

Note: Once the blood is collected in an Eppendorf tube, leave it at room temperature for around 20 min before placing on ice. Centrifuge the blood sample at 2,000 × *g* for 10 min in a pre-cooled centrifuge and then collect the supernatant as serum.

24. Collect the brown adipose tissue (BAT).
    a. Place the mouse on the dissection surface, in a prone position. Spray some alcohol on the shoulder area.

Note: The alcohol keeps the skin wet so that the hair and fur do not contaminate the tissue.

    b. Secure the mouse on the dissection foam using the pins.
    c. Make a transversal incision on the skin, in the middle of the back.
    d. Locate and expose the interscapular area.
    e. The interscapular BAT has the shape of a butterfly, with brown color (Figure 1B).
    f. Remove the adjacent white adipose tissue.
    g. Weigh the BAT.
    h. Place the BAT on a plate. Take a picture of the collected tissue.

i. Cut the sample into 3 pieces.
j. Put one piece in a cassette and then submerge the cassette in 10% Phosphate Buffered Formalin.
k. Put one piece in RNAlater for RNA extraction.
l. Put the other piece in a cryotube and store immediately on dry ice, placing them into a −80°C freezer when possible.
m. After 24 h, remove the cassette from the 10% Phosphate Buffered Formalin and transfer to 70% ethanol.

25. Collect the inguinal white adipose tissue.
    a. Peel the skin downwards and expose the groin area.
    b. Locate the anterior subcutaneous adipose tissue (Figure 1C).
    c. Use scissors and ring forceps to carefully remove the adipose tissue.
    d. Weigh the inguinal white adipose tissue.
    e. Place the inguinal adipose tissue on a plate and take a picture.
    f. Cut the sample into 3 pieces.
    g. Put one piece in a cassette and then submerge the cassette in 10% Phosphate Buffered Formalin.
    h. Put one piece in RNAlater for RNA extraction.
    i. Put the other piece in a cryotube and store it immediately on dry ice, then place it into a −80°C freezer when possible.
    j. After 24 h, remove the cassette from the 10% Phosphate Buffered Formalin and transfer to 70% ethanol.
26. Collect the epididymal white adipose tissue.
    a. Make an incision on the abdominal cavity to expose the testes area.
    b. Take a picture of the abdominal cavity to overview the tumor progression.
    c. The epididymal white adipose tissue is located next to the testes in male mouse (Figure 1D).
    d. Use forceps to lift the epididymal white adipose tissue and use scissors to carefully remove the adipose tissue.
    e. Weigh the epididymal adipose tissue.
    f. Place the epididymal adipose tissue on a plate and take a picture.
    g. Cut the sample into 3 pieces.
    h. Put one piece in a cassette and then submerge the cassette in 10% Phosphate Buffered Formalin.
    i. Put one piece in RNAlater for RNA extraction.
    j. Put the other piece in a cryotube and store it immediately on dry ice, then place it into a −80°C freezer when possible.
    k. After 24 h, remove the cassette from the 10% Phosphate Buffered Formalin and put it in 70% ethanol.
27. Collect the tumor tissue.
    a. Check whether there are tumor metastases in other organs in the abdominal cavity (Figure 1E).

    Note: There may be ascites in the abdominal cavity, especially when there are peritoneal metastases. Drain and estimate the volume of the ascites.

    b. Use forceps and scissors to carefully remove the tumor.

    Note: There may be some tumor tissue grown on the peritoneum wall. The primary tumor tissue and the tumor tissue on skin can be separated by scalpel or scissors.

    c. Weigh the tumor tissue.
    d. Place the tumor tissue on a plate and take a picture.
    e. Cut the sample into 4 pieces.

f. Put one piece in a cassette and then submerge the cassette in 10% Phosphate Buffered Formalin.
g. Put one piece in RNAlater for RNA extraction.
h. Put one piece in PBS for flow cytometry analysis.
i. Put the other piece in a cryotube and store it immediately on dry ice, and then put into a −80°C freezer when possible.
j. After 24 h, remove the cassette from the 10% Phosphate Buffered Formalin and transfer to 70% ethanol.

28. Collect the tibialis anterior (TA) muscle tissue.
    a. Separate the leg from the body and secure it with pins.
    b. Remove the skin and expose the hind limb muscle area (Figure 1F).
    c. Peel off the tendon of TA muscle.
    d. Use a blade to carefully separate the TA muscle from the surrounding muscle tissue.
    e. Weigh the TA muscle.
    f. Place the TA muscle on a plate and take a picture.
    g. Cut the sample transversely into 3 pieces. Put one piece in a cassette and then submerge the cassette in 10% Phosphate Buffered Formalin. Put other pieces in a cryotube and store it immediately on dry ice, and then place in a −80°C freezer when possible.

    Note: Cut the TA muscle at about 1/3 from the tendon side in a cross-sectional manner. Put the 1/3 portion in a cassette for embedding, orienting the tendon superior to the muscle. Collect the other 2/3 for storage.

    h. After 24 h, remove the cassette from the 10% Phosphate Buffered Formalin and put it in 70% ethanol.

29. Collect the gastrocnemius (GAS) muscle tissue.
    a. Secure the leg with pins to expose the GAS muscle facing-up (Figure 1G).
    b. Peel off the tendon of GAS muscle.
    c. Use a blade to carefully separate the GAS muscle from the surrounding muscle tissue.

    Note: The soleus (SOL) muscle is a strip-like muscle that is located closely under the GAS muscle. After peeling up the GAS muscle, remove the SOL muscle.

    d. Weigh the GAS muscle.
    e. Place the GAS muscle on a plate and take a picture.
    f. Cut the sample transversely into 3 pieces. Put one piece in a cassette and then submerge the cassette in 10% Phosphate Buffered Formalin. Put other pieces in a cryotube and store it immediately on dry ice, and put it in into a −80°C freezer when possible.

    Note: Cut the GAS muscle at about 1/3 from the tendon side in a cross-sectional way. Put the 1/3 portion in a cassette for embedding, orienting the tendon superior to the muscle. Collect the other 2/3 for storage.

    g. After 24 h, remove the cassette from the 10% Phosphate Buffered Formalin and put it in 70% ethanol.

Tissue sample processing, embedding, sectioning and H&E staining

Timing: 3 days

This section delineates the steps of processing the tissue samples.

30. Again, after 24 h, replace the 10% Phosphate Buffered Formalin with 70% alcohol.

31. Send the samples to a pathology core lab for processing, embedding, sectioning and H&E staining.

⚠ CRITICAL: When embedding the muscle tissue, the tissue should be embedded in a vertical direction, so that the sectioning area is facing down. This is critical because the sections will be used for the calculation of cross-sectional areas.

Data analysis of cross-sectional area of muscle fiber

Timing: 30–45 min/mouse

This section delineates the details of analyzing the cross-sectional areas of muscle fibers.

32. Measure the cross-sectional area of muscle fiber using ImageJ (version: v1.53t).
    a. Open the image of the H&E staining of cross-sectional areas of muscle tissue.
    b. Duplicate the original image.
    c. Use the scale bar as a standard to draw a line. Set scale under the menu of "Analyze". Set the known distance and change the unit of length. Then select "Global" and click "Set".
    d. Set the image type to 8-bit.
    e. Adjust the image threshold.
    f. Set the measurements under the "Analyze" menu. Select "Area" and "Limit to threshold".
    g. Select the wand tool and then select one cross-sectional area.

    Note: Sometimes the wand tool cannot recognize the area accurately. In this case, you must manually select the area. To do this, click the freehand selection, then draw a circle around the area.

    h. Select "Measure" under the "Analyze" menu.

    Note: A keyboard shortcut can be used by pressing "Control + M".

    i. Export the results for subsequent statistical analysis in GraphPad Prism 10.
33. Alternatively, we can measure the cross-sectional areas of the muscle fibers using HALO module which can detect the diameter and area of each fiber.

EXPECTED OUTCOMES

The protocol described above illustrates the procedure of establishing two cancer cachexia mouse models, including one from a human cell line and the other one from a mouse cell line. We also illustrate the steps to evaluate cancer cachexia, especially muscle wasting, which is examined by grip strength and cross-sectional area calculation. This model recapitulates the features of cancer cachexia in patients, therefore represents a valuable pre-clinical model for discovering novel therapeutic targets for cachexia. The model has been successfully applied for the study of cachexia in pancreatic cancer and several therapeutic targets have been identified.[1–4]

Analysis of therapeutic targets for cancer cachexia

This model can be utilized for evaluating the anti-cachexia effect of potential therapeutic targets of interest. It can be applied to multiple scenarios with minor modifications depending on the study aims. i.e., one can use stable cell lines or genetically modified mice to examine the role of a specific gene in cancer cachexia. We collect the serum, tumor tissue, adipose tissue and muscle tissue, which can be used for varieties of subsequent analyses, such as western blotting, immunohistochemistry (IHC) staining, single-cell sequencing and Enzyme-linked immunosorbent assay (Figure 2).

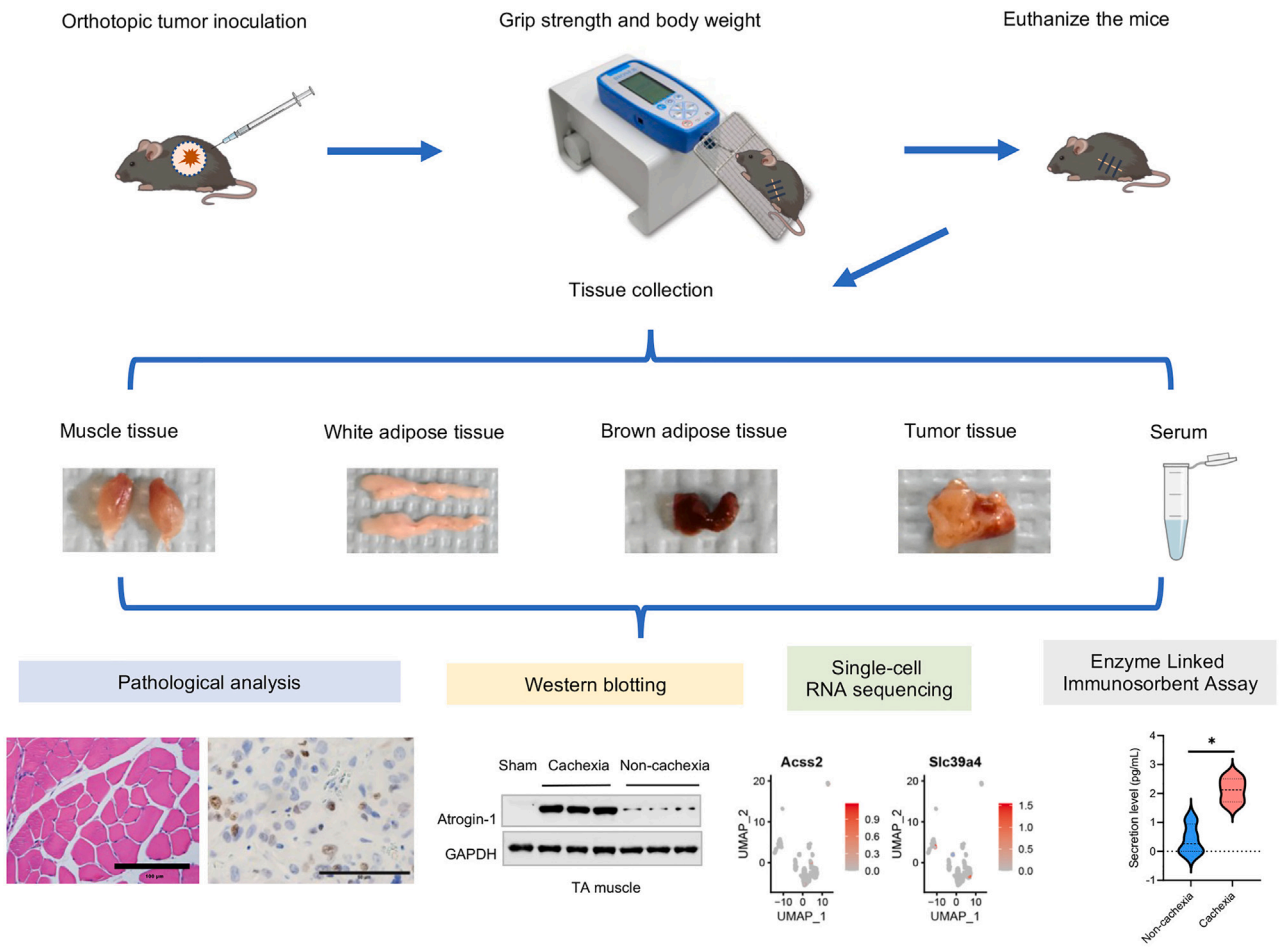

Figure 2. Establishment of cancer cachexia mouse model and tissue collection for subsequent analyses

Establish the orthotopic pancreatic cancer cachexia mouse model. Evaluate the grip strength. Monitor body weight. Collect the serum, muscle tissue, adipose tissue and tumor tissue for subsequent analyses, including pathological analysis of muscle and tumor tissue, western blotting of muscle tissue, single-cell RNA sequencing of tumor tissue and Enzyme Linked Immunosorbent Assay (ELISA) of serum. For the representative images of pathological analysis, the scale bars represent 100 μm and 50 μm, respectively. Data are represented as mean ± SD. *, $p < 0.05$ by t test.

Evaluating cancer cachexia associated muscle wasting

Using this protocol, we establish two pancreatic cancer cachexia mouse models that recapitulate features of cancer cachexia in patients. We will be able to evaluate cancer cachexia associated muscle wasting by measuring the cross-sectional areas of muscle fibers and grip strength (Figures 3A–3C).

Analysis of immune infiltration in tumor tissue through flow cytometry

Using this protocol, we are able to examine the infiltration of immune cells. We focused on macrophages in our study. On the day of tissue collection, tumor tissues were dissected and immediately processed for flow cytometry to examine the infiltration level of macrophages (Figure 3D).

QUANTIFICATION AND STATISTICAL ANALYSIS

Cross-sectional areas of muscle fibers were evaluated by ImageJ and the graphs were generated by GraphPad. The significance of grip strength examination was determined by unpaired t-test. All statistical analyses were performed using Prism 10 (GraphPad).

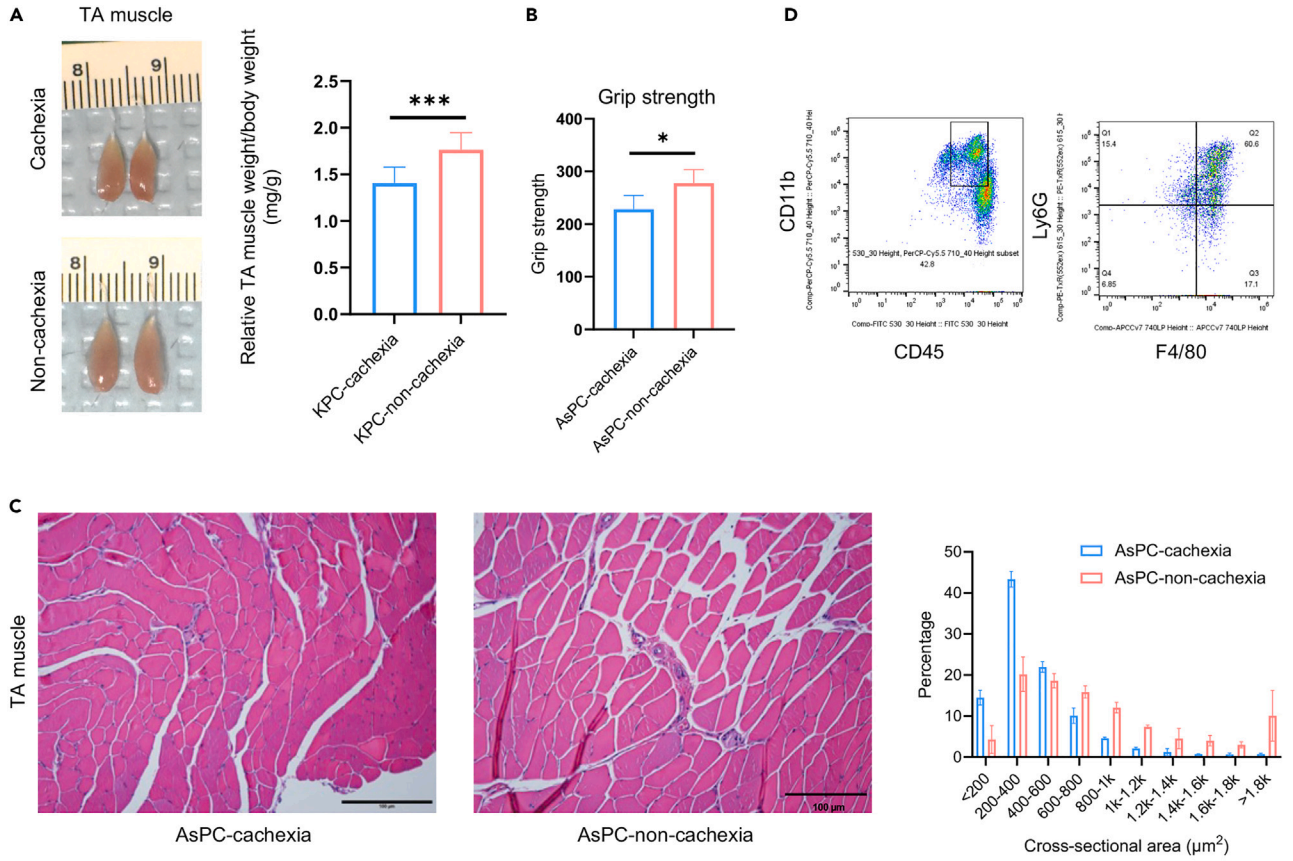

Figure 3. Analysis of muscle wasting and macrophage infiltration in tumor tissue

(A) Representative images of TA muscle and statistics analysis of muscle weight in mice allografted with KPC-cachexia or KPC-non-cachexia cells.

(B) Grip strength of mice xenografted with AsPC-cachexia or AsPC-non-cachexia cells.

(C) Representative images and statistics analysis of cross-sectional areas of GAS muscle fibers of mice xenografted with AsPC-cachexia or AsPC-non-cachexia cells. The scale bar is 100 μm.

(D) Gating strategy for flow cytometry. We examined the level of macrophages in tumor tissue by evaluating the level of $F4/80^+$ cells in $CD45^+CD11b^+$ cells. Data are represented as mean ± SD. *, $p < 0.05$, ***, $p < 0.001$ by t test.

LIMITATIONS

The selection of cancer cell line for tumor inoculation is critical, because some cancer cell lines cannot induce cachexia. The protocol may not be successful if other cancer cell lines are used. It is advised to test the cell lines in a preliminary study. Meanwhile, we restrict this protocol to the establishment of orthotopic mouse model for pancreatic cancer cachexia. The application of this procedure to other cancer types, such as colon cancer, stomach cancer, should be modified in order to fit in other settings.

TROUBLESHOOTING

Problem 1

The mice may not cooperate with the grip strength measurement (Related to the step "grip strength measurement post-surgery").

Potential solution

Place the mouse back to the cage for several minutes and then measure it again.

Problem 2

When measuring the cross-sectional area using ImageJ, the result shows "NaN" (Related to the step "data analysis of cross-sectional area of muscle fiber").

Potential solution

After adjusting the threshold, avoid click "Apply". Instead, click "set".

Problem 3

When measuring the cross-sectional area using ImageJ, the scale changed (Related to the step "data analysis of cross-sectional area of muscle fiber").

Potential solution

After setting the scale, make sure to click the "Global".

Problem 4

Images of the tissue samples are taken by a camera. Some images may look blurry (Related to the step "tissue collection").

Potential solution

The camera should be secured to a rack in order to make sure that the camera is taking pictures from the same distance. Meanwhile, we you must manually adjust the focal length of the camera, especially when switching from taking pictures of the whole mouse to taking pictures of the tissue.

Problem 5

There may be hemorrhaging during tumor inoculation (Related to the step "surgery").

Potential solution

Avoid the visible vessels on the pancreas. If there is hemorrhage, use sterile gauze pad to stop the hemorrhage.

Problem 6

The H&E staining of muscle tissue shows that some muscle fibers are not cut cross-sectionally, which may influence the accuracy of measuring the cross-sectional areas of the muscle fibers (Related to the step "data analysis of cross-sectional area of muscle fiber").

Potential solution

When cutting and embedding the muscle tissue, cut it cross-sectionally and make sure the cross-sectional area aligns parallelly to the section.

Problem 7

Some mice may have surgical complications, such as wound disruption. Although the prevalence is very low, it could be fatal if it is not found and treated in a timely manner (Related to the step "recovery after Surgery").

Potential solution

When making the incision on the left flank, avoid the area that is closed to the abdomen, because the mouse may be able to tear apart the suture later on. Meanwhile, when suturing the incision, make sure to suture both the peritoneum and the skin. Check the mice every 12 h within the 48 h post-surgery.

Problem 8

Tumor growth rate may be lower than expected and may vary dramatically even within the same group (Related to the step "surgery").

Potential solution

Keep the cell suspension on ice until tumor inoculation. Once the cell suspension is ready, perform the tumor inoculation as soon as possible, because cell viability will decline as time goes by. Before tumor inoculation, vortex the cells in medium strength.

Problem 9

The weight of muscle tissue and adipose tissue of both sides may vary greatly if the humidity of the room is low and the tissue is not weighted immediately. (Related to the step "tissue collection").

Potential solution

If the humidity of the room was low, the tissue weight may drop if not evaluated immediately. Weigh the tissue immediately.

Problem 10

The body weight and the grip strength of the mice may vary dramatically. (Related to the step "grip strength measurement post-surgery").

Potential solution

The body weight and the grip strength of the mice vary at different time points during the day. Evaluate the body weight and the grip strength of the mice at the same time of the day and make sure it is evaluated by the same person.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Min Li (Min-Li@ouhsc.edu).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Jingxuan Yang (Jingxuan-Yang@ouhsc.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This paper did not generate any original code. All software and algorithms used in this study have been listed in the key resources table with accession links.

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AUTHOR CONTRIBUTIONS

M. Li, Y.Z., Y.-P.L., Z.Z., and J.Y. developed the protocol; Z.Z. and J.Y. wrote the manuscript; M. Liu, Y.R., and X.S. validated the protocol; Y.C. and A.X.A. provided technical support; and M. Li and Y.Z. provided funding, resources, and supervision. All authors edited and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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