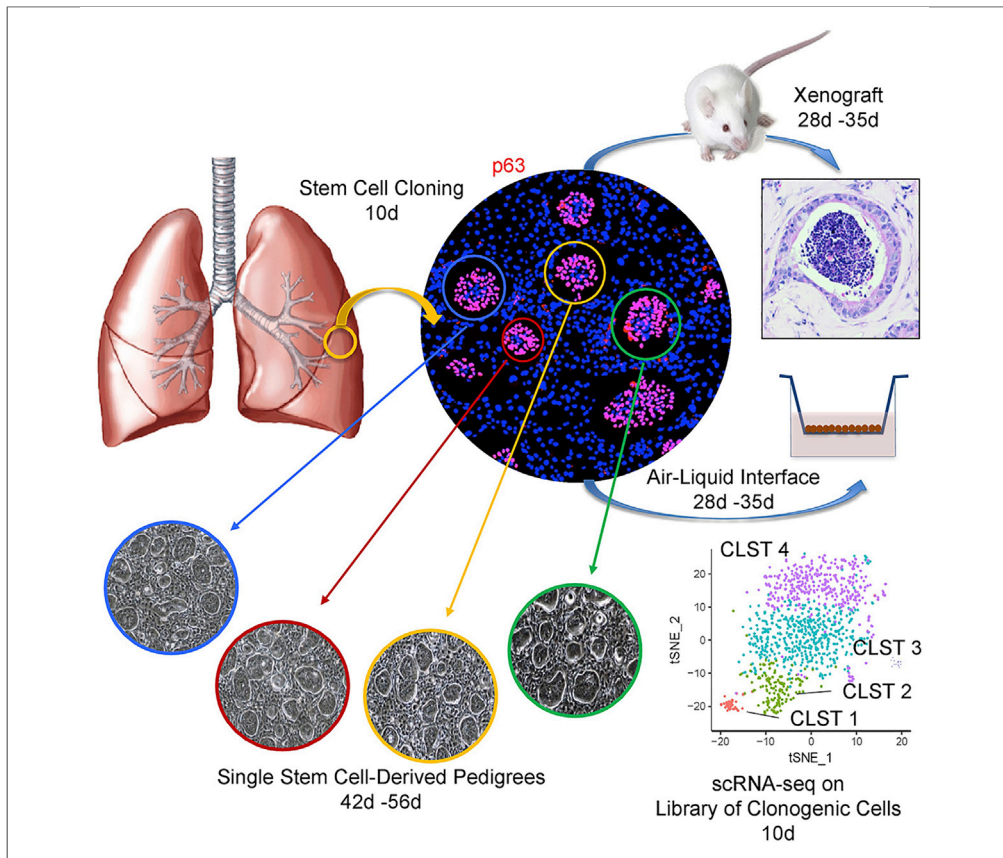


Protocol

Protocol for Cloning Epithelial Stem Cell Variants from Human Lung



The plurality of clonogenic cells derived from human lung includes a spectrum of diverse p63+ stem cells responsible for the regeneration of normal epithelial tissue and disease-associated metaplastic lesions. Here, we detail protocols for the cloning, expansion, and characterization of these stem cell variants, which in general assist in analyses of lung stem cell heterogeneity, genome editing, drug screening, and regenerative medicine.

Wei Rao, Suchan Niroula, Shan Wang, Matthew Vincent, Frank McKeon, Wa Xian

fdmckeon@uh.edu (F.M.)
wxian@uh.edu (W.X.)

HIGHLIGHTS

Protocol for generating libraries of p63+ clonogenic epithelial cells from lung

Normal lung dominated by type 1 clone, COPD lung by type 2, 3, and 4 clones

Type 2, 3, and 4 clones committed to discrete and pathogenic metaplasia

Protocol enables insights into disease, drug discovery, and regenerative medicine

Rao et al., STAR Protocols 1, 100063

September 18, 2020 © 2020

The Author(s).

[https://doi.org/10.1016/](https://doi.org/10.1016/j.xpro.2020.100063)

[j.xpro.2020.100063](https://doi.org/10.1016/j.xpro.2020.100063)



Protocol

Protocol for Cloning Epithelial Stem Cell Variants from Human Lung

Wei Rao,^{1,3} Suchan Niroula,¹ Shan Wang,¹ Matthew Vincent,² Frank McKeon,^{1,*} and Wa Xian^{1,4,*}¹Stem Cell Center, Department of Biology and Biochemistry, University of Houston, Houston, TX 77204, USA²Nüwa Medical Systems, Inc., Houston, TX 77479, USA³Technical Contact⁴Lead Contact*Correspondence: fdmckeon@uh.edu (F.M.), wxian@uh.edu (W.X.)
<https://doi.org/10.1016/j.xpro.2020.100063>

SUMMARY

The plurality of clonogenic cells derived from human lung includes a spectrum of diverse p63+ stem cells responsible for the regeneration of normal epithelial tissue and disease-associated metaplastic lesions. Here, we report protocols for the cloning, expansion, and characterization of these stem cell variants, which in general assist in analyses of stem cell heterogeneity, genome editing, drug screening, and regenerative medicine.

For complete details on the use and execution of this protocol, please refer to Kumar et al. (2011), Zuo et al. (2015), and Rao et al. (2020).

BEFORE YOU BEGIN

The epithelial tissue regeneration relies on the intrinsic ability of adult stem cells to self-renew and differentiate to various cell types appropriate for respective tissues. Several decades ago, Howard Green and colleagues pioneered adult stem cell-based regenerative medicine. They established a culture system dependent on Swiss 3T3-J2 murine fibroblast feeder cells for capturing p63+ epidermal stem cells and demonstrated these cells can be maintained in their most immature and clonogenic form *in vitro* and yield a stratified squamous epithelium upon engraftment (Rheinwald and Green, 1975; Green, 2008; Hirsch et al., 2017). We have leveraged the success of Green's system and established a new system that employs a custom media and 3T3-J2 feeder cells for the cloning and expansion of single stem cells from human lung.

⚠ **CRITICAL:** Human lung tissues must be obtained in compliance with institutional review board regulations and guidelines. All procedures used for handling human tissues should assume potential contamination of tissue with human pathogens (HBV, HCV, HIV, *C. difficile*, etc.).

General Laboratory Preparations

⌚ Timing: 60–90 min

1. Prepare media and solutions. Refer to “Materials and Equipment” for more details
2. Set water bath to 37°C
3. Warm media to 37°C



KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal CC10 Dilution factor: 1:100	Santa Cruz	Cat# sc-130411; RRID: AB_2183388
Rabbit polyclonal SFTPC Dilution factor: 1:100	Santa Cruz	Cat# sc-13979; RRID: AB_2185502
Rabbit polyclonal MUC5AC Dilution factor: 1:100	Santa Cruz	Cat# sc-20118; RRID: AB_2146854
Rabbit polyclonal AQP4 Dilution factor: 1:200	Sigma-Aldrich	Cat# HPA014784; RRID: AB_1844967
Rabbit polyclonal SFTPB Dilution factor: 1:200	Atlas Antibodies	Cat# HPA034820; RRID: AB_2674347
Mouse monoclonal acetylated tubulin Dilution factor: 1:500	Sigma-Aldrich	Cat# T7451; RRID: AB_609894
Mouse monoclonal p63 Dilution factor: 1:200	Abcam	Cat# ab735; RRID: AB_305870
Biological Samples		
Human Lung samples	UCONN Health; UTHSC; UIHC	NA
Chemicals, Peptides, and Recombinant Proteins		
DMEM without L-glutamine, without sodium pyruvate	Invitrogen	Cat# 11960-044
F12	Life Technologies	Cat# 11765-054
HEPES	Life Technologies	Cat# 15630-080
Gentamycin	Life Technologies	Cat# 15710-064
Fungizone	Life Technologies	Cat# 15290018
Bovine Calf Serum (BCS)	HyClone	Cat# SH130072.03
Fetal Bovine Serum (FBS)	HyClone	Cat# SH30396.03
Penicillin-streptomycin	Invitrogen	Cat# 15140-122
L-glutamine	Invitrogen	Cat# 25030-081
0.05% Trypsin-EDTA	Invitrogen	Cat# 25300-062
DPBS, no calcium, no magnesium	Invitrogen	Cat# 14190-144
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat# 41640
Feeder removal beads	Miltenyi Biotec	Cat#130-095-531
TrypLE™ Express Enzyme (1×), phenol red	Thermo Fisher	Cat#12605036
Trypan blue solution	Corning	Cat# 25-900-CI
Corning™ Matrigel™ GFR Membrane Matrix	Fisher Scientific	Cat# CB-40230
Collagenase Type IV	Life Technologies	Cat# 17104-019
StemECHO100	Nüwa Medical Systems, Inc.	http://www.stemecho.com
StemECHO™PU Expansion medium	Nüwa Medical Systems, Inc.	http://www.stemecho.com
PneumaCult-ALI Medium	STEMCELL Technologies	Cat# 05001

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Cell Lines		
3T3-J2	Kerafast	Cat# EF3003
Experimental Models: Organisms/Strains		
Mouse: NOD.Cg-Prkdc ^{scid} Il2rg ^{tm1Wjl} /SzJ	The Jackson Laboratory	Stock No:005557 NSG
Software and Algorithms		
SH800S Cell Sorter Software version 2.1	SH800S Cell Sorter	https://www.sonybiotechnology.com/us/instruments/sh800s-cell-sorter/software/
Other		
Inverted microscope	Nikon	Eclipse Ts2
CO ₂ incubator	Panasonic	Cat# KM-CC17RU2
Tissue culture biological safety cabinet	NuAire LabGard ES	Cat# NU-437
Student Dumont no. 5 forceps	Interfocus	Cat# 91150-20
Fine scissors straight large loops 10 cm	Interfocus	Cat# 14040-10
Pasteur pipettes	Scientific Laboratory Supplies	Cat# PIP4172
Sterile disposable scalpels	Dynarex	Cat# 4122
6 Well Falcon™ Polystyrene Microplates	Fisher Scientific	Cat# 08-772-1B
12 Well Falcon™ Polystyrene Microplates	Fisher Scientific	Cat# 08-772-29
24 Well Falcon™ Polystyrene Microplates	Fisher Scientific	Cat# 08-772-1
Nunc™ 384-Well, Nunclon Delta-Treated, Flat-Bottom Microplate	Fisher Scientific	Cat# 12-565-342
Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units with PES Membrane	Fisher Scientific	Cat# 09-741-03
Steriflip 50 mL filtration units	Millipore	Cat# C3238
100 μm cell strainer	Falcon	Cat# 08-771-19
Pre-separation filters (30 μm)	Miltenyi Biotec	Cat# 130-041-407
QuadroMACS Starting Kit (LS)	Miltenyi Biotec	Cat# 130-091-051
Transwell with 0.4μm pore polyester membrane insert	Fisher Scientific	Cat# 07-200-154
Cell Sorter	Sony	Cat# SH800S
X-ray irradiation machine	RadSource	Cat# RS1800
Mini shaking incubator	WISBiomed	Cat# BS-ICH1
Centrifuge	Eppendorf	Cat# 5810 R
Hemocytometer	Daigger	Cat# EF16034F
Cool Cell Freezing Container	Corning	Cat# 432008
-80°C freezer	VWR	Cat# 76307-942
Liquid nitrogen tank	VWR	Cat# 10027-532

MATERIALS AND EQUIPMENT

Preparation of Media and Buffers

△ **CRITICAL:** The preparation needs to be performed in a Class II biological hood with standard aseptic technique.

Murine 3T3-J2 Embryonic Fibroblast Culture Medium

Reagent	Amount	Final Concentration
DMEM	440 mL	n/a
BCS	50 mL	10%
Penicillin-streptomycin	5 mL	1%
L-glutamine	5 mL	1%

Filter the medium with a 0.22 μ m filter. The culture medium can be stored at 4°C for up to 1 month.

3T3-J2 Freezing Medium

Reagent	Amount	Final Concentration
DMEM	700 mL	n/a
BCS	200 mL	20%
DMSO	100 mL	10%

Filter the medium using a 0.22 μ m filter. Aliquot and store at –80°C for up to a year.

Tissue Processing Buffer

Reagent	Amount	Final Concentration
F12	914 mL	n/a
FBS	50 mL	5%
Penicillin-streptomycin	10 mL	1%
Gentamycin	10 mL	1%
HEPES	15 mL	1.5%
Fungizone	1 mL	0.1%

Filter the medium with a 0.22 μ m filter. The buffer can be stored at 4°C for up to 1 month.

Digestion Buffer

Measure and dissolve 1 mg collagenase in 1 mL tissue processing buffer. Filter the medium with a 0.22 μ m filter. Prepare fresh digestion buffer each time.

Neutralizing Medium

StemECHO100 is a ready-to-use neutralizing medium. The culture medium can be stored at 4°C for up to 1 month.

Stem Cell Growth Medium

To prepare complete growth medium, StemECHOTMPU Expansion Medium, add 0.25 mL StemECHObullet003 to 250 mL StemECHO103. Mix thoroughly and store at 4°C for up to 1 month.

Stem Cell Freezing Medium

Reagent	Amount	Final Concentration
StemECHO TM PU Expansion Medium	70 mL	n/a
FBS	20 mL	20%
DMSO	10 mL	10%

Filter the medium using a 0.22 μ m filter. Aliquot and store at –80°C for up to a year.

PneumaCult-ALI Media

Prepare the PneumaCult-ALI Media following the product instructions (StemCell Technologies).

STEP-BY-STEP METHOD DETAILS

The procedures described in this protocol can be conducted in a typical Class II biological hood using standard aseptic technique. Given their high clonogenicity and rapid proliferation, a single stem cell will yield more than 1 billion stem cells with 60–80 days in this system, which enables downstream applications in a timely manner (Figure 1). Importantly, the intrinsic “immortality” of lung stem cell variants that renders their unlimited proliferative potential is not due to mutations of pRB, CDKN2A, p53, or any of a host of tumor suppressor or proto-oncogenes. Moreover, lung stem cell variants in this system retain their capacity of multipotent, lineage-specified differentiation despite continuous cultivation of more than one year.

Reviving 3T3-J2 Cells from Frozen Stock

⌚ Timing: 4–5 days

1. Retrieve cryovials from cell storage deep freezer and thaw the vial containing 10^6 cells/1 mL in 37°C water bath.
2. Gently transfer cells with 1 mL pipette into a 50 mL Falcon tube and add 30 mL culture medium drop by drop while swirling the tube slowly.
3. After re-suspension, seed cells into a 150 mm tissue culture plate.
4. Gently shake the plates with cells in the hood to evenly distribute the cells and transfer the cells in an incubator at 37°C with 7.5% CO₂.
5. Change the medium the next day after thawing the frozen vial and then change medium every 2 days. In general, cells will be ready for passaging and expansion 3 days after thawing.

⚠ **CRITICAL:** Do not centrifuge to wash away DMSO. Change the medium the day after thawing the vial because the freezing medium contains DMSO that is harmful to cells. Passage the cells when they become 70~80% confluent. If 3T3-J2 cells have grown past confluence, abandon them and thaw a new vial.

Expanding 3T3-J2 Cells

⌚ Timing: 1 day

6. Transfer plates from the incubator and check the confluency and morphology of the 3T3-J2 cells under a microscope.

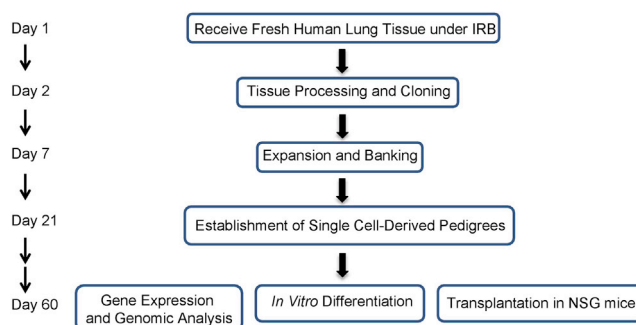


Figure 1. Workflow of Lung Stem Cell Isolation and Characterization Protocol

7. Move the plate gently into a cleaned hood, remove the medium, wash the plate with 20 mL of DPBS to remove any serum, and add evenly 5 mL of pre-warmed 0.05% Trypsin-EDTA to the plate.
8. Incubate the cells with trypsin in an incubator at 37°C with 7.5% CO₂ for 5–6 min.
9. Add 10 mL of warmed 3T3-J2 culture medium to neutralize the trypsin. Pipette up and down 10 times to make a single-cell suspension.
10. Spin down the cells at 300 g for 5 min, remove the supernatant carefully and resuspend the pellet into 5 mL of fresh 3T3-J2 culture medium. Count the cells using a hemocytometer and Trypan blue.
11. Seed 600,000–800,000 cells per plate in the amount of 20 mL 3T3-J2 culture medium, shake the plates to distribute the cells evenly and transfer the plates in the incubator at 37°C with 7.5% CO₂.

⚠ **CRITICAL:** It is important to strictly follow the seeding cell numbers recommended here; either higher or lower seeding density will affect the quality of the 3T3-J2 cells.

12. Every 2–3 days after passaging change the medium by removing the medium from the plate and adding 20 mL of fresh, pre-warmed medium.

Irradiating and Freezing Down 3T3-J2 Feeder Cells

⌚ Timing: 1 day

13. Repeat cell passaging procedure steps based on the need of cell number. Collect the cell pellets, resuspend the cells in 50 mL Falcon tubes containing 40 mL 3T3-J2 culture medium at the density less than 10⁷ cells/mL and leave on ice before irradiation.
14. Irradiate 3T3-J2 cells at 2,000 rad. We use X-ray irradiation machine (RadSource, RS1800, Cat No. 1087). 2,000 rad is calculated based on the irradiation standard to the machine.

⚠ **CRITICAL:** Although the irradiation and mitomycin-C (MC) treatment seem to be qualitatively equivalent, some studies suggest that irradiation is more suitable and efficient than MC treatment for the preparation of nonreplicating feeder cells. (Roy et al., 2001; Fleischmann et al., 2009; Llames et al., 2015). However, we have not directly compared these treatments in our laboratory so it remains unclear whether MC treatment is suitable to prepare feeder cells for lung stem cell cloning.

15. After irradiation, put the container on the ice and immediately count viable cell numbers with a hemocytometer and Trypan blue.
16. Spin down cells at 300 g for 5 min at 4°C and resuspend in freezing medium very gently to freeze down 1 × 10⁷ cells/vial. Use 0.6 mL freezing medium per vial.

⚠ **CRITICAL:** 3T3-J2 cells are extremely fragile after irradiation. Thus, cells need to be handled with extreme care. Do not pipet up and down to mix the cells. Swirl the container of cells very gently for mixing.

17. Put the cells in a Cool Cell Freezer box at –80°C for at least 24 hrs for gradual freezing. Transfer the frozen vials to a liquid nitrogen tank or deep freezer next day for long-term storage.

Preparing Feeder for Stem Cell Culturing

⌚ Timing: 1 day

Table 1. The Seeding Density of Feeder Cells and Lung Stem Cells in Different Cell Culture Vessels

Cell Culture Vessel	Approx. Growth Area (cm ²)	Recommended Culture Volume (mL)	Recommended No. of Feeder Cells	Recommended Seeding Density of Stem Cells
6 well	9.5	2	1,500,000–1,800,000	50,000–100,000
12 well	3.8	1	600,000–720,000	15,000–30,000
24 well	1.9	0.5	300,000–400,000	8,000–15,000
48 well	0.95	0.25	120,000–150,000	5,000–7,000
96 well	0.32	0.125	50,000–60,000	1,000–3,000
384 well	0.056	0.05	10,000–15,000	500–1,000
100 mm dish	55	12	9,000,000–11,000,000	500,000–1,000,000
150 mm dish	152	30	25,000,000–30,000,000	1,200,000–2,500,000
12 well Transwell	1.12	well 1.5, insert 0.5	800,000–1,000,000	400–600,000
24 well Transwell	0.33	well 0.7, insert 0.2	300,000–400,000	200–300,000

18. Revive and seed irradiated 3T3-J2 cells in the presence of 3T3 culture medium and shake the plates with cells to evenly distribute the cells (see [Table 1](#) for recommended cell numbers and culture medium volume) and transfer the plates in an incubator at 37°C with 7.5% CO₂.
19. Change with 3T3-J2 culture medium next day.

Cloning Stem Cell Variants from Human Lung

⌚ **Timing:** 10 days

⚠ **CRITICAL:** Fresh human normal or diseased lung tissue should be preserved in cold tissue processing buffer for short-term storage or shipping and be processed within 48 hrs for optimal yield. If lung tissues are properly minced and frozen in the presence of stem cell freezing medium, the lung stem cells can be derived from these frozen tissues at a later time. We have successfully derived cells from tissues that have been frozen for 2 years. There is no obvious difference in the yield of clonogenic cells among different sections of the lung. In this protocol we used distal lung tissue, but this protocol is also applicable to proximal lung tissue. The improper storage or insufficient digestion will lead to unsatisfying yield of healthy and clonogenic human cells in this system.

20. Wash 1 cm³ of distal lung tissue with 30 mL cold tissue processing buffer in 50 mL Falcon tube, centrifuge at 300 g for 10 min at 4°C, and then carefully remove the medium without disturbing the tissue. Repeat the washing procedure three times.
21. Carefully remove the tissue and put it into a 150 mm tissue culture dish. Mince the tissue between two feather scalpels until the tissue resembles a paste. The whole cutting process takes about 10–15 min.

⚠ **CRITICAL:** This mincing step is critical and will determine cloning efficiency. Thus, try to mince the tissue as thoroughly as possible. To keep the tissue from drying, add a few drops of tissue processing buffer during the process.

22. Digest minced tissue in digestion buffer (10 mL digestion buffer per 1 cm³ lung tissue) for approximately 60 - 90 min in a 37°C rocker set to 150 rpm. Every 10 min, pipette the mixture up and down to break up any aggregated clumps.

Table 2. The Seeding Density of Primary Lung Cells on 3T3-J2 Feeder Cells

Culture Plate Format	6 Well	12 Well	24 Well
No. of cells	1,000,000–2,000,000	500,000–1,000,000	200,000–400,000
Volume of medium	2 mL	1 mL	0.5 mL

23. After digestion, add 20 mL cold tissue processing buffer to the tubes containing digested tissue. Then mix the contents by inverting the tubes ten times.
24. After mixing, pipet the solutions through a 100 μ m cell strainer and collect the pass-through suspension in a new 50 mL Falcon tube.
25. Add tissue processing buffer to this filtered cell suspension until the total volume is 45 mL, centrifuge at 300 g for 20 min at 4°C, remove the buffer carefully and repeat washing steps 6 more times at 300 g for 10 min.
26. After the final wash, lyse the erythrocytes in the cell pellets by adding 5 mL ACK lysing buffer and incubating for 5 min at 20–22°C. Add 35 mL of tissue processing buffer and spin down at 300 g for 10 min at 4°C. Wash one more time with 10 mL cold neutralization medium.
27. Following the last centrifugation, resuspend the cell pellet in 1 mL of stem cell growth medium completely by pipetting up and down for ten times. Count the primary cells using hemocytometer. Seed the cells into the tissue culture dish pre-seeded with irradiated 3T3-J2 feeder cells (see [Table 2](#) for the seeding density of primary cells).
28. Change stem cell growth medium every 2 days.

△ CRITICAL: Plates seeded with irradiated 3T3-J2 feeder cells need to be used within 5 days. Using the aged feeder plates will lead to the loss of stemness in these lung stem cell variants.

Passaging Human Lung Stem Cell Variants

⌚ **Timing:** 2–4 h

29. When small and round lung stem cell colonies with relatively large nucleus and high nucleus/cytoplasm ratio are visible under the microscope (takes about 7–10 days, [Figure 2](#)), wash the plate with DPBS twice, add 1 mL TrypLE and incubate at 37°C in the incubator with 7.5% CO₂ for 15 min. Check the cells once or twice and, if they are not totally dissociated, incubate another 5–10 min until cells are fully detached from the plate.
 30. Pipet the cells gently up and down 5–10 times to further dissociate them into single cells.
 31. Add 2 mL warmed neutralizing medium quickly into the well-trypsinized cells and pipet up and down 20 times. Pipet the cells through a 30 μ m pre-separation filter, centrifuge them at 300 g for 6 min at 4°C and then discard the supernatant.
- △ CRITICAL:** Do not add neutralization medium dropwise as cells will aggregate. Pipet vigorously to break down cell clusters into single cells.
32. Resuspend the cell pellets into 6 mL stem cell growth medium, count the cells, and seed them onto a plate that was pre-seeded with irradiated 3T3-J2 feeder cells (see [Table 1](#) for recommended seeding density).
 33. Change the medium every 2 days. In about 2–3 days, individual lung stem cell colonies will be observed. In about 4–6 days, the stem cell culture plate should be ready for passaging, freezing down, single-cell cloning or single-cell RNA sequencing.

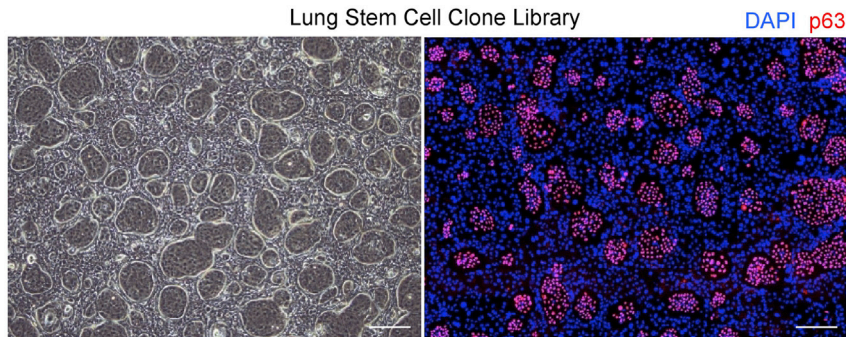


Figure 2. Cloned Lung Stem Cells Are p63 Positive

Left: A representative bright field image of the lung stem cell clones generated from resected human distal lung tissue. The image was taken 7 days after dissociated lung epithelial cells were plated on 3T3-J2 feeder cells before being passaged, and p63+ stem cells were selectively formed colonies in this culture system. Scale bar, 100 μ m. Right: A representative immunofluorescence image of the lung stem cell libraries stained with antibody to p63 showing uniform nuclear staining within each p63+ stem cell colony. These are dissociated lung epithelial stem cells after 7 days growth before being passaged. Scale bar, 100 μ m. The p63 negative cells are the 3T3-J2 feeder cells.

- △ **CRITICAL:** This system can support lung stem cell immaturity for at least 25 passages (250 days continuous propagation). We recommend performing single-cell cloning and single-cell RNA sequencing as early as possible, preferably at passage 2.

Establishing Single Stem Cell-Derived Pedigrees

⌚ **Timing:** 6–8 weeks

34. After 2 days post seeding at a density of 100,000 cells per well of 6-well plate, the stem cell clone library culture is approximately 60% confluent and each colony has about 50 cells. Trypsinize the stem cell clones following steps mentioned above, pipette the neutralized cells up and down to achieve a single-cell suspension, and pass them through a 30 μ m pre-separation filter.
35. Centrifuge at 300 g for 5 min and remove the 3T3-J2 feeder cells using QuadroMACS Starting Kit (LS) following manufacturer's protocol. Resuspend the cell pellet in 1 mL stem cell growth medium.
36. Use a cell sorter to sort single cells to individual wells of a 384-well plate previously seeded with feeder cells (Figure 3).
37. Change medium every 3–5 days. It will take around 7–10 days to observe colonies in many wells of the plate.
38. Two weeks after plating, gently wash the wells having colonies with 75 μ L DPBS twice, add 30 μ L TrypLE, incubate for 15 min or even longer in the incubator at 37°C with 7.5% CO₂.
 - △ **CRITICAL:** Check each well during trypsinization and make sure that all colonies are completely dissociated before neutralizing.
39. Gently pipet up and down 5 times for each well. Neutralize the TrypLE by adding 50 μ L warmed stem cell growth medium quickly into each well and pipet up and down another 10 times to dissociate colonies.
 - △ **CRITICAL:** Change tips for each well and pipet carefully to avoid aerosols and cross-contamination.

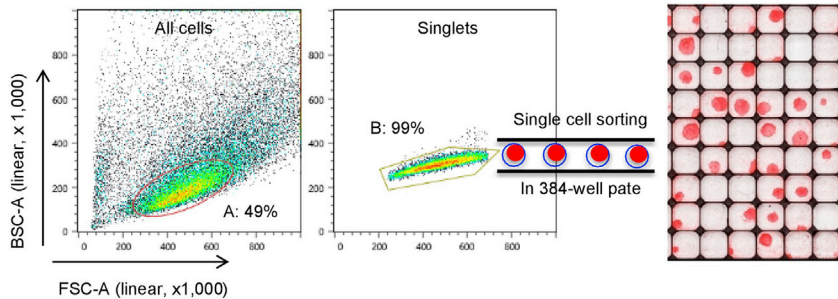


Figure 3. Schematic of Generating Single-Cell-Derived Pedigrees of Lung Stem Cells

Single cells are sorted into each well of 384-well plate and majority of them can be expanded to unique pedigrees.

40. For each well of dissociated cells, transfer the cell suspension into one well of 24-well plate each that has been seeded with feeder cells. Each well will be marked as a single-cell-derived clone and can be expanded into billions of cells in a few weeks.

Characterization of Individual Clones *In Vitro*

⌚ Timing: 4–5 weeks

Expansion of individual stem cell clones for molecular genetics and functional analyses *in vitro* (air-liquid interface, ALI) and *in vivo* (xenografts) is essential to deconstruct the heterogeneity among the epithelial stem cells of the lung and airways. Growing individual clones at ALI allows assessment of the *in vitro* differentiation potential of each clone.

41. Expand individual clones in one well of a 6-well plate pre-seeded with feeder cells. Aspirate the culture medium, rinse with DPBS thoroughly, and add 1 mL TrypLE™ Express Enzyme (1×) for 10–20 min in the incubator at 37°C with 7.5% CO₂.
42. Pipette up and down 5–10 times. Neutralize with 2 mL stem cell neutralizing medium, vigorously pipette up and down and pass through a 30 μm pre-separation filter to achieve a single-cell suspension. Remove mouse feeder cells using QuadroMACS Starting Kit.
43. Count the cells and seed 200,000–300,000 cells in 200 μL complete Stem Cell growth medium per well of a 24-well Transwell insert. Add 700 μL complete stem cell growth medium into the lower chamber of the insert.
44. Incubate the Transwell insert in a 37°C incubator with 7.5% CO₂ for 3–4 days until confluency, and change medium of both upper and lower chambers every other day.
45. At confluency, remove the medium of the upper chamber of the insert by carefully pipetting to create ALI culture. Change the medium of the lower chamber into PneumaCult-ALI Media, and keep for an additional 21 days in the incubator at 37°C with 7.5% CO₂ to induce complete differentiation.

Characterization of Individual Clones *In Vivo*

⌚ Timing: 4–5 weeks

To assess the pathogenic potential of the lung epithelial clones, such as neutrophilic inflammation and fibrosis, subcutaneous transplantation of them into highly immunodeficient NSG (NODscidIL2-ranull) is performed (Figure 4 and Video S1). A detailed phenotypic and molecular characterization of the stem cell variants in chronic obstructive pulmonary disease (COPD), for instance, has been presented (Rao et al., 2020).

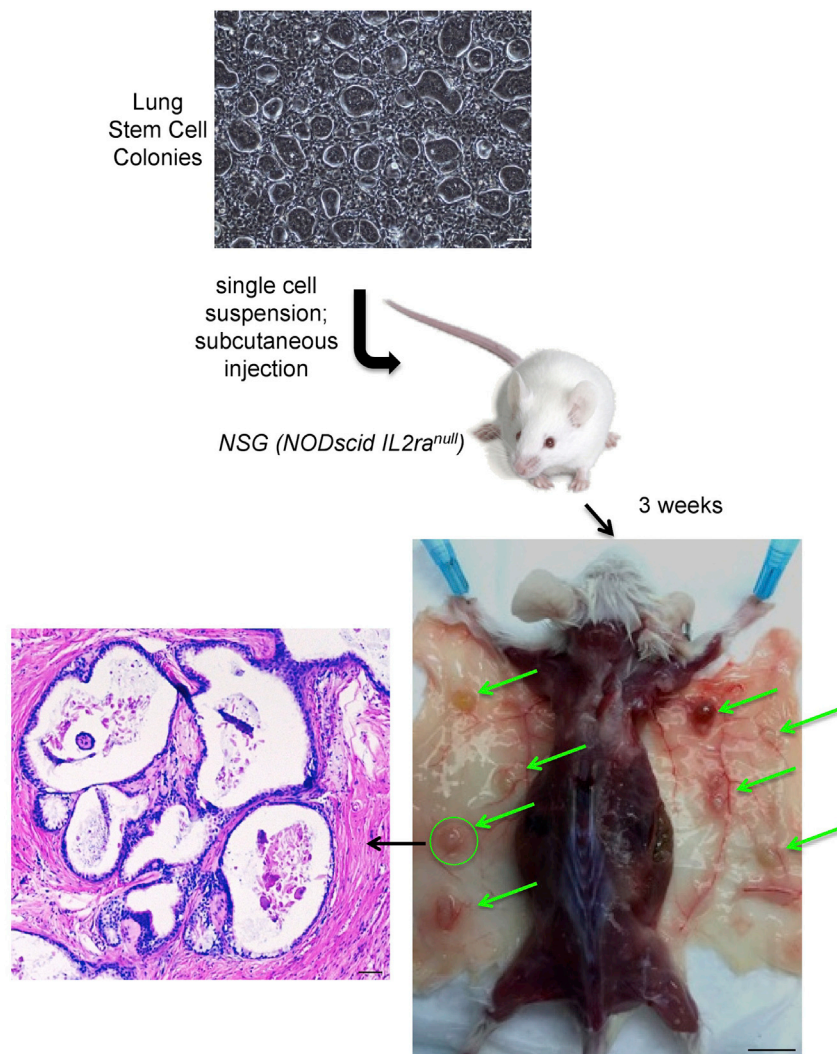


Figure 4. Work Flow of Subcutaneous Transplantation of Lung Stem Cells in NSG Mice

Lung stem cells in colonies (scale bar, 100 μ m) are trypsinized to single cells, mixed with Matrigel and subcutaneously transplanted into appropriately anesthetized 8-week-old male or female NSG mice. At 3 weeks post-transplant, mice are euthanized by CO₂ and the xenograft nodules harvested (scale bar, 1 cm), embedded, and analyzed by H&E staining (scale bar, 100 μ m).

46. Expand individual clone in three wells of a 6-well plate pre-seeded with feeder cells.
47. At 4 to 5 days post seeding, the stem cells in each well should reach 90% confluency in a 6-well plate. Trypsinize and collect the cell pellets following the same procedure as described above.
48. Resuspend the cell pellets in 50 μ L serum-free F12 medium and keep the suspension on ice.
49. Mix approximately 1×10^6 lung stem cells with 50 μ L (1:1) growth factor-reduced Matrigel, and subcutaneously inject the cells into the back of 8- to 10-week-old NSG mice of either sex following inhalation anesthetic using isoflurane gas (Video S1).

△ **CRITICAL:** In this step, both cell suspensions and GFR Matrigel should be kept on ice before injection. It is absolutely essential to use growth factor-reduced Matrigel as non-reduced contains factors that might affect complete differentiation of the cells. The differentiation fates of these clones in xenografts have been proved to be

remarkably stable to 250 days of continuous propagation *in vitro*, suggesting that the passages of these clones will unlikely affect the outcome of the xenograft experiment.

50. Clearly label the exact spots where cells were injected with a permanent marker.
51. 3 weeks after the injection, euthanize the mice, collect nodules, and fix them in 4% PFA for 24 hrs at 4°C (Figure 4).
52. Process the nodules as a small tissue sample in a tissue processor, embed in paraffin blocks, cut sections, and characterize the histology using recommended antibodies.

△ **CRITICAL:** It is important to keep the xenografts in the mice for at least 3 weeks before collecting to allow the stem cells to differentiate properly.

EXPECTED OUTCOMES

Using this protocol, libraries of epithelial stem cell variants from human normal lung or COPD lung have been established. This library comprises multiple types of p63+ stem cells that are committed to distinct lineages (e.g. Clusters 1–4; Rao et al., 2020). Single stem cell-derived pedigrees can be established and further characterized *in vitro* (molecular genetics, air-liquid interface assays, etc.) and *in vivo* (mouse xenograft assay). These established pedigrees are also suitable for the applications including RNA or DNA sequencing, genome editing, drug screening and stem cell-based regenerative medicine.

QUANTIFICATION AND STATISTICAL ANALYSIS

We provide the seeding density of irradiated 3T3-J2 cells in various types of tissue culture dishes in order to generate the highest quality of feeder seeded plates (Table 1). In addition, we provide the seeding density of lung stem cells for the optimal culture condition of maintaining stemness of these cells (Table 2).

LIMITATIONS

We have successfully derived and cultured stem cell variants from lungs of a large number of donors and observed very similar efficiency of cloning and long-term culturing independent of donor sex and age. The condition of the 3T3-J2 feeder layer can play a defining role in the success of human lung stem cell derivation, and this condition is ultimately dependent on adhering to rigid parameters of 3T3-J2 growth and expansion as defined in this protocol. Not every investigator in the laboratory can or will work within these parameters. Another important limitation of this method is the tendency of lung stem cells to spontaneously differentiate if colonies are allowed to merge to confluence. Thus, to maintain the stemness of lung stem cells, the seeding density and confluency of the cultures need to be strictly monitored. In addition, lung stem cells tend to differentiate if they are seeded as clusters of cells instead of single cells during passaging. Thus, thorough trypsinization and filtration or flow-sorting before seeding is essential to maintain the potential of these cells. While we have endeavored to control the culture conditions, we note that these media require fetal bovine serum, a variable whose impact is difficult to estimate but lot numbers should be monitored. Finally, it is critical to ensure the quality of lung stem cells prior to seeding them on *Transwell* membranes for ALI differentiation, transplanting them as xenografts, or subjecting them to genome editing protocols.

An important consideration in employing this technology is that the initial "libraries" of clonogenic cells from the lungs are complex and comprised of heterogeneous stem cells with respect to their fate commitment. Thus from COPD lungs, four major clone types were identified, all of which expressed high levels of the p63 transcription factor, a master regulator of all stratified epithelial stem cells (Senoo et al., 2007). Apart from this similarity, the four major classes of stem cells show distinct and absolute fate commitments (Cluster 1: distal airway: Club cells, type I and II pneumocytes; Cluster 2: goblet cell metaplasia; Cluster 3: squamous cell metaplasia; Cluster 4; inflammatory

cell metaplasia; Rao et al., 2020). Given this complexity, and the possibility that one clone type might display proliferation advantages over another, it is likely that long-term expansion of the libraries could alter the clone distribution. We therefore recommend that analyses such as single-cell RNA sequencing or the generation of single-cell-derived clones be performed at early-passage stages, preferably at passage 2 or 3 of the library.

TROUBLESHOOTING

Problem

3T3-J2 cells lose contact inhibition and continue to proliferate at high density resulting in a loss of lawn quality.

Potential Solution

Contact inhibition is a feature of the 3T3-J2 line that makes these cells suitable to use as feeder layers for cloning stem cells. Growth at high densities will select for those that have lost this property. If this happens, it is better to discard the cells and start over with a new, early-passage vial of 3T3-J2.

Problem

Increased saturation density of 3T3-J2 cells

Potential Solution

Typically, 150 mm dish of 3T3-J2 cells yields 8–10 million cells. If you get around 15–20 million cells, your 3T3-J2 cells have been grown improperly and, as detailed above, have lost contact inhibition. This will adversely affect your stem cell culture. In this case, it is highly recommended that you thaw a new vial of 3T3-J2 cells.

Problem

Poor health of feeder cells

Potential Solution

The condition of feeder cells strongly depends on the quality of the 3T3-J2 culture. Ensure irradiation is performed on the collected 3T3-J2 cells as soon as possible. The cell suspension should be placed on ice at all times. After irradiation, the freezing medium must be added dropwise to the cells and gently mixed.

Problem

Stem cells lose clonogenicity.

Potential Solution

Loss of clonogenicity indicates a loss of stemness. This happens if the cells start to differentiate (Figure 5). To ensure immaturity of cells, the seeding density guidelines should be strictly followed (Table 1). In addition, care must be taken to not let the cells grow to confluency as this will trigger differentiation. In case the clonogenicity is significantly reduced, it is better to start a new culture from early-passage stem cells, preferably from passage 2 or 3.

Problem

ALI-derived epithelium is damaged.

Potential Solution

The loss of structural integrity may be a result of bad condition of seeded lung stem cells or damage induced during medium changes. The seeding density of lung stem cells on Transwell filters should be strictly adhered to (Table 1). The apical medium should be carefully removed after the culture attains confluency. If leakage in the ALI is observed, the experiment should be started again.

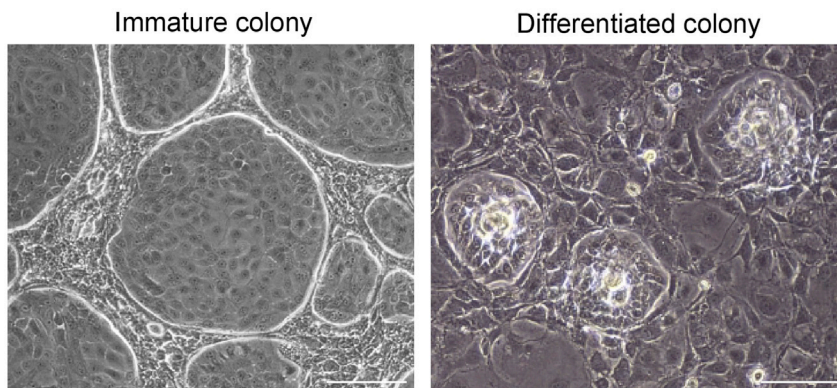


Figure 5. Inappropriate Lung Stem Cell Differentiation

Comparison between immature lung stem cell clones (left) and clones undergoing spontaneous differentiation (right). Scale bar, 100 μm . The images were taken at 7 days after the lung stem cells were seeded onto lawns of 3T3-J2 feeder cells. If the procedures described in this protocol are followed strictly, more than 80% of the stem cell colonies remain immature as shown in the left panel. If more than 50% of the colonies display the morphology shown in the right panel, the culture should be discarded.

Problem

Xenograft nodule shows incomplete differentiation.

Potential Solution

The timing of *in vivo* differentiation is key for appropriate differentiation. Based on our experience, 3 weeks seem to be ideal to collect the xenografts. One or two weeks of differentiation may not be sufficient for terminal differentiation. In addition, if nodules are left for longer than a month, the epithelia in these xenografts may diminish.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Wa Xian (wxian@uh.edu).

Materials Availability

The unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and Code Availability

This protocol does not include any datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.xpro.2020.100063>.

ACKNOWLEDGMENTS

This work was supported by grants from the Cancer Prevention Research Institute of Texas (CPRIT; RR150104 to W.X. and RR1550088 to F.M.), the National Institutes of Health (1R01DK115445-01A1 to W.X., 1R01CA241600-01 and U24CA228550 to F.M.), the US Dept. of Defense (W81XWH-17-1-0123 to W.X.), and the American Gastroenterology Association Research and Development Pilot Award in Technology (to W.X.). W.X. and F.D.M. are CPRIT Scholars in Cancer Research. We thank all the members in the Xian-McKeon laboratory for helpful discussions and support. We thank H. Green and J. Rosen for advice and support.

AUTHOR CONTRIBUTIONS

Experimental design and conception were done by W.X., M.V., W.R., and F.D.M. Human lung stem cell cloning and analysis *in vitro* and *in vivo* were performed by W.R. and S.N. S.W. and W.R. performed bioinformatics analyses. W.X., W.R., S.N., and F.D.M. wrote the manuscript with input from all other authors. W.X. and F.D.M. supervised the research.

DECLARATION OF INTERESTS

W.X., F.D.M., W.R., S.W., and M.V. have filed patents related to technologies used in the present work. M.V., F.D.M., and W.X. have financial interests in Nüwa Medical Systems, Inc., Houston, TX, USA, a trademark of Tract pharmaceuticals, Inc., Houston, TX, USA.

REFERENCES

- Kumar, P.A., Hu, Y., Yamamoto, Y., Hoe, N.B., Wei, T.S., Mu, D., Sun, Y., Joo, L.S., Dagher, R., Zielonka, E.M., et al. (2011). Distal airway stem cells yield alveoli *in vitro* and during lung regeneration following H1N1 influenza infection. *Cell* 147, 525–538.
- Rao, W., Wang, S., Duleba, M., Niroula, S., Goller, K., Xie, J., Mahalingam, R., Neupane, R., Liew, A.A., Vincent, M., et al. (2020). Regenerative metaplastic clones in COPD lung drive inflammation and fibrosis. *Cell* 181, 848–864.
- Senoo, M., Pinto, F., Crum, C.P., and McKeon, F. (2007). p63 is essential for the proliferative potential of stem cells in stratified epithelia. *Cell* 129 (3), 523–536. <https://doi.org/10.1016/j.cell.2007.02.045>.
- Zuo, W., Zhang, T., Wu, D.Z., Guan, S.P., Liew, A.A., Yamamoto, Y., Wang, X., Lim, S.J., Vincent, M., Lessard, M., et al. (2015). p63(+)Krt5(+) distal airway stem cells are essential for lung regeneration. *Nature* 517, 616–620.
- Rheinwald, J.G., and Green, H. (1975). Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 6, 331–343.
- Green, H. (2008). The birth of therapy with cultured cells. *BioEssays* 30, 897–903.
- Hirsch, T., Rothoef, T., Teig, N., Bauer, J.W., Pellegrini, G., De Rosa, L., Scaglione, D., Reichelt, J., Klausegger, A., Kneisz, D., et al. (2017). Regeneration of the entire human epidermis using transgenic stem cells. *Nature* 551, 327–332.
- Roy, A., Krzykwa, E., Lemieux, R., and Néron, S. (2001). Increased efficiency of gamma-irradiated versus mitomycin C-treated feeder cells for the expansion of normal human cells in long-term cultures. *J. Hematother. Stem Cell Res.* 10, 873–880.
- Fleischmann, G., Müller, T., Blasczyk, R., Sasaki, E., and Horn, P.A. (2009). Growth characteristics of the nonhuman primate embryonic stem cell line cjes001 depending on feeder cell treatment. *Cloning Stem Cells* 11, 225–233.
- Llames, S., García-Pérez, E., Meana, Á., Larcher, F., and del Río, M. (2015). Feeder layer cell actions and applications. *Tissue Eng. Part B Rev.* 21, 345–353.