Identification of a novel epithelial-like bone marrow cell population that can repopulate the airway epithelium

Amy P. Wong[1], Armand Keating[2], Wei-Yang Lu[3], Pascal Duchesneau[1], Xinghua Wang[2], Adrian Sacher[1], Jim Hu[4], Thomas K. Waddell[1].

Supplemental Figure Legends

Supplemental Figures

Figure 1: CCSP expression in human volunteer bone marrow cells.

(A-E) CCSP positive cells can be found in the bone marrow of 4 of 5 human volunteers. Grey histogram = isotype stain, Solid black line = CCSP stain. (F) Real time PCR amplification curve show detection of CCSP mRNA in all bone marrow samples. No amplification was observed in non-template control. Equal concentration (10ng) of cDNA was loaded in each well. Isolated human lung cells were used as positive control for PCR.

Figure 2: Mouse MSC express classical stromal cell markers but not hematopoietic markers.

Murine MSC was used as positive and negative controls for immunostaining. Scale bars = 20 microns. Original magnification x40

Figure 3: Mouse MSC and fibroblast cell line NIH3T3 cells do not coexpress stromal and hematopoietic cell markers.

Mouse MSC do not co-express CD90/CD105 and CD45. Fibroblasts do not express CD90/CD105 or CD45. Scale bars = 20 microns. Original magnification x40

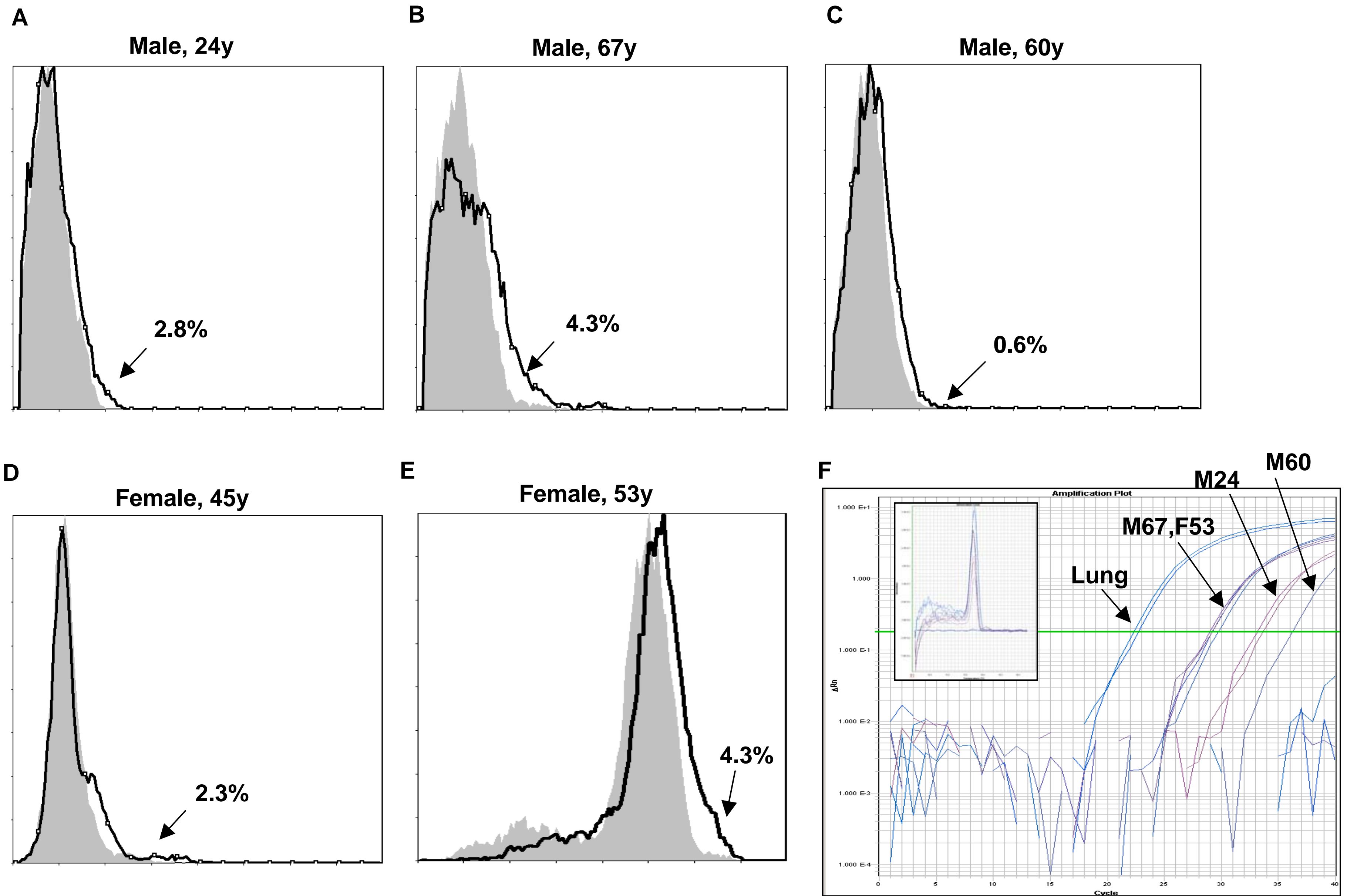
Figure 4: Most CCSP+ cells cultured under air liquid interface for 4 weeks lose CCSP expression and gain other epithelial markers.

White arrow points to a cell that does not express CCSP. Red arrow points to a cell that express CCSP but not other epithelial proteins. Scale bars = 20 microns.

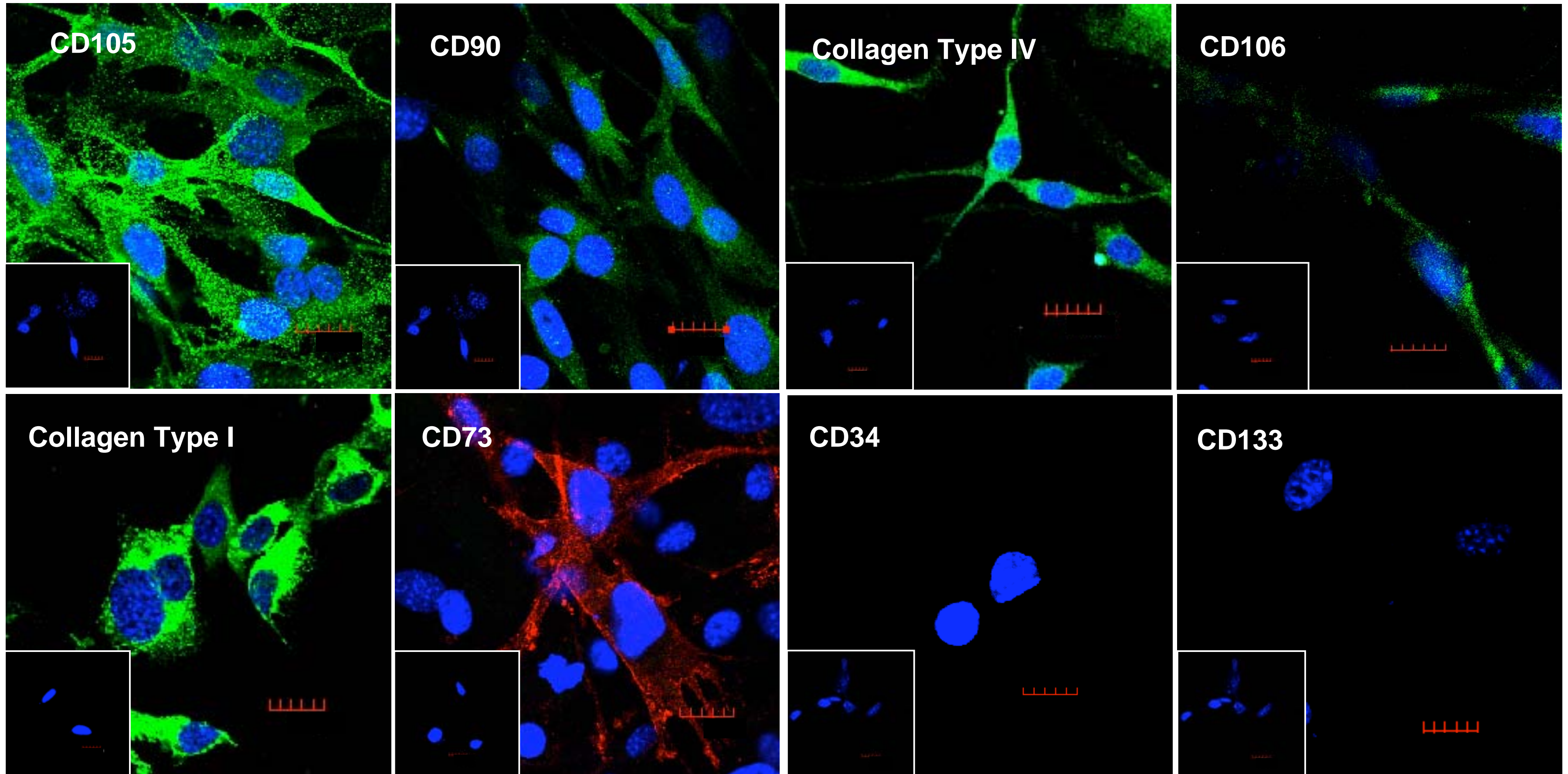
Figure 5: Real time PCR of CCSP^{+} and CCSP^{-} cells for epithelial genes immediately after FACS sorting.

GAPDH was used as housekeeping gene for normalization of expression levels. Each bar represents normalized relative levels compared to TEC.

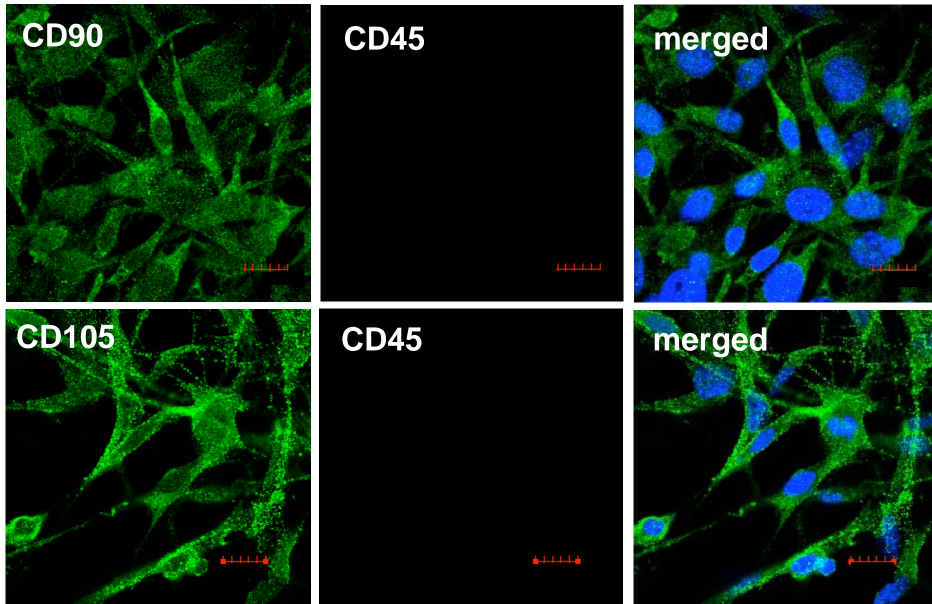
Supplementary Figure 1

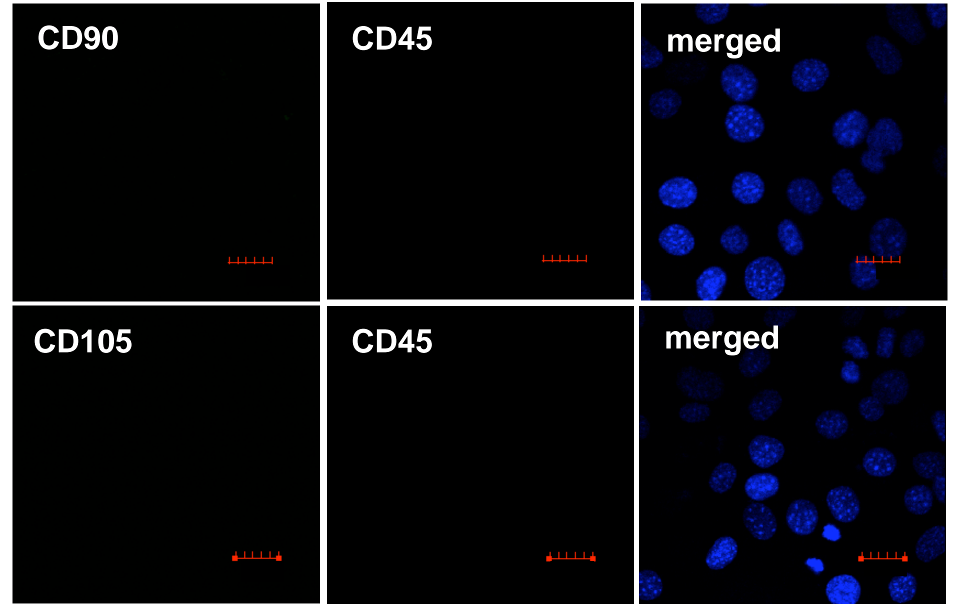


Supplementary Figure 2

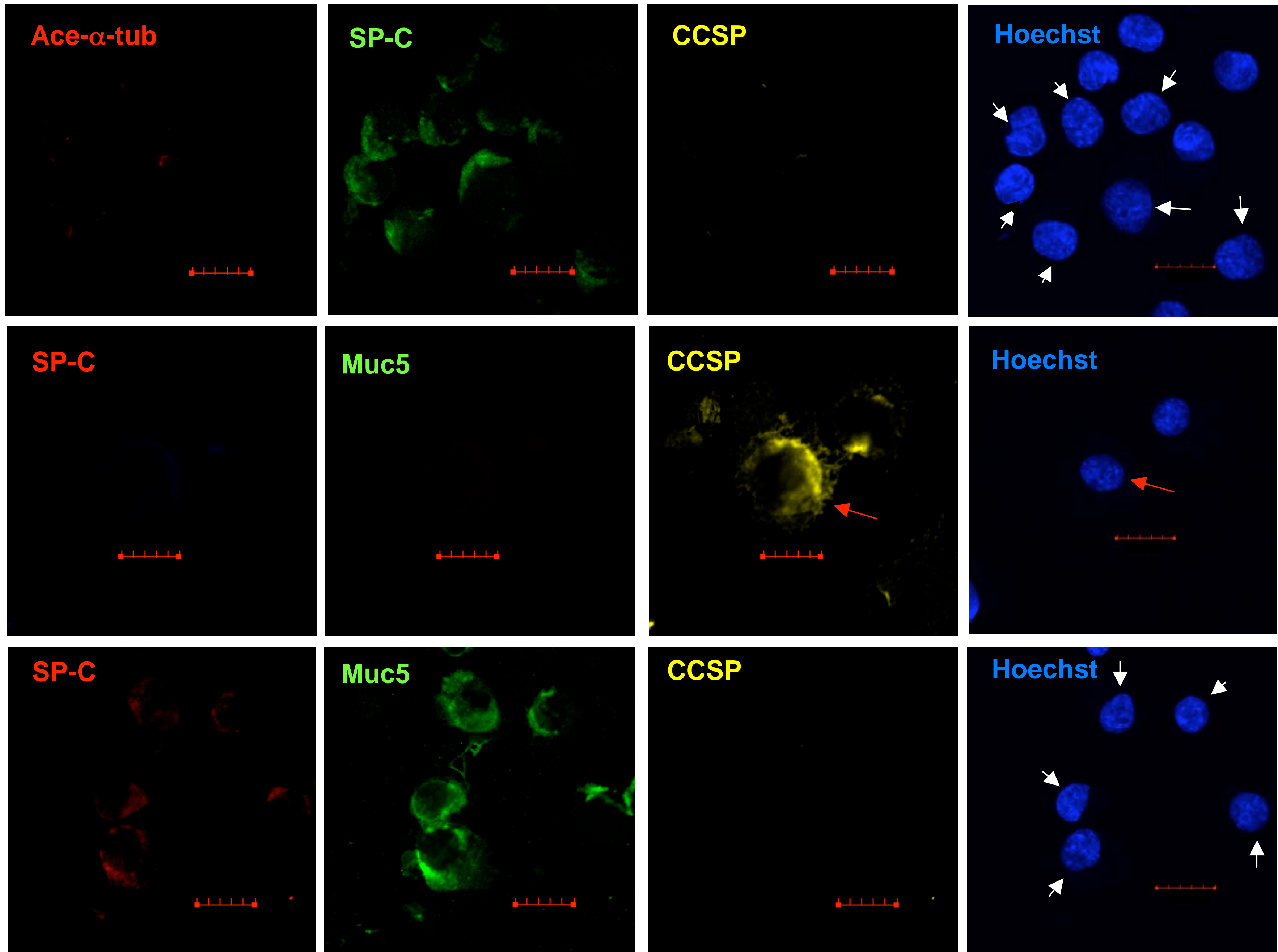


Supplementary Figure 3

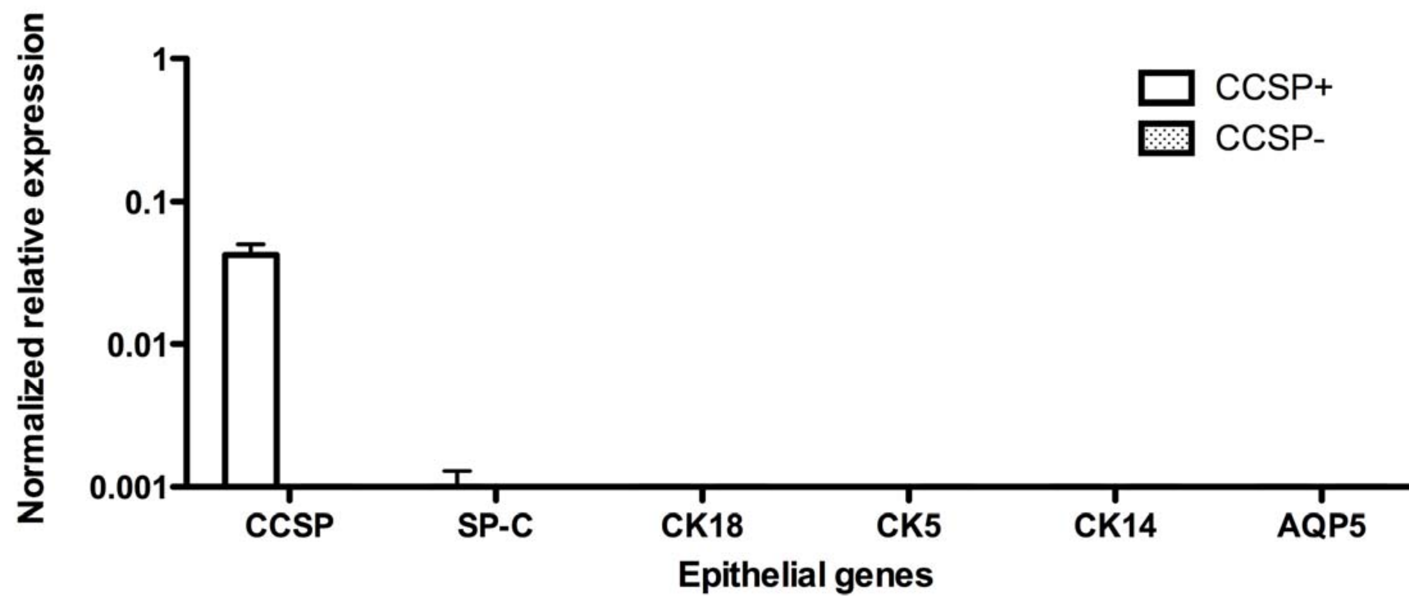




Supplementary Figure 4



Supplementary Figure 5



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Supplemental Data

Additional Methods

Bone marrow harvest and culture

BMC were harvested as previously described (1). Briefly, whole bone marrow was harvested aseptically by flushing femurs and tibiae of donor mice with cold DMEM supplemented with 10% FBS (Invitrogen) and 1% penicillin-streptomycin (Gibco) using a 23 gauge needle. BMC were plated on standard plastic tissue culture flasks at a density of $5x10^5$ cells/cm^2 and the medium was changed every 2-3 days. Non-adherent cells were gradually washed away with successive media changes. The remaining plastic-adherent BMC were cultured for 7 days in DMEM containing 10% FBS and 1% penicillin-streptomycin before use as BMC in vivo.

Fluorescence activated cell sorting and cell labeling

Freshly isolated BMC and cultured, plastic-adherent BMC from naphthalene-injured and non-injured mice (5×10^5 cells per sample) were stained with rabbit anti-mouse CCSP (1:1000, Upstate Labs) in FACS buffer containing 2% BSA in PBS for 30 minutes on ice after blocking with buffer containing 5% normal goat serum and 2% BSA to block non-specific binding. After primary antibody treatment, the cells were washed and incubated with AlexaFluor 488 secondary IgG (1:500, Molecular Probes) on ice for 30 minutes. Relative expression was compared to isotype controls for all staining. Dead cells were labeled with propidium iodine staining (1:100) on ice for 5 minutes. A FACS calibur flow cytometer and CXP Software (Becton-Dickinson) were used to assess expression of these markers. Similar results were obtained with primary anti-CCSP antibodies from Abnova (cat #H00007356-M01).

Plastic-adherent BMC cultured for 7-14 days were sorted by CCSP cell surface expression using the MoFlo Cell Sorter (Becton Dickinson). The staining protocol was followed as above. After sorting, the cells were cultured for 4 days prior to labeling with CMTMR (CellTracker™ Orange, Invitrogen) according to the manufacturer's protocol. They were injected via either the intra-tracheal or intravenous route into naphthalene-injured recipients (10^6 cells/mice). In addition, a small fraction (25%) of sorted CCSP$^+$ cells were mixed with the CCSP$^-$ population and co-injected as a "mixed" population.

Co-culture Study

Female CCSP$^-$ BMC were sorted and plated on 35 cm^2 dishes for 24 hours. Male CCSP$^+$ cells were then added to some cultures containing female CCSP$^-$ cells in a 1:4 (CCSP$^+$:CCSP$^-$) concentration. "Day 0" cells were immediately harvested after mixing and analyzed using flow cytometry for CCSP and real-time PCR for CCSP mRNA and SRY gDNA. "Day 14" cells were cultured for 14 days and harvested for flow cytometry and PCR.

Real-time PCR analysis

Total RNA was prepared from sorted BMC using the RNeasy Kit (Qiagen). For RNA, reverse transcription for first-strand cDNA was generated using Superscript II (Sigma) according to manufacturer's protocol. Real-time PCR (SYBR green detection method; Applied Biosystems) was performed for amplification of the following genes: CCSP (forward primer 5'- ATCTGCCCAGGATTTCTTCA-3' and reverse primer 5'-TCTTGCTTACACAGAGGACTTGTT-3'), K5 (forward primer 5'-

GGGAAGGAGTTGGACCAGTC-3’ and reverse primer 5’-CTTAGCCCGCTACCCAAACC-3’), AQP5 (forward primer 5’-AGCCTTATCCATTGGCTTGTC-3’ and reverse primer 5’-TGAGAGGGGCTGAACCGAT-3’), K14 (forward primer 5’-AGCGGCAAGAGTGAGATTTCT-3’ and reverse primer 5’-CCTCCAGGTTATTCTCCAGGG-3’), K18 (forward primer 5’-GACATCCGCGCCCAGTAT-3’ and reverse primer 5’-TCGGCAGACTTGGTGGTG-3’), SP-C (forward primer 5’-GCTCATGGGCCTCCACAT-3’ and reverse primer 5’-TGGAGCCGATGGAAAAGG-3’), CFTR (forward primer 5’-TCAAGCCGCGTTCTTGATAA-3’ and reverse primer 5’-AATGTGCCAAGGCAAGTCCT-3’), ENaC (forward primer 5’-TGCAGTGTGACCAACTACAAG-3’ and reverse primer 5’-TCTCGAAGATCCAATCCTGGG-3’), E-cadherin (forward primer 5’-GTCTACCAAAGTGACGCTGAA-3’ and reverse primer 5’-GGGTACACGCTGGGAAACAT-3’).

The real-time PCR conditions and instrumentation was as follows: 50˚C for 2 min; 95˚C for 10 min; 35 cycles at 94˚C for 15 sec; 60˚C for 1 min followed by dissociation using the ABI7900HT robot and analyzed with SDS 2.0 software. Gene expression of the male sex-determining gene SRY (forward primer 5’-GGGATGCAGGTGGAAAAGC-3’ and reverse primer 5’-GTGACACTTTAGCCCTCCGAT-3’) was used to quantify level of donor cell in the host lung. A standard curve was generated by spiking female lungs with increasing numbers of male cells. GAPDH (forward primer 5’-TGTGTCCGTCGTGGATCTGA-3’

and reverse 5'-GATGCCTGCTTCACCACCTT-3') was determined to be the most stable housekeeping gene by geNorm program therefore it was used as the housekeeping gene to normalize gene expression levels using REST-384 (relative expression software tool (2) available at www.Gene-Quantification.com) program. Normalized mRNA or gDNA levels are expressed as "relative" to the positive control samples (airway epithelial cells or male lung).

Western blot analysis

Cells (2x10^6/ml) and homogenized lung tissue were lysed for western blot analysis using RIPA buffer (1X PBS, 1% NP-40 substitute, 0.5% SDS, one Complete Mini EDTA-free protease inhibitor tablets (Roche) per 10ml, 4 mM Na_3VO_4, 4 mM NaF) for 1 hour on ice. The supernatants were resolved by 12.5% SDS-PAGE and assessed for CCSP (07-623, Upstate) protein levels.

Immunofluorescence

Assessment of epithelial marker expression

Briefly, adherent cells were fixed with 4% paraformaldehyde (PFA), blocked with 5% goat serum and 2% BSA in PBS containing 0.5% Triton X-100 for 1 hour, and incubated with primary antibodies overnight at 4˚C. Secondary antibodies AlexaFluors 350, 488, 532, 633 or 647 (depending on the species in which the primary antibody was raised, see figures) were applied after 3 successive washes with PBS and incubated at room temperature for 1 hour. For CD90/105 and CD45 double staining, goat anti-rat secondary was added to the cells for 1 hour followed by extensive washing and staining with rat anti-CD45-PE. In some cases, nuclei were visualized with Hoechst dye (1:5000,

Molecular Probes) for 5 minutes. Slides were mounted with immunofluorescent mounting medium. As controls for the antibodies, airway epithelial, $CCSP^+$, and $CCSP^-$ cells were stained with appropriate non-specific IgG isotypes.

Assessment of donor cells in recipient lungs

Recipient lungs were fixed at constant pressure (20 cm H_2O) with 10% formalin. Paraffin-embedded sections (5 μm thick) were prepared and stained for CCSP using an antibody kindly provided by Dr. Barry Stripp, Duke University. Sections were deparaffinized through xylene and graded alcohol washes. Antigen retrieval was done by treating the slides with Digest-All pepsin (Zymed laboratories) for 10 minutes at 37ºC. Non-specific binding were blocked with 5% BSA for 3 hours. Primary antibody mix (anti-CCSP: 1:10,000 and AlexaFluor 488-conjugated anti-GFP (Molecular Probes): 1:500) was added and incubated overnight at 4ºC followed by secondary antibody anti-goat AlexaFluor 532 for double immunofluorescence microscopy. For triple immunofluorescence microscopy, goat anti-CCSP was incubated overnight at 4ºC by secondary donkey anti-goat AlexaFluor 532 for 1 hour at room temperature. After extensive washes with PBS and 0.3% Triton X-100, slides were blocked with 5% goat serum followed by incubation with AlexaFluor 488-conjugated anti-GFP and anti-CK18 (RDI) overnight at 4ºC and secondary anti-mouse AlexaFluor 647. Nuclei were visualized with Hoechst stain (1:5000) and mounted with immunofluorescent mounting media (Dako). Isotype controls were used for non-specific binding. Images were acquired using an Olympus FluoView 1000 Confocal Imaging System (Olympus) with a pinhole diameter of 80 μm. Background noise was reduced using Kalman filter setting of 12.

Resolution was improved with sequential imaging and an imaging speed of 8um/us.

Epithelial differentiation assay

Air-liquid interface cultures

To determine whether $CCSP^+$ cells could give rise to other epithelial cell lineages, plastic-adherent BMC were sorted into $CCSP^+$ and $CCSP^-$ cells after 7 days in culture. After an additional 2 weeks they were then cultured in air-liquid interface conditions for 4 weeks as previously described (3). TEC were harvested by 0.15% pronase digestion overnight at 4°C followed by DNase I digestion on ice for 10 minutes. Cells were plated on plastic culture plates for 3 hours to allow fibroblasts to adhere. The non-adherent cells populations were then seeded onto rat collagen type IV (BD Biosciences) -coated transwell membranes (Costar) and cultured for 2 weeks in MTEC medium containing 10 mg/ml insulin, 5 mg/ml transferring, 0.1 mg/ml cholera toxin, 25 ng/ml epidermal growth factor, 0.03 mg/ml bovine pituitary extract and 5% FBS in DMEM-F12 mixture until confluency. After 2 weeks in submerged culture, only the bottom of the transwell membrane were exposed to media. $CCSP^+$ or $CCSP^-$ BMC were cultured under ALI in media containing 75% MTEC and 25% DMEM. Cell suspensions were prepared by cytospin for immunofluorescence staining. TEC were used as positive control for the assay and staining.

Perforated patch clamp recording

Perforated patch-clamp recordings were carried out on $CCSP^-$ and $CCSP^+$ cells using an Axopatch-1D amplifier (Axon Instruments). Perforated patch recording was used because this approach would not alter intracellular metabolism, thus not interrupting the metabolism-controlled ion conductance such as CFTR. All the electrical signals were digitized, filtered (1 kHz) and acquired online using Clampex software and analyzed offline using Clampfit software (Axon Instruments). Data analysis was performed using the Clampfit program (pClamp 8.1; Axon Instruments). BMC were rinsed with and bathed in the standard extracellular solution (ECS) containing (in mM) 145 NaCl, 1.3 $CaCl_2$, 5.4 KCl, 25 HEPES and 10 mM glucose (kept at 32°C). The solution filling the patch electrode consisted of (in mM) 150 Cs-gluconate, 10 HEPES, 2 $MgCl_2$, 1 $CaCl_2$, and the pore-forming agent gramicidin (50 μg ml^{-1}, Sigma-Aldrich Corp). The pH of ECS and ICS was adjusted to 7.4 and 7.3 respectively, and the osmolarity was corrected to a range of 310–315 mOsM. Patch electrodes (3–5 M) were constructed from thin-walled glass (1.5 mm diameter; World Precision Instruments) using a two-stage puller (PP-830, Narishige). Recordings started under voltage-clamp mode and membrane perforation was observed as a constant decrease in serial resistance after electrode seal. In most recordings, the resistance declined to a value ranging from 30 to 35 M within 5–8 min after the electrode seal. Recordings showing a sudden change in the resistance were abandoned and not used for the study. The total transmembrane conductance at different levels of membrane potential (V_M) was revealed by a voltage-ramp, and the CFTR- and ENaC-mediated conductance was determined by addition of forskolin (3μM), glybenclamide (100μM) and/or amiloride (100μM), to the bath solution. Glybenclamide

and amiloride were focally applied to the recorded cell using a multibarreled perfusion system (SF-77B, Warner Instruments). Total transmembrane conductance was revealed by a voltage-ramp (a steady voltage change from -100 to 100 mV within 1.5 s).

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