Online Supplemental Data

Assessing Human Airway Epithelial Progenitor Cells for Cystic Fibrosis Cell Therapy

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Running Title: Assessing Progenitors for CF Cell Therapy

Grant/Funding: Cystic Fibrosis Foundation RANDEL15XX0, RANDEL17XX0, BOUCHE15R0 and NIH P30DK065988.

Supplemental Materials

Table E1. Non-CF donor demographics; all non-smokers. CVA = cerebrovascular accident; ICH = intracranial hemorrhage.

Donor	Sex	Age	Race	Cause of Death
DONOR 1	F	17	White	Head trauma
DONOR 2	F	55	White	Head trauma
DONOR 3	M	43	White	CVA/ICH
DONOR 4	M	34	Black	CVA/ICH
DONOR 5	F	46	White	CVA/ICH
DONOR 6	F	15	White	Head trauma
DONOR 7	M	54	White	CVA/ICH
DONOR 8	F	36	White	Head trauma
DONOR 9	F	53	White	Head trauma
DONOR 10	M	14	White	Head trauma
DONOR 11	F	42	Black	Head trauma
DONOR 12	M	55	Black	Head trauma
DONOR 13	F	46	White	Head trauma
DONOR 14	F	23	Black	Anoxia/CVA
DONOR 15	M	43	Asian	CVA/ICH
DONOR 16	M	32	White	Head trauma

Methods E1. Extended Materials and Methods.

Flow Cytometry and Cell Sorting. HBECs were passaged using Accutase (Innovative Cell Technologies AT104-500) and then washed and resuspended in Cell Staining Buffer (Biolegend 420201) containing FITC-labeled anti-EpCAM (Biolegend 324204; 1:20) and PE-labeled anti-NGFR (Biolegend 345106; 1:100) antibodies (5 X 10^5-1 X 10^6 cells/tube). After 20-minutes on ice, cells were washed and resuspended in HBSS + BSA + HEPES + Pen/Strep + 10 μM Y-27632 containing Pacific Blue Annexin V (Biolegend 640918; 1:500) and Sytox Blue (Invitrogen S34857; 1:1000). All populations were analyzed by flow cytometry using single-color controls for compensation, but only D1P1 cells were sorted for NGFR positivity. Subsets of NGFR- and NGFR+ D1P1 cells were seeded at 1-5 x 10^3 cells per 100-mm dish, cultured using the CRC method, and passaged at 70-90% confluence. Analysis and sorting were performed using a Sony SH800 cytometer. FlowJo V10 was used to obtain fluorescence intensity and population density data.

Whole-mount immunostaining and imaging. ALI cultures were fixed for 30 minutes in 4% formaldehyde in PBS on day 24-32 and stained for α-tubulin (Millipore MAB1864; 3ug/mL), MUC5AC (ThermoScientific 45M1; 4ug/mL), Phalloidin (F-actin) (Invitrogen A22287), and DNA (Hoechst 33342, Invitrogen) using species-specific secondary antibodies. Subsets of P1 and CRC P1 cultures, each seeded with 2 x 10^5 HBECs, were stained with antibodies against FOXI1 (Sigma HPA071469; 1ug/mL) and CFTR (A570; 1:250), obtained from the CF Foundation CFTR Antibody Distribution Program (Figure 7).

Table E2. The percentage of NGFR+ cells and median fluorescence intensity (MFI) of candidate cell populations and donors used. Values are the median [first quartile – third quartile].

Population	% NGFR$^+$	MFI	Donors
D1P1	50.3 [44.4–56.5]	243 [204–303]	14 – 16
P1	68.5 [34.5–72.5]	450 [320–477]	
P3	71.8 [61.7–74.3]	363 [339–501]	
P5	35.6 [21.7–38.5]	459 [383–461]	
CRC P1	90.2 [81.7–93.2]	2310 [2240–2950]	
CRC P3	93.7 [92.8–94.7]	3040 [2500–3590]	
CRC P5	86.7 [85.5–87.1]	936 [826–971]	

Table E3. Colony forming efficiency (CFE) and relative cell number as determined by ddPCR for NGFR+, NGFR-, and NGFR- cCRC populations and the donors used. Values are the median [first quartile – third quartile].

Population	CFE (%)	Donors	Relative Cell #	Donors
NGFR+	6.8 [5.0–10.9]	2-3, 5, 12	14.0 [12.4–18.6]	2-3, 8
NGFR-	2.2 [1.6–8.6]		3.7 [1.8–4.7]	2-3, 8
NGFR- cCRC	5.6 [3.0–15.2]		10.4 [7.9–11.7]	2-3, 5

Table E4. Ussing chamber results from the competitive repopulation assay with NGFR+, NGFR-, and NGFR- cCRC cells. $V_{baseline}$ in mV, $R_{baseline}$ in $\Omega \cdot cm^2$. All other values in $\mu A/cm^2$. Values are the median [first quartile – third quartile].

	$V_{baseline}$	$I_{baseline}$	$R_{baseline}$	$KBR_{baseline}$	ΔAmil	ΔFSK_{peak}	$\Delta FSK_{plateau}$	ΔCFTRinh-172	ΔUTP	Donors	N
NGFR+	-(2.7[3.1–2.4])	4.4[3.5–6.2]	295[259–377]	11.5[9.8–13.9]	-(7.1[7.7–3.7])	6.4[4.3–9.1]	3.8[3.1–5.8]	-(7.1[7.7–5.4])	7.5[6.8–9.0]	2-3, 8	9
NGFR-	-(1.8[2.3–1.0])	2.9[2.2–3.2]	264[236–421]	5.9[5.7–7.4]	-(2.7[3.3–2.2])	0.2[-0.3–0.4]	0.3[-0.2–0.7]	-(1.4[2.2–1.0])	11.8[7.6–14.8]	2-3, 5, 8	7
NGFR- cCRC	-(2.4[3.9–2.1])	4.7[3.6–5.6]	298[290–357]	8.9[7.2–10.3]	-(2.7[3.0–2.0])	4.5[2.8–5.3]	2.8[1.9–3.7]	-(6.6[7.1–5.4])	14.1[10.5–16.8]	2-3, 5	9

Table E5. The percentage of NGFR+ cells and median fluorescence intensity (MFI) of NGFR+ and NGFR- cCRC cells. Values are the median [first quartile – third quartile].

Population	% NGFR+	MFI	Donors
NGFR+ cCRC	83.6 [77.2–85.7]	1760 [1330–1840]	14 – 16
NGFR- cCRC	81.8 [81.5–84.6]	1560 [991–1670]	

Table E6. Colony forming efficiency (CFE) of candidate cell populations and donors used. Values are the median [first quartile – third quartile].

Population	CFE (%)	Donors
D1P1	13.5 [5.1 – 18.1]	1 – 8, 13
P1	23.0 [11.2 – 34.7]	1 – 4, 6 – 8, 13
P3	8.1 [3.6 – 10.3]	1 – 4, 6 – 8, 13
CRC P1	19.1 [8.9 – 27.5]	1 – 9, 13
CRC P3	11.8 [8.1 – 17.4]	2 – 4, 6, 7, 9, 11, 13

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Table E7. Ussing chamber results from the competitive repopulation assay. $V_{baseline}$ in mV, $R_{baseline}$ in Ω·cm^2. All other values in µA/cm^2. Values are the median [first quartile – third quartile].

		$V_{baseline}$	$I_{baseline}$	$R_{baseline}$	$KBR_{baseline}$	ΔAmil	ΔFSK_{peak}	$\Delta FSK_{plateau}$	ΔCFTRinh-172	ΔUTP	N
UNCCF7T		-(1.4[2.0–1.1])	3.1[2.3–3.6]	232[180–315]	6.8[5.8–8.2]	-(3.4[4.6–2.6])	0.1[0.0–0.3]	-0.3[-0.5–0.0]	-(0.8[1.1–0.6])	12.1[7.5–17.3]	98-99
D1P1	**1%**	-(3.6[4.7–2.0])	5.2[4.1–6.7]	322[253–381]	10.5[7.4–15.2]	-(3.6[4.6–2.8])	3.9[1.1–6.6]	3.1[0.9–3.9]	-(6.3[9.3–4.5])	9.3[6.5–13.2]	20-21
	5%	-(5.3[7.8–3.3])	7.6[6.2–0.0]	321[272–405]	16.3[13.4–21.2]	-(6.3[8.0–3.8])	13.0[2.9–17.2]	7.7[2.4–11.7]	-(14.4[19.9–12.1])	9.4[5.6–12.4]	20-21
	10%	-(5.7[7.4–3.4])	7.7[6.0–11.8]	329[263–349]	17.9[14.7–27.8]	-(6.9[9.4–4.2])	14.9[6.1–21.8]	9.5[4.4–14.3]	-(18.8[22.4–10.2])	6.8[5.3–9.2]	20-21
	100%	-(6.0[14.0–5.5])	7.8[6.2–16.4]	392[354–438]	27.0[18.0–43.8]	-(12.1[14.5–3.9])	17.3[3.6–41.6]	8.8[2.1–16.8]	-(28.1[36.6–12.0])	3.9[3.1–4.4]	8
P1	**1%**	-(2.5[3.1–1.7])	3.7[3.1–5.0]	282[219–345]	11.2[9.2–14.3]	-(4.4[7.6–3.6])	5.2[1.4–6.8]	3.2[0.2–5.1]	-(5.4[10.7–4.4])	8.6[5.4–12.3]	23
	5%	-(3.7[6.5–2.7])	7.3[4.4–9.1]	292[192–354]	20.1[14.6–22.6]	-(5.2[9.8–4.2])	11.3[5.2–17.7]	5.9[1.9–12.6]	-(14.0 [19.8–13.0])	6.4[4.9–9.9]	24
	10%	-(3.8[6.7–2.8])	6.4[4.8–9.3]	313[253–345]	19.0[15.4–27.0]	-(6.3[12.6–5.2])	17.9[7.9–26.0]	11.8[4.3–21.1]	-(19.2[22.3–14.7])	7.3[4.2–8.6]	23
	100%	-(6.0[12.1–3.2])	10.8[7.6–12.6]	302[151–390]	33.0[21.5–36.5]	-(8.2[26.1–6.2])	16.4[13.1–28.4]	8.0[6.7–19.2]	-(26.6[31.5–17.4])	3.8[2.7–6.4]	12
P3	**1%**	-(1.8[2.4–1.2])	3.3[2.6–4.0]	235[191–314]	5.7[5.1–7.0]	-(2.8[3.4–2.2])	0.3[0.0–0.8]	0.0[-0.2–0.4]	-(1.9[2.3–1.0])	11.5[6.5–15.1]	23
	5%	-(2.8[3.5–1.9])	5.1[2.8–7.2]	280[224–310]	8.3[5.8–11.5]	-(2.7[3.5–2.0])	1.0[0.2–4.1]	0.4[-0.2–3.2]	-(4.8[6.7–2.1])	9.9[5.8–13.1]	21-22
	10%	-(3.1[4.1–1.7])	4.7[3.2–8.0]	273[232–326]	10.4[6.5–13.5]	-(3.3[4.1–2.3])	3.4[0.7–10.2]	1.4[0.1–6.6]	-(7.5[10.9–2.8])	10.2[5.5–12.2]	20-21
	100%	-(5.3[7.3–3.1])	8.7 [6.0–18.6]	282[165–380]	23.0[14.3–30.4]	-(2.8[4.9–2.2])	5.7[3.0–33.0]	1.0[-0.2–30.9]	-(27.1[32.7–16.8])	4.5[2.9–5.6]	12
CRC P1	**1%**	-(3.2[3.6–2.3])	4.8 [4.1–6.9]	259[211–360]	14.4[11.6–17.5]	-(4.9[8.7–3.6])	7.3[2.0–11.8]	3.6[0.0–9.2]	-(10.0[13.5–5.8])	10.3[7.0–16.4]	23
	5%	-(5.8[7.2–4.0])	7.3 [6.2–10.8]	344[269–409]	21.6[19.4–26.5]	-(6.9[13.9–4.9])	15.4[2.6–25.5]	9.4[-0.1–17.7]	-(15.9[23.7–10.9])	7.7[5.5–10.8]	23
	10%	-(4.7[8.3–3.5])	7.7 [5.2–13.5]	316[245–393]	23.7[16.6–28.4]	-(8.5[14.8–5.3])	18.2[6.9–28.5]	12.1[3.4–20.8]	-(17.6[24.3–15.0])	6.7[5.7–10.9]	29
	100%	-(7.0[16.9–4.7])	12.3 [8.8–23.1]	313[299–334]	34.1[21.5–43.4]	-(7.8[25.5–5.7])	14.0[5.7–30.0]	5.5[1.3–22.1]	-(25.1[29.0–14.8])	4.3[3.3–5.1]	18
CRC P3	**1%**	-(2.1[3.0–1.7])	5.1 [3.8–6.6]	218[181–271]	10.6[8.9–14.0]	-(3.1[5.5–2.1])	1.5[0.7–3.8]	0.3[-0.2–1.5]	-(6.6[7.7–3.9])	14.7[9.5–16.9]	21
	5%	-(3.2[4.0–2.3])	7.1 [4.9–9.4]	252[203–276]	16.7[12.1–20.0]	-(3.7[6.9–2.6])	3.7[1.3–9.0]	0.9[-0.4–5.5]	-(13.6[15.2–7.3])	12.5[5.4–13.8]	21
	10%	-(3.9[5.4–2.8])	7.3 [4.7–10.8]	274[245–319]	17.5[14.6–21.4]	-(3.7[7.9–2.9])	6.2[1.9–10.9]	2.1[-0.9–6.2]	-(14.7[18.3–8.8])	10.8[4.1–12.9]	21
	100%	-(3.9[7.1–2.2])	6.9 [4.8–10.4]	362[220–434]	21.4[17.7–26.1]	-(5.2[12.4–3.2])	9.0[4.8–15.2]	3.9[0.7–9.6]	-(17.1[26.5–13.9])	5.7[1.5–6.7]	18

Table E8. Relative cell number in chimeric ALI cultures as measured by ddPCR from the competitive repopulation assay and donors used. Values are the median [first quartile – third quartile].

Population	1% Seeded	5% Seeded	10% Seeded	Donors
D1P1	20.1 [10.8–31.3]	56.0 [41.8–67.0]	70.4 [47.9–83.8]	1 – 7
P1	18.1 [11.9–26.3]	47.5 [37.7–65.5]	67.4 [59.7–80.3]	1 – 7
P3	1.1 [0.4–3.6]	4.4 [2.3–16.7]	20.7 [4.3–34.7]	1 – 7
CRC P1	29.3 [20.1–38.7]	65.6 [50.4–75.2]	77.5 [65.3–86.0]	1 – 7
CRC P3	14.9 [9.3–18.6]	40.4 [31.3–53.3]	62.3 [54.0–70.2]	2 – 4, 6, 9, 11

Table E9. Colony forming efficiency (CFE) and relative cell number in chimeric ALI cultures seeded with HBECs grown using the CRC method with NIH3T3J2 or MRC5 cells. P1 and P3 populations were studied, the donors used is indicated, and cell percentage was determined by ddPCR. Values are the median [first quartile – third quartile].

Population	CFE (%)	Donors	Relative Cell #			Donors
CRC P1	14.0 [3.7–24.8]	6, 7, 9, 11	**1% Seeded**	**5% Seeded**	**10% Seeded**	
MRC5 P1	11.5 [4.9–17.2]					
CRC P3	11.8 [10.4–15.6]		15.2 [3.8–18.4]	42.3 [16.6–51.7]	66.2 [27.1–69.0]	6, 7, 9, 11
MRC5 P3	12.9 [4.5–17.9]		10.8 [8.2–14.8]	33.0 [26.1–38.0]	55.8 [46.7–59.0]	

Grant/Funding: Cystic Fibrosis Foundation RANDEL15XX0, RANDEL17XX0, BOUCHE15R0 and NIH P30DK065988.

Table E10. Ussing chamber data from the studies where MRC5 cells replaced NIH3T3J2 cells in the CRC technique. CRC P1 and P3 populations were studied. $V_{baseline}$ in mV, $R_{baseline}$ in Ω·cm^2. All other values in μA/cm^2. Values are the median [first quartile – third quartile].

		$V_{baseline}$	$I_{baseline}$	$R_{baseline}$	$KBR_{baseline}$	ΔAmil	ΔFSK_{peak}	$\Delta FSK_{plateau}$	ΔCFTRinh-172	ΔUTP	N
MRC5 P1	1%	- (4.0[4.7–3.3])	8.2[6.9–9.9]	232[188–259]	20.2[18.5–21.5]	-(7.5[8.2–7.1])	3.8[1.6–4.8]	0.4[-0.5–1.0]	-(10.3[11.0–9.3])	14.3[10.4–25.5]	6
	5%	-(8.5[15.2–6.8])	11.7[7.1–22.4]	375[306–441]	31.3[23.1–39.3]	-(9.1[10.1–7.9])	7.5[4.6–8.9]	3.6[-0.3–4.1]	-(18.4[25.0–14.9])	9.4[8.3–12.2]	6
	10%	-(7.0[11.2–6.4])	13.3[6.6–17.7]	324[248–371]	33.7[23.0–35.6]	-(10.2[11.2–7.9])	6.0[4.0–9.1]	2.3[0.3–4.8]	-(17.9[20.2–14.5])	9.7[7.4–12.4]	6
	100%	-(10.1[16.5–5.1])	10.7[6.7–18.5]	398[361–461]	33.0[24.7–39.3]	-(11.7[12.5–9.8])	9.2[6.6–12.1]	6.1[0.7–7.9]	-(20.8[26.7–16.1])	4.8[3.9–5.7]	6
MRC5 P3	1%	-(2.1[2.6–0.9])	4.1[3.3–5.5]	194[90.8–249]	9.5[5.4–12.0]	-(4.7[5.3–4.0])	0.6[0.2–2.3]	-0.4[-0.8–0.5]	-(4.0[4.7–2.9])	13.5[3.2–17.5]	12
	5%	-(4.2[5.2–2.0])	7.3[6.2–9.7]	227[129–278]	15.4[13.5–18.1]	-(4.9[6.3–3.5])	2.6[1.2–5.2]	0.1[-0.7–3.0]	-(11.0[12.9–6.3])	10.1[3.3–13.5]	12
	10%	-(4.0[4.7–2.0])	8.4[6.4–10.0]	250[97.8–263]	18.2[15.4–20.9]	-(5.0[6.2–3.9])	3.9[1.4–9.8]	1.3[-0.5–5.4]	-(12.5[14.3–8.3])	7.4[3.3–10.2]	10
	100%	-(4.9[6.6–1.9])	9.1[8.0–12.7]	256[111–423]	18.2[17.4–24.6]	-(3.8[6.3–3.3])	5.6[3.0–9.5]	2.6[1.7–7.3]	-(17.6[28.6–11.5])	3.5[1.1–7.1]	6

Grant/Funding: Cystic Fibrosis Foundation RANDEL15XX0, RANDEL17XX0, BOUCHE15R0 and NIH P30DK065988.

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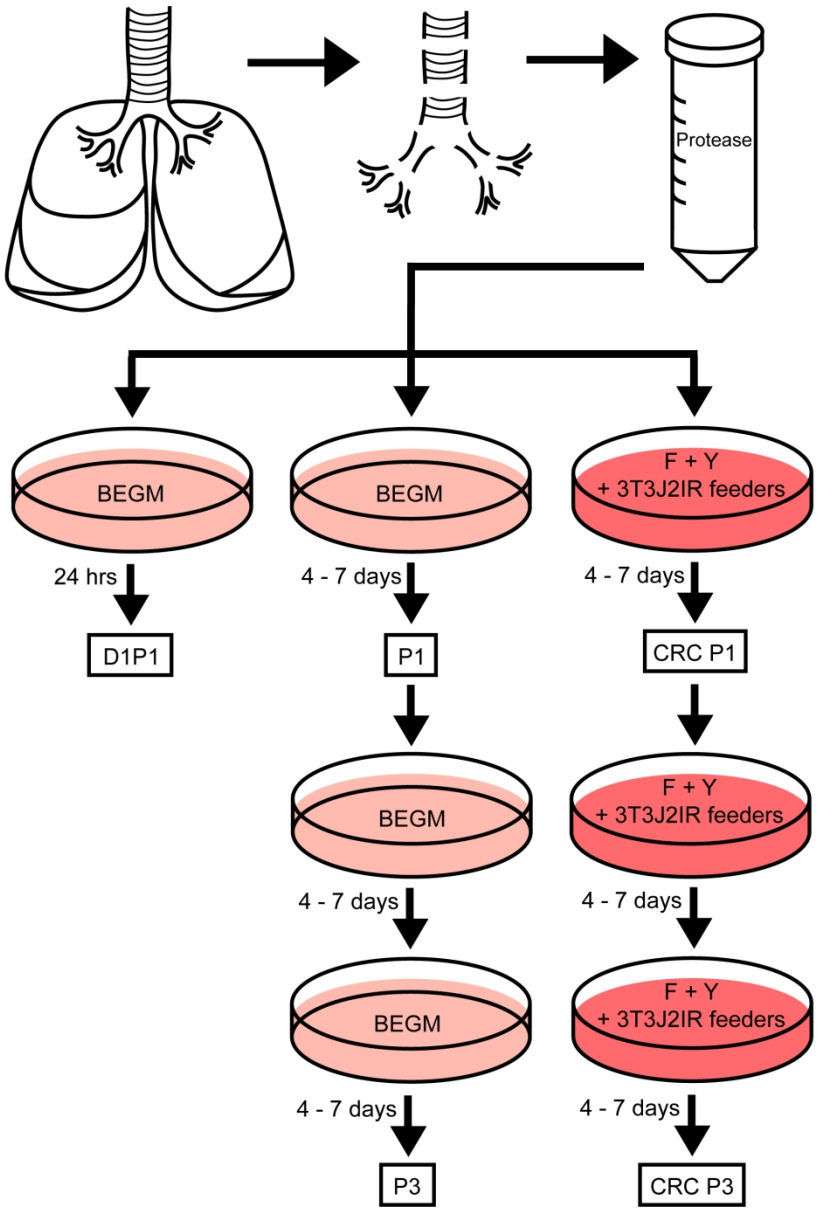

Figure E1: Schematic illustration of five candidate HBEC populations. Primary human bronchial epithelial cells (HBECs) were collected from donor lungs by dissecting large airways and digesting with protease as previously described (1). HBECs were then cultured on collagen-coated plastic dishes in bronchial epithelial growth media (BEGM) and passaged after one day (D1P1) or 4-7 days to passage 1 (P1) or an additional 4-7 days to P3. HBECs were also grown using the conditionally reprogrammed cell (CRC) culture method as described previously (2, 3) to P1 and P3. For one experiment, HBECs were further passaged to P5 and CRC P5 (Figure 1).

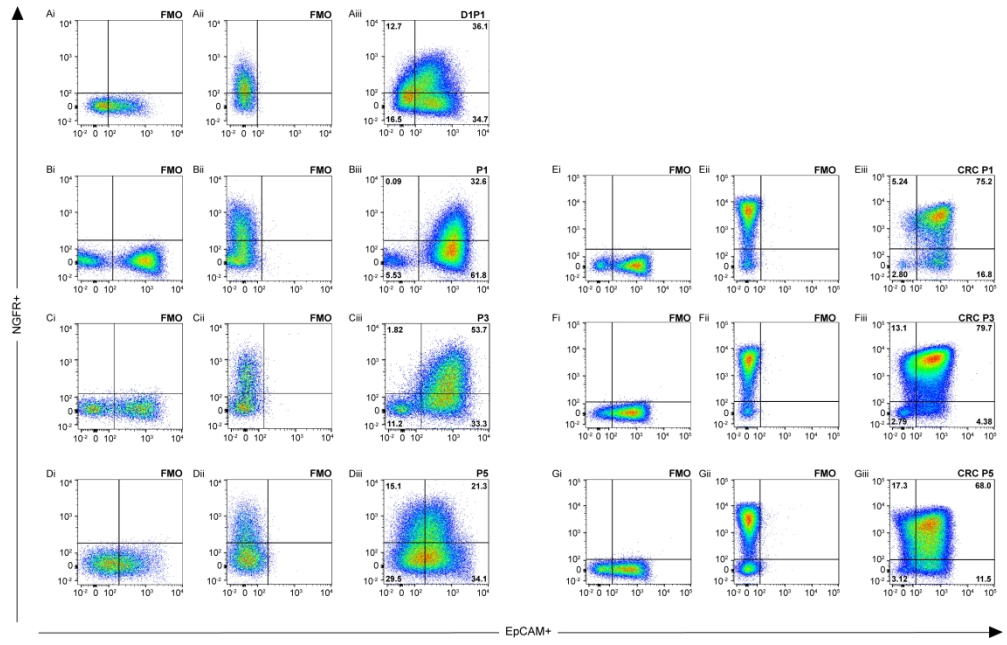

Figure E2: Gating strategy for flow cytometry plots. Fluorescence minus one (FMO) controls were run for each population: (A) D1P1, (B) P1, (C) P3, (D) P5, (E) CRC P1, (F) CRC P3, and (G) CRC P5 and for each fluorophore to define the positive populations for (Ai-Gi), EpCAM-FITC and (Aii-Gii) NGFR-PE. These gates were applied to samples stained with all fluorophores (Aiii-Giii) to quantify the percentage of NGFR+, EpCAM+ cells.

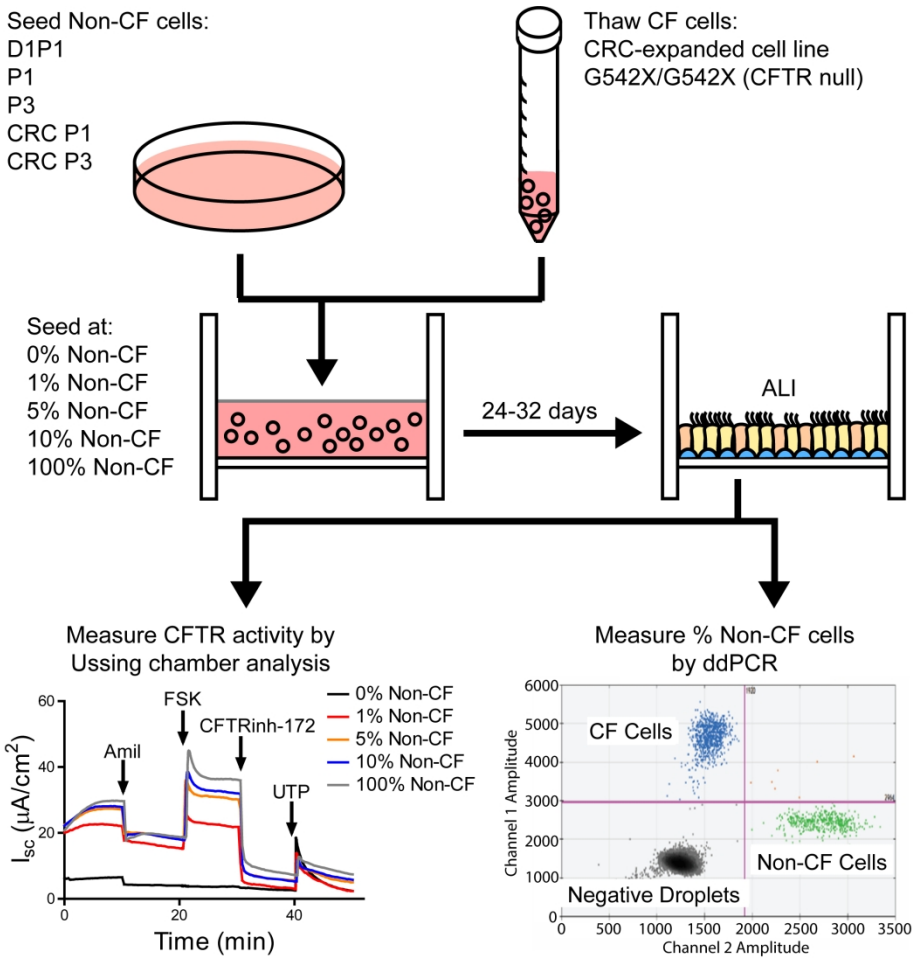

Figure E3: Schematic illustration of the competitive repopulation assay. Non-CF primary HBECs were expanded to generate five candidate populations: D1P1, P1, P3, CRC P1, and CRC P3. Each candidate population was mixed with a CRC-expanded CF cell line (G542X/G542X) or the parent HBECs at 0%, 1%, 5%, 10%, or 100% non-CF cells and seeded on a porous membrane. Cultures were grown at an air-liquid interface (ALI) for 24-32 days before functional evaluation in Ussing chambers. After Ussing studies, ALI cultures were analyzed by droplet digital PCR (ddPCR) to determine the percentage of CF and non-CF cells.

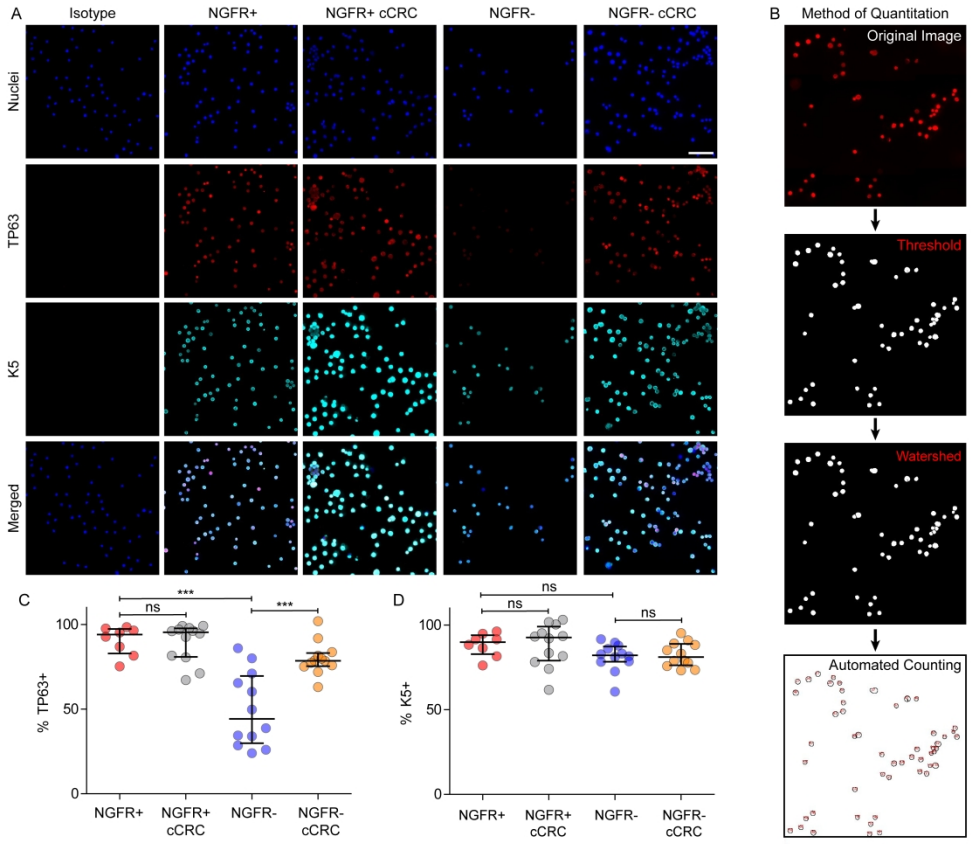

Figure E4: CRC culture rescues TP63 expression in NGFR- HBECs. (A) Representative immunofluorescence images of NGFR+, NGFR+ cCRC, NGFR-, and NGFR- cCRC cells from a single donor. Cells were stained with antibodies against the basal cell markers TP63 and K5 or with an isotype control. Scale bar = 50 μm. (B) Method of cell quantitation. Images were analyzed in ImageJ software by setting a consistent threshold for all conditions, applying a watershed algorithm to distinguish between neighboring nuclei, and then counting using the built-in particle analyzer. Percentage of (C) TP63+ and (D) K5+ cells. Biological N = 2-3. Four representative fields (766 x 766 μm) per cytospin slide were quantified. *** $P < 0.001$, ns = non-significant.

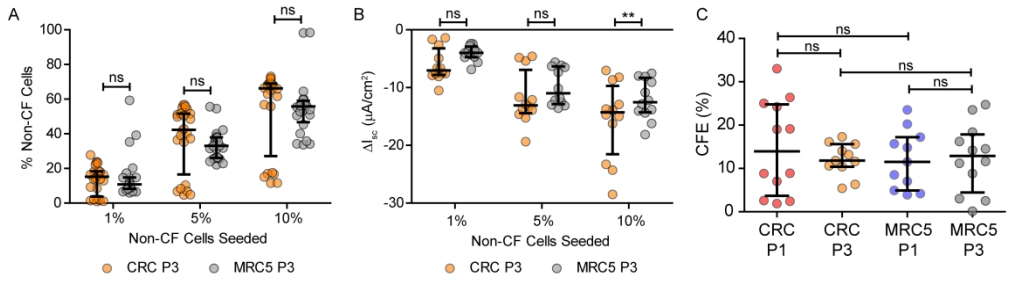

Figure E5: Human-derived feeders can replace murine feeders in CRC expansion of lung progenitors. Comparison of lung progenitors expanded using the CRC method or a modified CRC method with MRC5 feeder cells. (A) Percent non-CF cells in competitive repopulation assay on day 24-32 in ALI culture (Table E9). (B) Average short circuit current response to CFTRinh-172 for NIH3T3J2 CRC or MRC5 CRC P3 populations at doses of 0-10% (Table E10). (C) Colony forming efficiency (CFE) of 3T3J2 CRC or MRC5 CRC P1 and P3 populations (Table E9). Biological N = 4 donors; 3 replicates per donor. ** P<0.01, ns = non-significant.