Cell Reports, Volume *25*

Supplemental Information

Long-Term *In Vitro* Expansion of Epithelial Stem Cells Enabled by Pharmacological Inhibition of PAK1-ROCK-Myosin II and TGF-β Signaling

Chengkang Zhang, Hyung Joo Lee, Anura Shrivastava, Ruipeng Wang, Travis J. McQuiston, Sharon S. Challberg, Brian A. Pollok, and Ting Wang

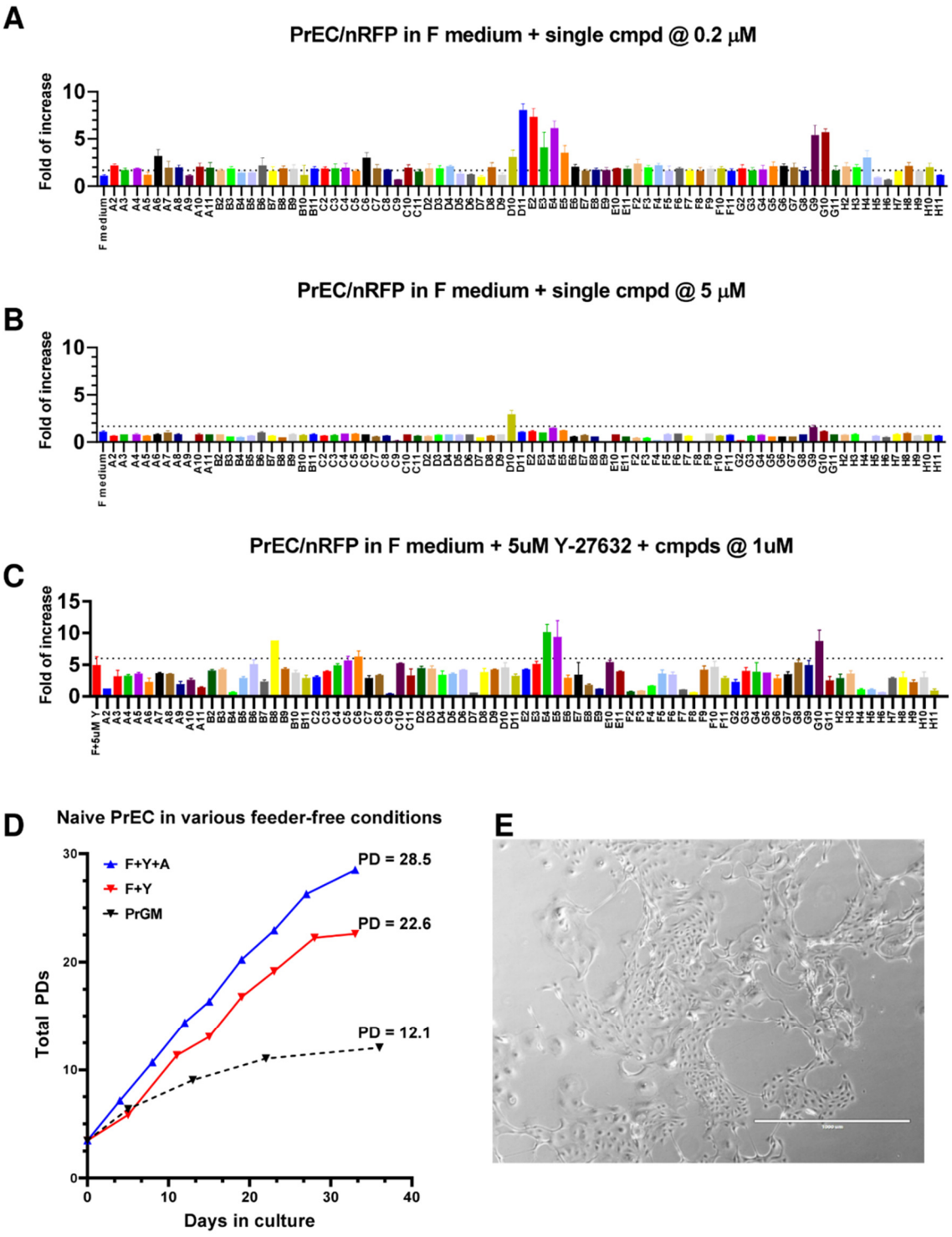


Figure S1.

Figure S1: TGF-β and ROCK inhibition extended the proliferation of epithelial cells in the F medium. Related to Figure 1. **A-B**. Proliferation of PrEC/nRFP cells in the F medium supplemented with each small molecule at 0.2 μM or 5 μM in the absence of feeder cells (data are represented as mean ± SD, n=3). **C**. Proliferation of PrEC/nRFP cells in the F medium supplemented with 5 μM Y-27632 and each small molecule at 1 μM (data are represented as mean ± SD, n=3). **D.** Population doublings (PDs) of naïve PrEC in PrGM (Lonza), F+Y (F medium supplemented

with 5 μM Y-27632) and F+Y+A (F medium supplemented with 5 μM Y-27632 and 1 μM A83-01) in the absence of feeder cells. **E.** Prostate epithelial cells in F+Y+A (passage 8), most of the cells exhibited differentiated morphology.

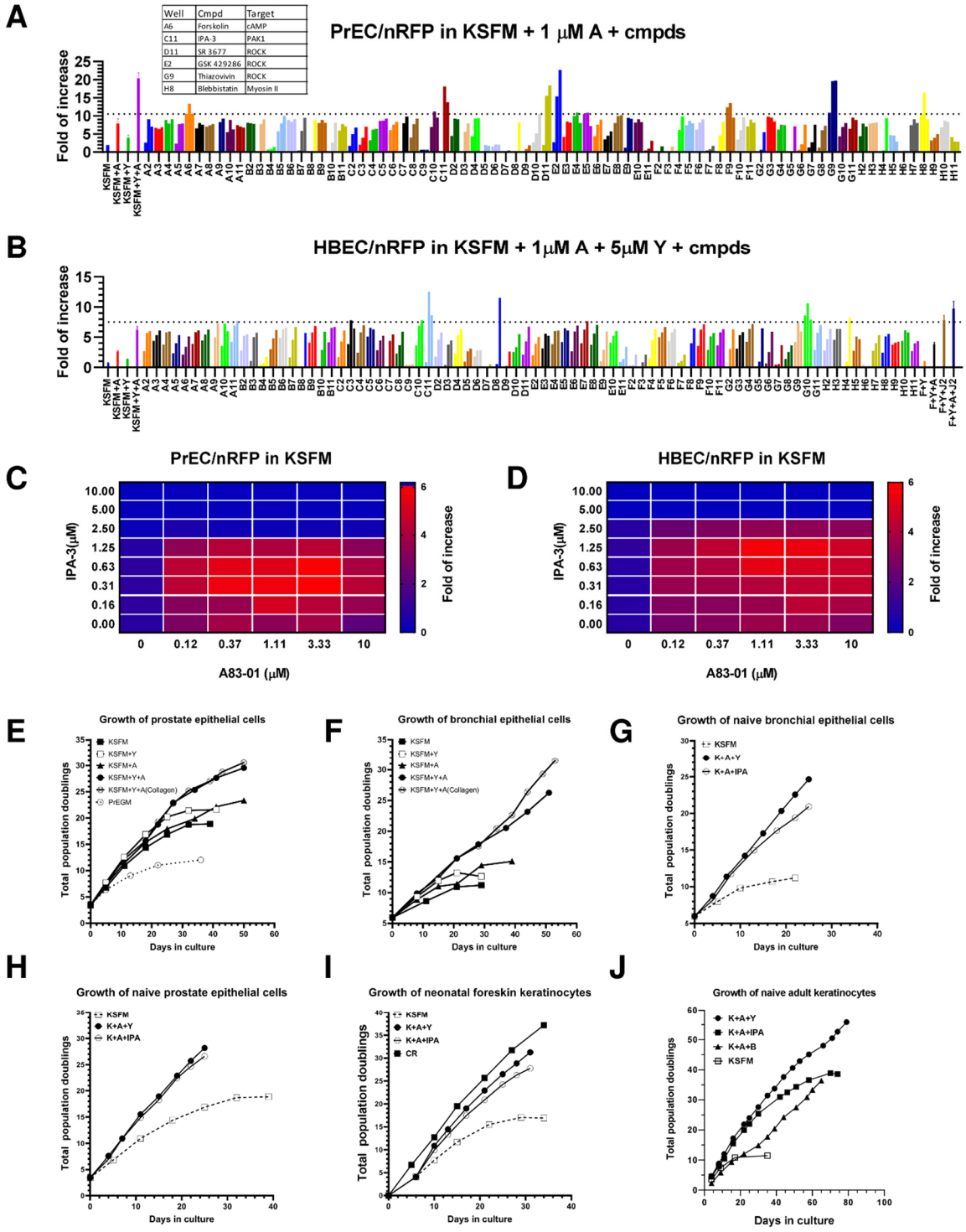


Figure S2.

Figure S2: TGF-β and PAK1-ROCK-Myosin II inhibition extended the proliferation of epithelial cells in KSFM. Related to Figure 1. **A.** Proliferation of late-passage PrEC/nRFP cells in KSFM supplemented with 1 μM A83-01 and each small molecule at 0.2 μM, 1 μM or 5 μM (dose escalation from left to right, data are represented as mean ± SD, n=3). **B.** Proliferation of late-passage HBEC/nRFP cells in KSFM supplemented with 1 μM A83-01 and 5 μM Y-27632 and each small molecule at 0.2 μM, 1 μM or 5 μM (dose escalation from left to right, data are represented as mean ± SD, n=3). **C-D**. Titration of A83-01 and IPA-3 on the growth of PrEC/nRFP or HBEC/nRFP cells in

KSFM (average of 4 replicates). **E-F.** Population doublings of PrEC or HBEC in PrGM (Lonza); KSFM; KSFM supplemented with 5 µM Y-27632 (KSFM+Y); KSFM supplemented with 1 µM A83-01 (KSFM+A); KSFM supplemented with 5 µM Y-27632 and 1 µM A83-01 on regular tissue culture vessel (KSFM+A+Y); or KSFM supplemented with 5 µM Y-27632 and 1 µM A83-01 on collagen I coated culture vessel (KSFM+A+Y(collagen)). **G-J**. Population doublings of naïve bronchial epithelial cells, prostate epithelial cells, foreskin keratinocytes and adult keratinocytes in different conditions. KSFM; K+Y+A, KSFM with 1 µM A83-01 and 5 µM Y-27632; K+A+IPA, KSFM with 1 µM A83-01 and 0.2 µM IPA-3; K+A+B, KSFM with 1 µM A83-01 and 2 µM blebbistatin; CR, conditional reprogramming method.

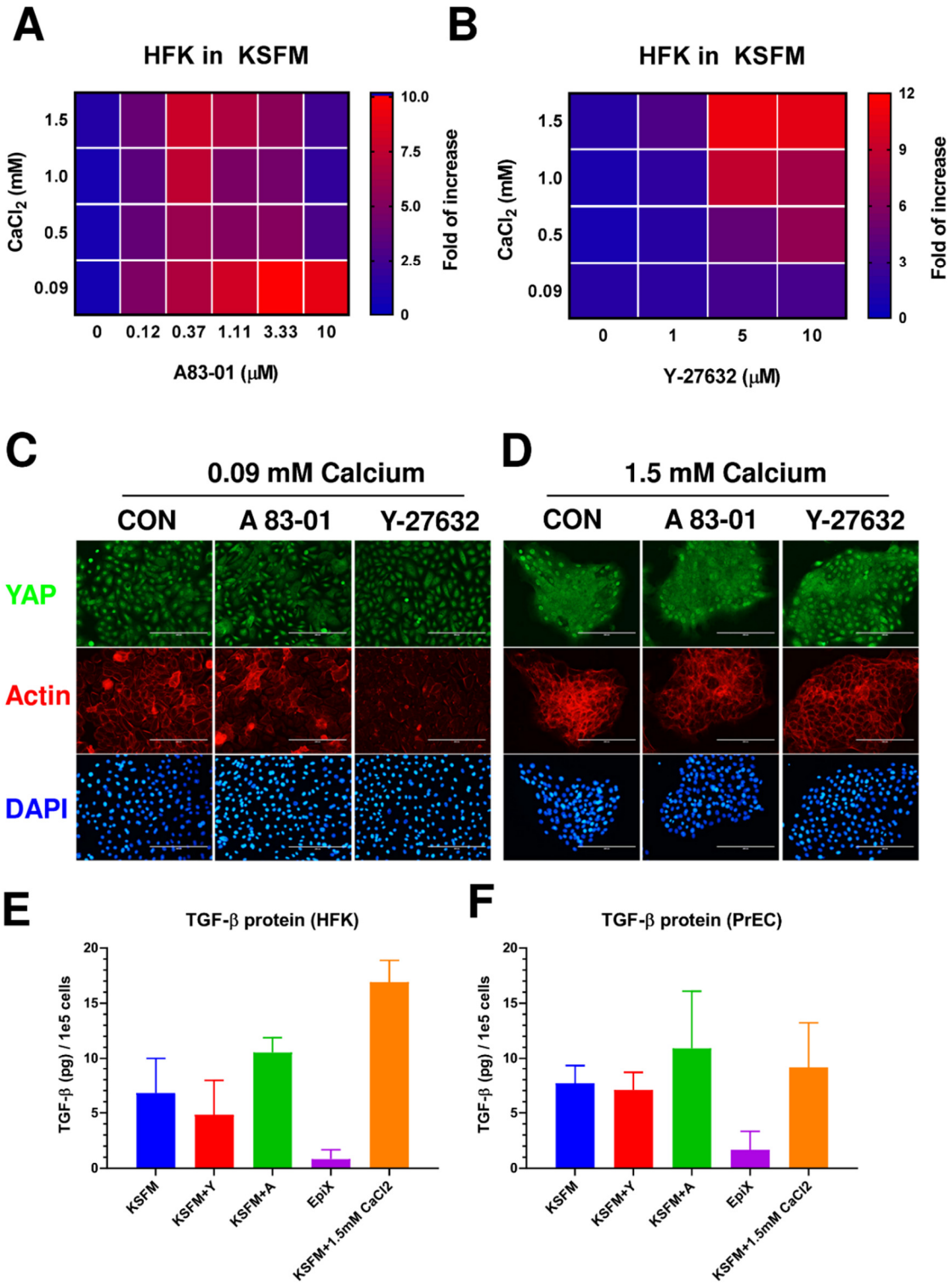


Figure S3

Figure S3: Nuclear translocation of YAP and reduced TGF-β protein expression by the epithelial cells cultured in the EpiX medium. Related to Figure 1. **A.** Interaction between A83-01 and $CaCl_2$ on the proliferation of HFK cells in KSFM (average of 4 replicates). **B.** Interaction between Y-27632 and $CaCl_2$ on the proliferation of HFK cells in KSFM (average of 4 replicates). Elevated $CaCl_2$ concentration decreased the proliferation-promoting effect of A83-

01 but boosted the effect of Y-27632. **C, D.** Immunofluorescence staining of YAP using an antibody and actin cytoskeleton using Phalloidin in HFK cultured in KSFM (90 µM $CaCl_2$) or KSFM plus 1.5 mM $CaCl_2$. **E-F.** Active TGF-β expressed by HFK or PrEC cells cultured in KSFM, KSFM with 5 µM Y-27632 (KSFM+Y), KSFM with 1 µM A83-01 (KSFM+A), EpiX (KSFM + 1 µM A83-01 + 5 µM Y-27632 + 3 µM Isoproterenol), or KSFM with 1.5 mM $CaCl_2$. TGF-β was expressed by HFK and PrEC in KSFM, and the TGF-β protein level was greatly reduced in the EpiX medium (ELISA assay data are represented as mean ± SD, n=3 replicates) .

A

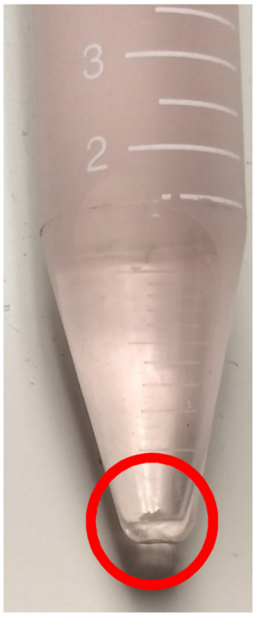
Cell Type	Donor Age	Culture Medium	PDs
HFKn	neonatal	KSFM	17
		KSFM+A+Y	71
		KSFM+A+IPA	42
		KSFM+A+B	31
		KSFM+A+GSK	38
	neonatal	KSFM+A+Y(*)	94
HEKa	adult	KSFM	12
		KSFM+A+Y	56
		KSFM+A+B	39
		KSFM+A+GSK	36
HMEC	adult	KSFM	12
		KSFM+A+Y	44
PrEC	adult	KSFM	19
		PrGM	12
		KSFM+A	23
		KSFM+Y	22
		KSFM+A+Y	43
		KSFM+A+IPA	33
HBEC	adult	KSFM	11
		KSFM+A	20
		KSFM+Y	19
		KSFM+A+Y	48
		KSFM+A+IPA	44
DHBE-CF	adult	KSFM+A+Y	52

B

Sample No.	Age/ Sex	Viable cells	Days to 50 million cells
CFNB02F	16/F	36,000	18 (P3)
CFNB03M	19/M	48,000	19 (P3)
CFNB04M	18/M	231,000*	19 (P3)
CFNB05M	15/M	36,000	22 (P3)
CFNB06M	18/M	59,500	20 (P3)
CFNB07F	20/F	400,000*	23 (P3)
CFNB08F	17/F	232,000*	25 (P4)

* The presence of blood cells might inflate total cell counts in these samples

C



D

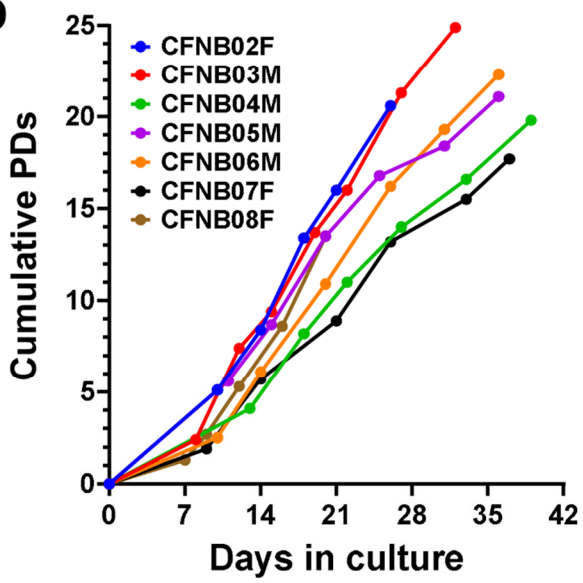


Figure S4

Figure S4: Propagation of primary epithelial cells from various tissues in the EpiX medium. Related to Figure 1. **A.** Various epithelial cells generally reach <20 PDs in the media recommended by the suppliers. The same cells could be expanded for more than 40 PDs in the EpiX medium. *, keratinocytes isolated in the EpiX medium reached more PDs than the ones purchased from commercial source. The cells are obtained from ThermoFisher (HFKn and HEKa) or Lonza (HMEC, PrEC, HBEC and DHBE-CF). HFKn, neonatal human foreskin keratinocyte. HEKa, adult human

epidermal keratinocyte. HMEC, human mammary epithelial cells (female). PrEC, human prostate epithelial cells. HBEC, human bronchial epithelial cells. DHBE-CF, diseased human bronchial epithelial cells from a cystic fibrosis patient. KSFM, Keratinocyte-SFM (ThermoFisher). PrGM, Prostate Epithelial Cell Growth Medium (Lonza). A, ALK5 inhibitor A83-01. Y, ROCK inhibitor Y-27632. B, Myosin II inhibitor blebbistatin. IPA, PAK1 inhibitor IPA-3. GSK, ROCK inhibitor GSK-429286. **B.** Expansion of primary nasal epithelial cells from nasal brushing samples obtained from 7 CF patients (all have homozygous ΔF508 mutations). More than 50 million nasal epithelial cells can be generated in 3–4 weeks. **C.** Nasal brushing samples collected from a CF patient. **D.** Expansion curves of 7 CF nasal epithelial cell strains in the EpiX medium over 5 weeks.

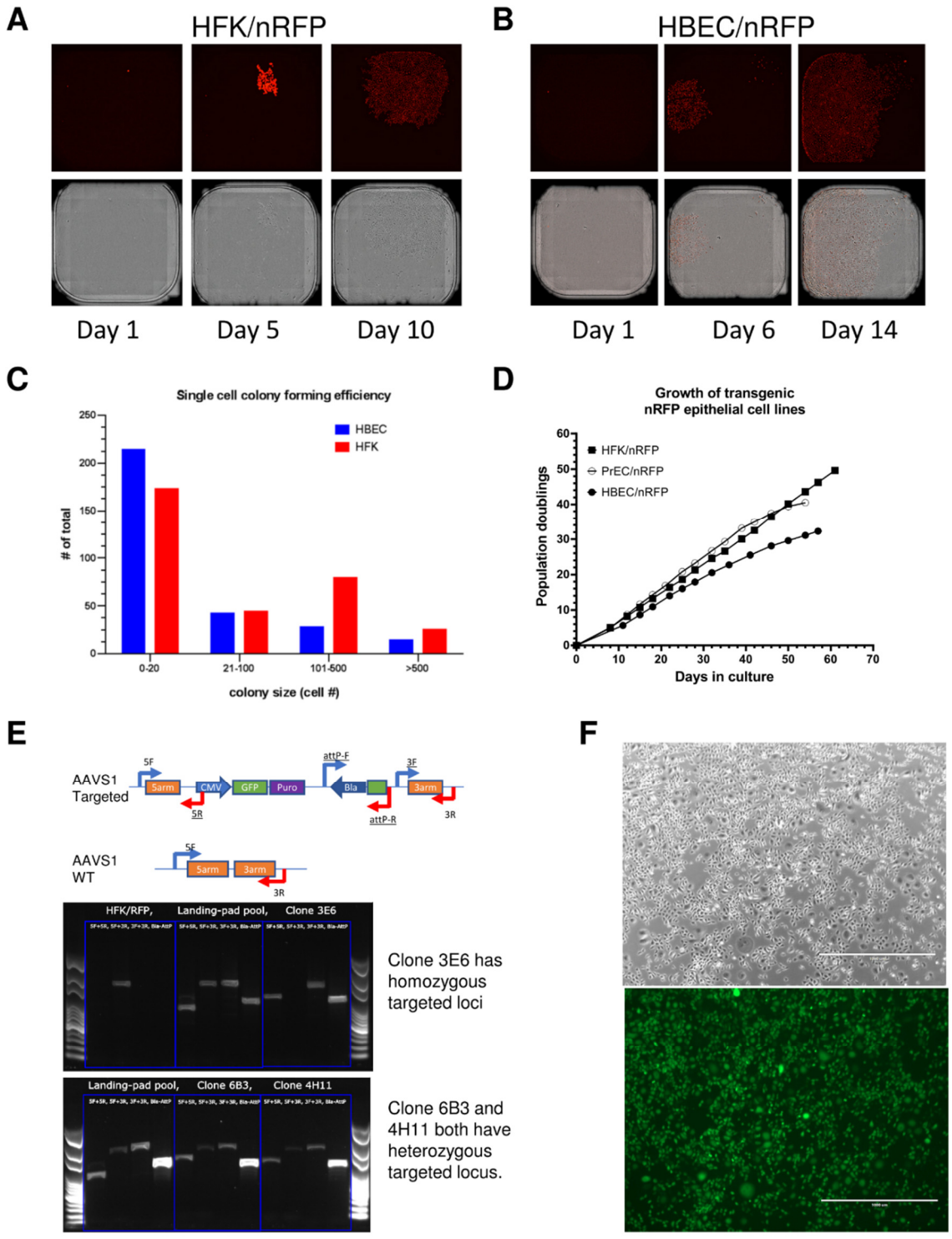
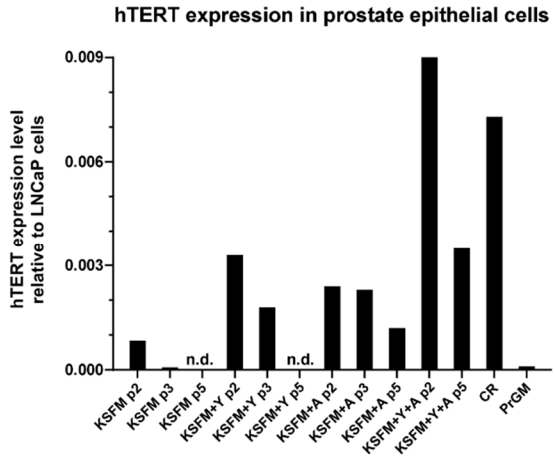


Figure S5.

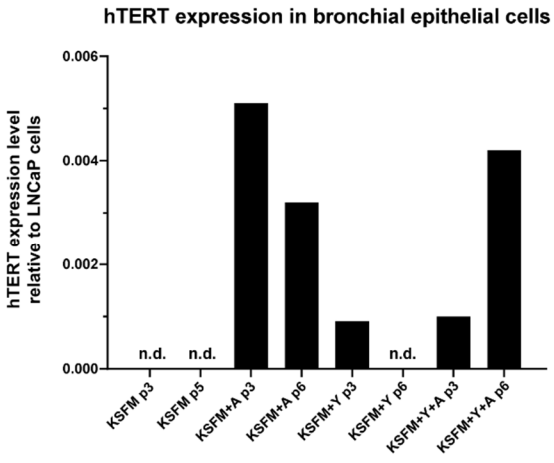
Figure S5: EpiX medium supported single cell cloning and genetic engineering by CRISRP/Cas9. Related to Figure 1. **A. B.** Limited dilution of HFK/nRFP and HBEC/nRFP cell lines in 384-well plate. One cell grew up into a colony in about 2 weeks. **C.** Single cell cloning efficiency of HFK/nRFP and HBEC/nRFP cell lines in the EpiX medium. The number of cells in each colony were counted after 10 days. **D.** PrEC, HBEC and HFK were engineered to

express nRFP via lentivirus transduction and continued to expand for more than 30 PDs in the EpiX medium. **E.** Introducing a GFP-expression cassette into the AAVS1 locus of HFK by CRISRP/Cas9 RNP. Primers used to confirm the targeting events were depicted. Genomic DNA PCR were used to confirm the correct targeting of GFP-expression cassette into the AAVS1 locus. Both AAVS1 loci were targeted in Clone 3E6, and one locus was targeted in clones 6B3 and 4H11. **F.** Morphology of HFK expressing GFP from the AAVS1 locus (clone 6B3) under phase contrast and fluorescence microscopy.

A



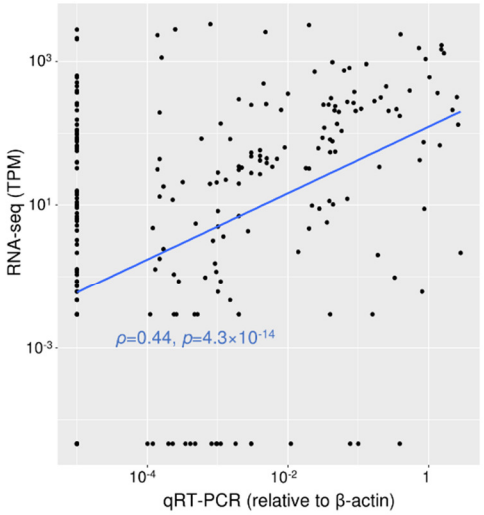
B



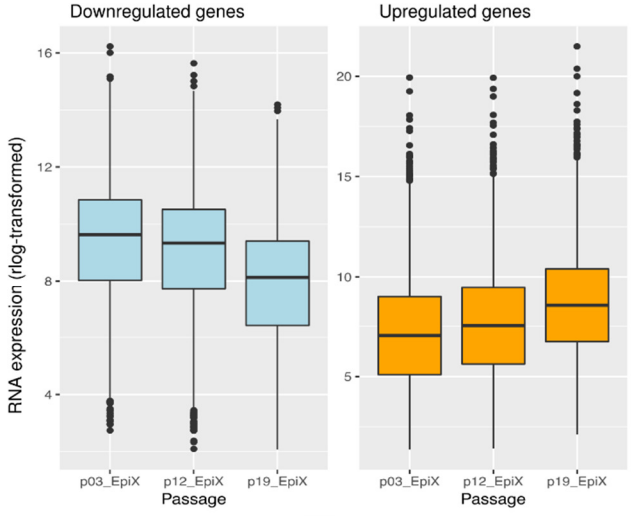
C

Gene Name	Description	HFKn in KSFM		HFKn in EpiX		
		P2	P6	P2	P13	P23
AKT1	Protein Kinase B	0.009	0.2	0.003	0.003	0.003
ATM	ATM Serine/Threonine Kinase	0.007	0.04	0.002	0.004	0.002
CDKN2A	p16, INK4A	0.002	0.04	0.001	0.006	0.004
GADD45A	Growth arrest and DNA-damage-inducible, alpha	0.01	0.07	0.002	0.003	0.008
GLB1	Galactosidase, beta 1	0.004	0.02	0.005	0.004	0.005
PLAU	Plasminogen activator, urokinase	0.02	0.07	0.001	0.002	0.001
SERPINE1	Plasminogen activator inhibitor 1	0.04	0.1	0.002	0.002	0.003

D



E



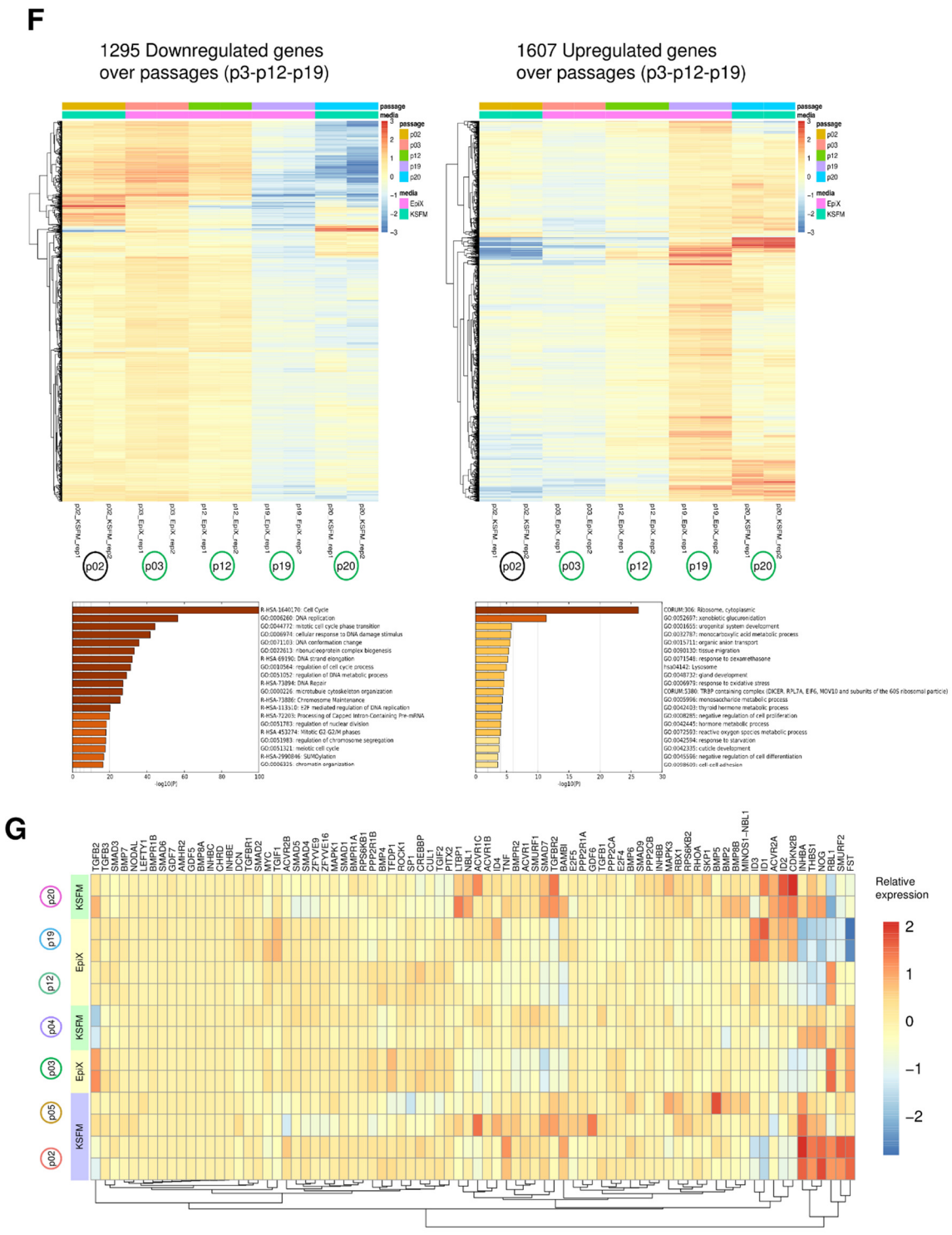


Figure S6.

Figure S6: Transcriptome dynamics of epithelial cells expanded in the EpiX medium. Related to Figure 2. **A. B.** qRT-PCR analysis of *TERT* expression in human prostate epithelial cells and bronchial epithelial cells cultured under different conditions. Prostate cancer cell line LNCaP was used as control for the expression of TERT, which was set as 1. n.d. not detected. **C.** qRT-PCR analysis of several stressed-related genes expression in HFK cultured in the EpiX medium or KSFM. The gene expression level was expressed as relative to that of β-actin, which was set as

1. **D.** Recapitulation of qRT² Profiler PCR Array results by RNA-Seq data. Each dot represented the expression of a gene in one matched condition. Each gene had five matched conditions: P2 in KSFM, P5 or P6 in KSFM, P2 or P3 in EpiX, P12 or P13 in EpiX, and P19 or P23 in EpiX. X-axis represented qRT-PCR expression value relative to the housekeeping gene β-actin expression level, which could be found in Table S2. Y-axis represented TPM value from RNA-Seq, which could be found in Table S2. Pseudocount 10^{-5} was added to each value and log-transformed. Pearson correlation coefficient was calculated and shown with *p*-value. **E.** Gradual changes of gene expression in the increasing passages in the EpiX medium. The expression levels of differentially expressed genes in P12 passage was intermediate between P3 and P19 passages. Y-axis represented rlog-transformed read counts. **F.** The heatmap of the expression levels of differentially expressed genes over increased passages in EpiX medium and gene ontology and pathway enrichment analysis of downregulated and upregulated genes by metascape tool. **G.** Heatmap of the expression levels of genes in KEGG human TGF-β signaling pathway among different culture conditions.

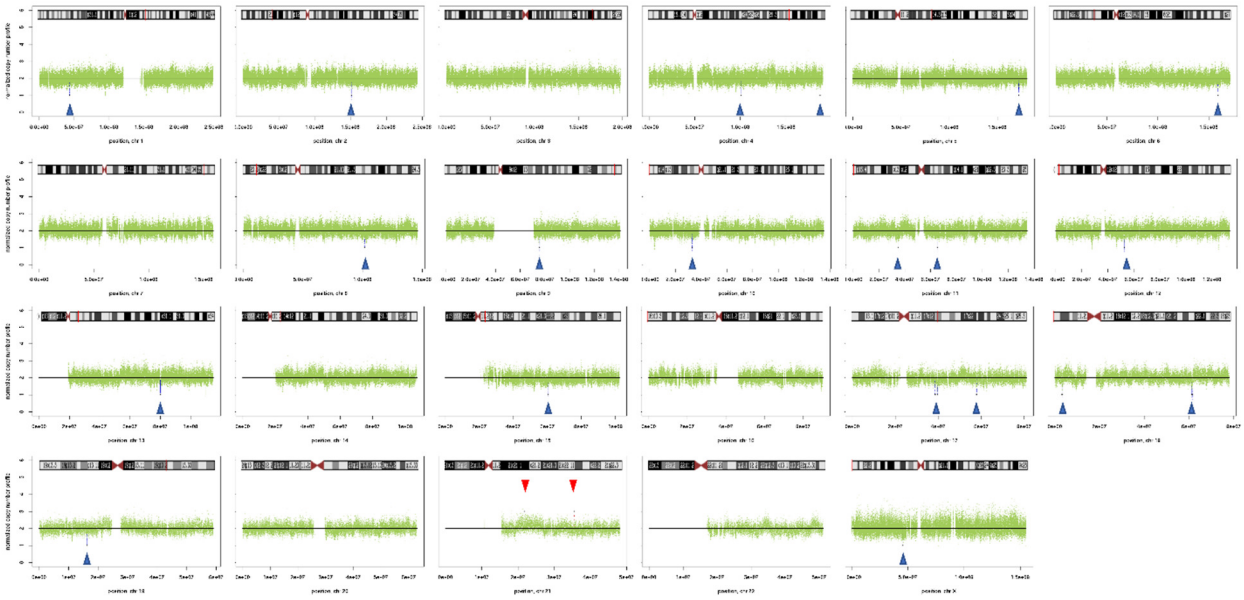


Figure S7.

Figure S7: Locations of copy number variations (CNV) that were identified the CF sample by whole genome sequencing. Related to Figure 3 and Table S4. Blue triangles indicated the locations of losses in CNV, and red triangles indict the locations of gains in CNV.

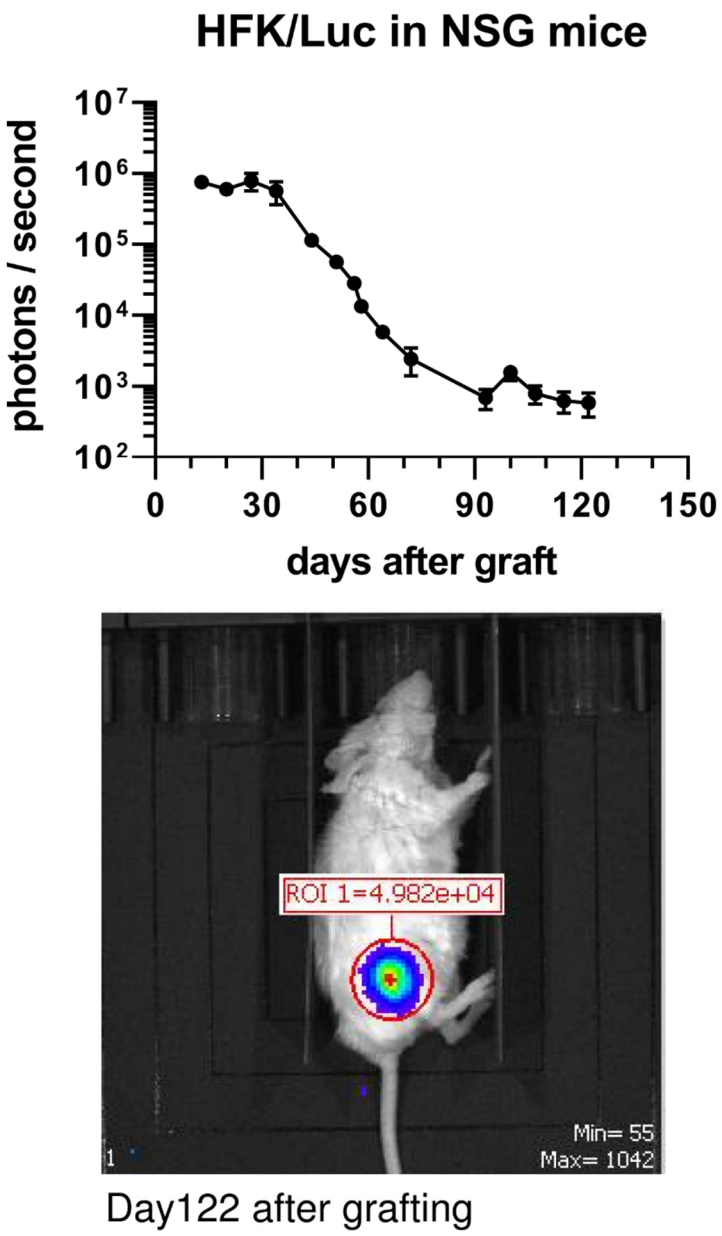


Figure S8.

Figure S8: *in vivo* luminescence imaging of HFK expressing firefly luciferase (HFK/Luc) that were injected subcutaneously into NSG mice. Related to Figure 4. Luminescence signal was readily detected 122 days after the HFK cells were injected into the NSG mouse, suggesting that EpiX-expanded HFK retained self-renewal ability *in vivo*.

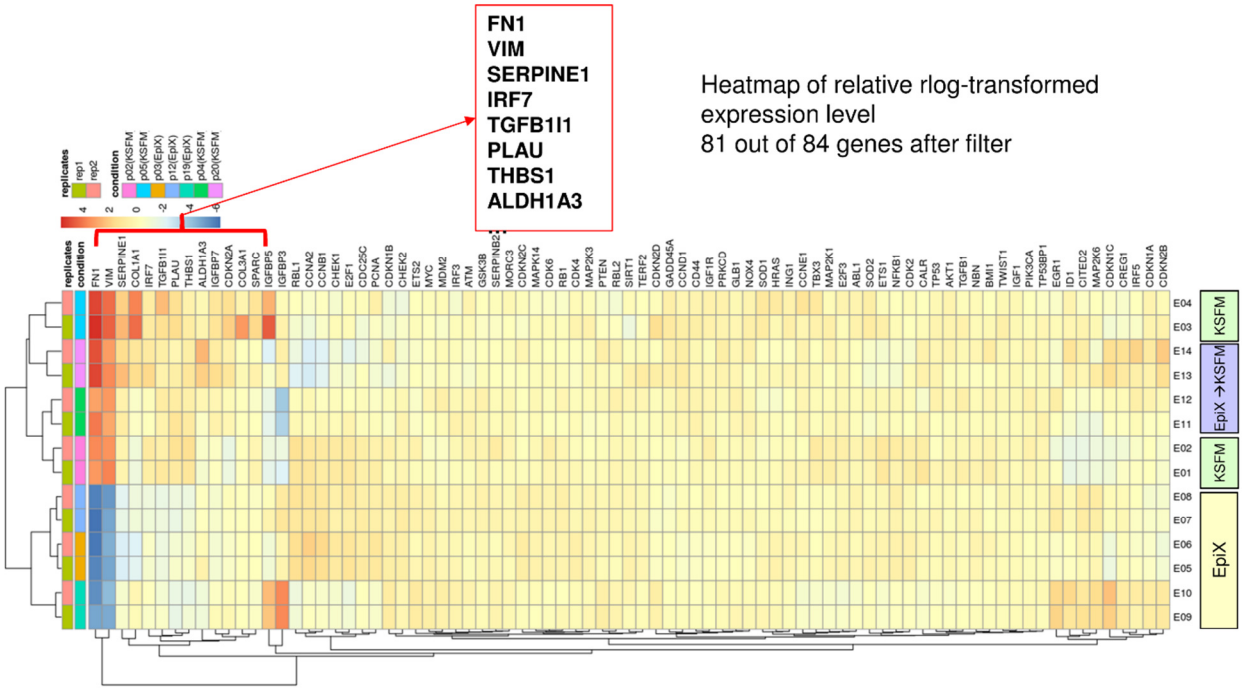


Figure S9.

Figure S9: Heat-map of gene expressions associated with cellular senescence (genes included in the RT^2 Profiler™ PCR Array). Related to Figures 2 and 6.

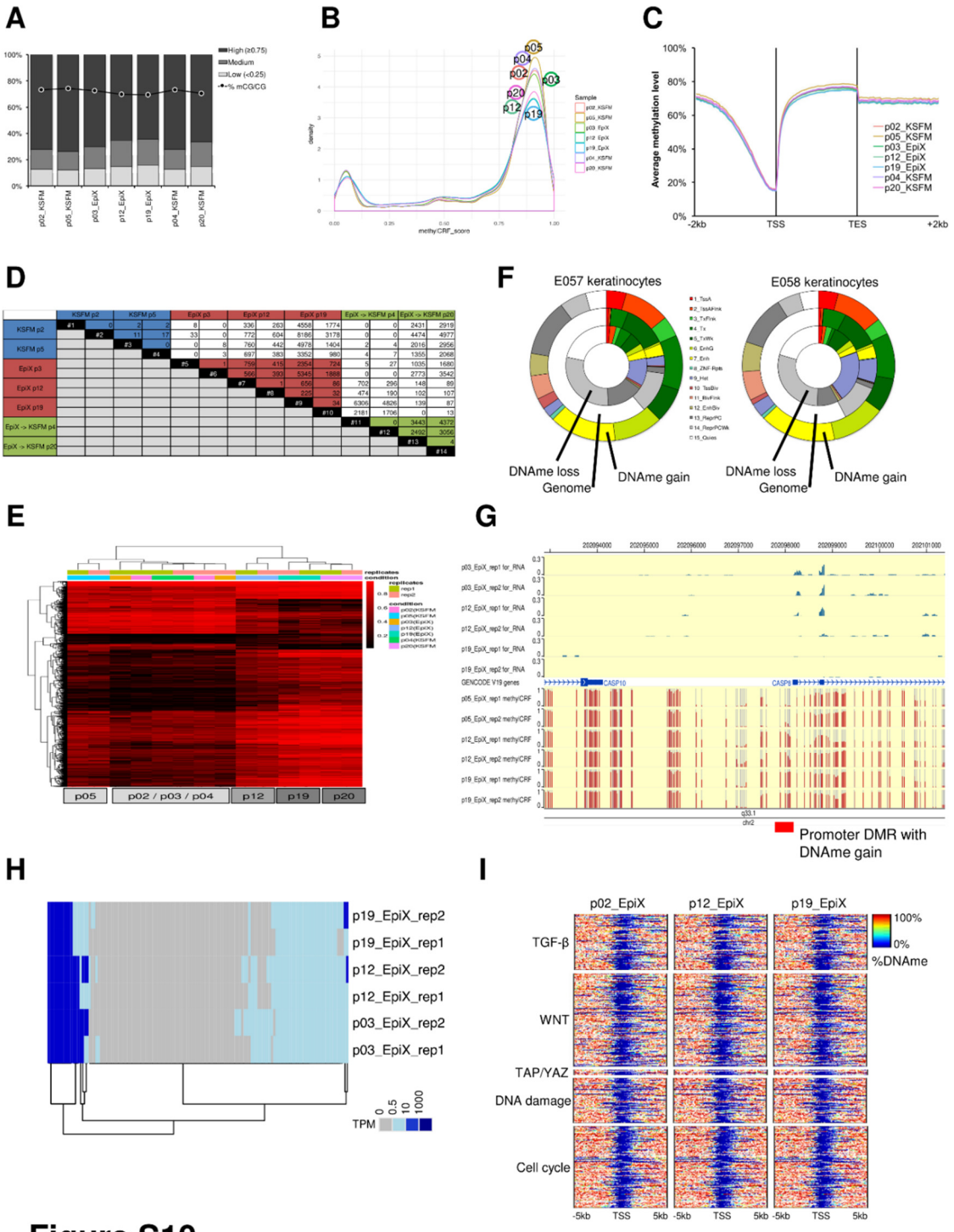


Figure S10.

Figure S10: DNA methylation changes of HFK expanded in the EpiX medium. Related to Figure 6. **A.** The average DNA methylation levels of all CpGs estimated by methylCRF (line plot) and the percentage of CpGs with high, medium, and low methylation levels (bar plot). **B.** The methylation level distribution of all CpGs. The CpG methylation levels showed bimodal distribution with two peaks of low (0-10%) and high (80-100%) methylation levels. **C.** The average DNA methylation levels over genic and 2kb neighboring regions. TTS, transcription start site. TES, transcription end site. **D.** Number of DMRs identified in all pair-wise comparisons. **E.** The heatmap of

DNA methylation levels of all 2419 DMRs across passages. **F.** The chromatin states of DMRs using chromHMM 15 core states in two keratinocytes (E057 and E058). **G.** The epigenome browser view of DMRs located on the promoter of the gene CASP8. The gene expression level was downregulated as the promoter gained DNA methylation level across passages. **H.** The heatmap of expression levels of genes with promoter methylation changes. Gene expression levels were considered as not detected (grey, TPM < 0.5), low (light blue, TPM of 0.5 to 10), medium (blue, TPM of 10 to 1000), or high (dark blue, TPM ≥ 1000) according to its TPM value. **I.** The heatmap of DNA methylation levels of promoters and its 5kb-neighboring regions of various pathway related genes. TSS, transcription start site.

Table S7: Prediction of cumulative population doublings. Related to Figure 6.

CpG site	1	2	3	3	4	5	6	Actual cPDs	Predicted cPDs
ID	cg02332525	cg17453778	cg03891191	cg03891191	cg01459453	cg01999333	cg16431978		
Associated gene	GRM7	CASR	PRAMEF2	PRAMEF13	SELP	CASP14	KRTAP13-3		
hg19 coordinates	chr3:69031 52-6903154	chr3:121902 560-121902562	chr1:129169 97-12916999	chr1:134525 81-13452583	chr1:169599 211-169599213	chr19:15162 622-15162624	chr21:31797 931-31797933		
p02_KSFM _rep1	0.04	0.05	0.86	0.32	0.90	0.90	0.83	7.5	0.0
p02_KSFM _rep2	0.04	0.04	0.90	0.32	0.90	0.90	0.79		-1.0
p05_KSFM _rep1	0.04	0.04	0.84	0.32	0.90	0.84	0.83	17.6	1.6
p05_KSFM _rep2	0.04	0.04	0.86	0.32	0.90	0.77	0.83		2.8
p03_EpiX_ rep1	0.04	0.04	0.86	0.32	0.87	0.85	0.83	14.2	0.4
p03_EpiX_ rep2	0.04	0.04	0.86	0.32	0.87	0.84	0.83		0.7
p12_EpiX_ rep1	0.04	0.04	0.86	0.32	0.85	0.84	0.79	45.7	0.8
p12_EpiX_ rep2	0.04	0.04	0.86	0.32	0.90	0.44	0.83		10.7
p19_EpiX_ rep1	0.04	0.04	0.87	0.32	0.88	0.08	0.46	73.0	22.6
p19_EpiX_ rep2	0.04	0.04	0.84	0.32	0.88	0.08	0.43		23.7
p04_KSFM _rep1	0.04	0.04	0.90	0.32	0.88	0.49	0.85	15.6	7.9
p04_KSFM _rep2	0.04	0.04	0.86	0.32	0.88	0.08	0.83		19.0
p20_KSFM _rep1	0.04	0.05	0.86	0.32	0.88	0.08	0.46	71.3	23.1
p20_KSFM _rep2	0.04	0.04	0.84	0.32	0.88	0.08	0.83		19.5
E057_meth ylCRF	0.04	0.04	0.91	0.33	0.90	0.90	0.92		-2.9
E058_meth ylCRF	0.04	0.04	0.60	0.33	0.90	0.90	0.92		5.4
E058_WG BS	0.00	0.00	0.43	ND	0.87	1.00	0.95		2.0