



# Expansion of airway basal epithelial cells from primary human non-small cell lung cancer tumors

DOI:  
[10.1002/ijc.31383](https://doi.org/10.1002/ijc.31383)

**Document Version**  
Accepted author manuscript

[Link to publication record in Manchester Research Explorer](#)

**Citation for published version (APA):**  
Crosbie, P. (2018). Expansion of airway basal epithelial cells from primary human non-small cell lung cancer tumors. *International Journal of Cancer*. <https://doi.org/10.1002/ijc.31383>

**Published in:**  
International Journal of Cancer

**Citing this paper**  
Please note that where the full-text provided on Manchester Research Explorer is the Author Accepted Manuscript or Proof version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version.

**General rights**  
Copyright and moral rights for the publications made accessible in the Research Explorer are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

**Takedown policy**  
If you believe that this document breaches copyright please refer to the University of Manchester's Takedown Procedures [<http://man.ac.uk/04Y6Bo>] or contact [openresearch@manchester.ac.uk](mailto:openresearch@manchester.ac.uk) providing relevant details, so we can investigate your claim.





# Supporting your research with our capabilities

BD Accuri™ C6 Plus Personal Flow Cytometer

BD FACSCelesta™ Cell Analyzer

BD LSRFortessa™ X-20 Cell Analyzer


BD FACSMelody™ Cell Sorter

One of the largest portfolios of reagents

**Learn more >**



# Expansion of airway basal epithelial cells from primary human non-small cell lung cancer tumors

Robert E. Hynds<sup>1,2</sup> , Assma Ben Aissa<sup>3</sup>, Kate H.C. Gowers<sup>4</sup>, Thomas B. K. Watkins<sup>2</sup>, Leticia Bosshard-Carter<sup>1,4</sup>, Andrew J. Rowan<sup>2</sup>, Selvaraju Veeriah<sup>1</sup>, Gareth A. Wilson<sup>2</sup>, Sergio A. Quezada<sup>3\*</sup>, Charles Swanton<sup>1,2\*</sup> on behalf of the TRACERx consortium and Sam M. Janes<sup>4\*</sup>

- 1) CRUK Lung Cancer Centre of Excellence, UCL Cancer Institute, University College London, London, U.K.
- 2) Translational Cancer Therapeutics Laboratory, The Francis Crick Institute, London, U.K.
- 3) Cancer Immunology Unit, UCL Cancer Institute, University College London, London, U.K.
- 4) Lungs for Living Research Centre, UCL Respiratory, University College London, London, U.K.

## \* Correspondence

### Professor Sam M. Janes

Lungs for Living Research Centre, UCL Respiratory, 5 University Street, London, WC1E 6JF, U.K.

Email: [s.janes@ucl.ac.uk](mailto:s.janes@ucl.ac.uk)

Phone: +44 (0) 203 108 7746

### Professor Charles Swanton

The Francis Crick Institute, 1 Midland Road, London, NW1 1AT, U.K.

Email: [charles.swanton@crick.ac.uk](mailto:charles.swanton@crick.ac.uk)

Phone: +44 (0) 207 2693463

### Dr. Sergio Quezada

Cancer Immunology Unit, UCL Cancer Institute, 72 Huntley Street, London, WC1E 6JD, U.K.

Email: [s.quezada@ucl.ac.uk](mailto:s.quezada@ucl.ac.uk)

Phone: +44 (0) 207 679 0743

**Keywords:** lung cancer, cell culture, epithelial cells, basal cells, stem/progenitor cells

**Conflict of Interest:** The authors declare no conflicts of interest.

**Word Count:** 2330

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1002/ijc.31383

**Abstract**

Pre-clinical non-small cell lung cancer (NSCLC) models are poorly representative of the considerable inter- and intra-tumor heterogeneity of the disease in patients. Primary cell-based *in vitro* models of NSCLC are therefore desirable for novel therapy development and personalized cancer medicine. Methods have been described to generate rapidly proliferating epithelial cell cultures from multiple human epithelia using 3T3-J2 feeder cell culture in the presence of Y-27632, a RHO-associated protein kinase (ROCK) inhibitor, in what are known as “conditional reprogramming conditions” (CRC) or 3T3+Y. In some cancer studies, variations of this methodology have allowed primary tumor cell expansion across a number of cancer types but other studies have demonstrated the preferential expansion of normal epithelial cells from tumors in such conditions. Here, we report our experience regarding the derivation of primary NSCLC cell cultures from 12 lung adenocarcinoma patients enrolled in the Tracking Cancer Evolution through Therapy (TRACERx) clinical study and discuss these in the context of improving the success rate for *in vitro* cultivation of cells from NSCLC tumors.

## Introduction

Progress in normal epithelial biology has shown that the addition of a RHO-associated protein kinase (ROCK) inhibitor<sup>1,2</sup> to traditional keratinocyte growth medium and mitotically inactivated 3T3-J2 murine embryonic feeder cell co-culture<sup>3</sup> can increase the number of cells expanded *in vitro* and help to initiate cultures from small samples. Traditionally, the primary culture of human cancer cells has been challenging, with few tumors amenable to culture on plastic, so this protocol, known as “conditional reprogramming” or “3T3+Y”, has naturally attracted attention in the cancer community. To date, variants of this protocol allowed cancer cell cultures to be established across multiple cancer types including lung, prostate, pancreas and colon<sup>4-6</sup>.

In non-small cell lung cancer (NSCLC), a number of reports demonstrate successful primary tumor cell culture using fibroblast co-culture and ROCK inhibition<sup>7-10</sup>. On the other hand, others have found that normal epithelial cells are preferentially expanded in these conditions<sup>11,12</sup>. For example, Sette and colleagues recently showed strong selection for normal, non-tumorigenic airway epithelial cells and/or no tumor cell expansion from 19 surgically resected NSCLC tumors of mixed histological subtypes, 4 pulmonary metastases, 2 lung cancer brain metastases and 1 patient-derived xenograft (PDX) model<sup>13</sup>.

Here, we present cell culture outcomes for 12 lung adenocarcinoma patients enrolled in the on-going Tracking Cancer Evolution through Therapy (TRACERx) clinical study<sup>14,15</sup>. We found that contaminating normal epithelial cells were the predominant cell type present in early passage cultures, although tumor cells were cultured in one case. We discuss these findings with a view to improving the efficacy of primary cell culture methodologies for early stage NSCLC tumors.

## Materials and Methods

### *Cell Culture*

Ethical approval for generating patient-derived models was obtained through the Tracking Cancer Evolution through Therapy (TRACERx) clinical study (REC reference 13/LO/1546). 3T3-J2 mouse embryonic fibroblast feeder cells were cultured in DMEM (Invitrogen) containing 1X penicillin/streptomycin (Gibco) and 9% bovine serum (Invitrogen). Feeder layers were prepared by inactivating confluent 3T3-J2 fibroblasts in medium containing 0.4 µg/ml mitomycin C (Sigma Aldrich) for two hours and re-plating these at 20,000 cell/cm<sup>2</sup> in fibroblast growth medium. Feeder cells were allowed to adhere overnight before tumor-derived cells were added. Tumors were received on ice in transport medium consisting of MEM alpha medium (Gibco) containing 1X penicillin/streptomycin (Gibco), 1X gentamicin (Gibco) and 1X amphotericin B (Fisher Scientific) after fresh dissection. Tumors were digested to single cell suspensions in serum-free RPMI medium (Gibco) containing 0.25 U/ml liberase TM (Roche) and 0.1 mg/ml DNase I (Sigma) in a waterbath at 37°C for two hours with regular agitation. Tumor-derived cells were plated on 3T3-J2 feeder layers in epithelial cell culture medium containing EGF and Y-27632 (3T3+Y) as previously described<sup>16,17</sup>.

Airway spheroids, or 'tracheospheres', were generated using a previously published protocol<sup>16</sup>.

### *Histology*

Samples were fixed in 4% PFA for 30 minutes at room temperature (tracheospheres<sup>16</sup>) or overnight at 4°C (subcutaneous tumors and primary tissue) and processed for paraffin embedding according to standard protocols. 4 µm formalin-fixed, paraffin-embedded (FFPE) sections were hematoxylin and eosin (H&E) stained using an automated tissue processor (Tissue-Tek). 40x images were acquired using a NanoZoomer 2.0HT whole slide imaging system (Hamamatsu Photonics). For immunofluorescence, slides were dewaxed using an automated protocol and antigen retrieval was performed using citrate buffer (pH 6.0). Primary antibodies were anti-MUC5B (Sigma HPA008246), anti-ACT (Sigma T6793) and anti-p63 (Abcam ab53039). Species-appropriate secondary antibodies were conjugated to Alexa Fluor dyes (Molecular Probes; 1:500). Images were acquired using a Zeiss LSM700 confocal microscope.

### *Immunocytochemistry*

Culture-expanded cells were seeded into 8-well, collagen-coated chamber slides (Ibidi) with feeder cells and expanded for four days. Cells were washed with PBS and fixed with 4% PFA at room temperature for 30 minutes. Cells were stored at 4°C in PBS until staining. Cells were permeabilized and blocked for one hour in PBS containing 10% FBS and 0.01% Triton X-100 (Fisher Scientific). Primary antibodies were anti-pan-keratin (Dako M3515; 1:500), anti-keratin 5 (Abcam ab52635; 1:200), anti-p63 (Abcam ab124762; 1:300) and anti-TTF-1 (Abcam ab76013; 1:200). These were incubated overnight at 4°C in block buffer without Triton X-100. Cells were washed three times with PBS before addition of species-appropriate secondary antibodies (Alexa Fluor; Molecular Probes; 1:500) at room temperature for two hours in block buffer without Triton X-100.

### *Subcutaneous xenografts*

Animal studies were approved by the University College London Biological Services Ethical Review Committee and licensed under UK Home Office regulations. Six-to-eight week-old male immunocompromised NOD/SCID/gamma (NSG) mice were purchased from Charles River, kept in individually ventilated cages under specific pathogen-free conditions and had *ad libitum* access to both sterile food and autoclaved water. To generate subcutaneous tumors, mice were anaesthetized using 2-4% isoflurane, the right flank was shaved and cleaned before 200 µl growth-factor reduced Matrigel containing  $1 \times 10^6$  cultured cells was injected subcutaneously. Animals were observed during recovery, then regularly monitored for tumor growth. Experiments lasted for 3 months or were terminated before tumors reached 1.5 cm<sup>3</sup> in volume.

### *Next-generation sequencing (NGS)*

NGS of a TruSeq custom amplicon for lung cancer panel that comprises 107 hotspot amplicons from 15 genes was performed using the MiSeq system (Illumina). The NGS amplicon library preparation was performed using 125 ng DNA as input for patient tissue and cell cultures derived from patient-matched tumors. The resulting sequence library was normalized and pooled prior to sequencing on a MiSeq instrument according to the manufacturer's instructions (Illumina). We used a MiSeq Reagent Kit v2 (300 cycles) with 2 ×

150 paired-end sequencing design according to the manufacturer's instructions (Illumina). The human hg19 genome assembly was used to align the paired-end raw reads.

The variant allele frequencies of 24 SNPs previously identified by Pengelly et al<sup>18</sup> were extracted for each sequenced cell culture and compared to previously sequenced tumor regions from the same patient in order to confirm that no sample swaps had occurred between patients.

SAMtools mpileup<sup>19</sup> (0.1.19; base phred score > 20 and read mapping quality > 20, BAQ computation disabled), VarScan2<sup>20</sup> (v2.4.1; includes running fpfilter.pl) and MuTect<sup>21</sup> (v1.1.7) were used to locate non-reference positions in cell culture, tumor and germline samples. To reduce false positive variant calls, additional filtering was performed. A variant allele frequency of 5% was required with a somatic p-value  $\leq 0.01$ , sequencing depth in each region was required to be  $\geq 30$  and  $\geq 10$  sequence reads had to support the variant call. In contrast, the VAF in the germline data had to be  $\leq 1\%$  whilst the number of reads supporting the variant had to be  $< 5$ . Finally, variants designated as 'germline' by VarScan2 from all regions in all 100 patients within the TRACERx exome cohort<sup>14</sup> were combined so that every germline variant detected in the cohort had an associated TRACERx population frequency. SNVs were filtered if they were found to have  $> 1\%$  frequency in the TRACERx germline cohort.



## Results

We attempted cell culture from 12 patients (median age = 75 years, range = 58-90 years), whose surgically resected primary lung adenocarcinoma (LUAD) tumors (stage II [n=9] or III [n=3]) are being analyzed in the on-going Tracking Cancer Evolution through Therapy (TRACERx) clinical study<sup>14,15</sup>, using a published protocol<sup>17</sup>. We obtained primary epithelial cell cultures that could be passaged from 10 (83.3%) of these.

Tumor-derived cells were morphologically indistinguishable from normal human airway epithelial cells in 3T3+Y culture and expressed proteins associated with airway basal cells, such as keratin 5 and the transcription factor p63 (Figure 1A). The LUAD-associated protein TTF-1 (also known as homeobox protein NKX-2.1) was expressed by cultured cells (Figure 1A; 3/3) but is also expressed by normal airway basal cells. To morphologically distinguish contaminating basal cells from tumor cells, we grew four of the cell cultures in 3D differentiation conditions<sup>16</sup>, expecting that cells would form either hollow normal airway 'tracheospheres' or solid tumor spheroids<sup>22</sup>. Cells underwent lumen formation and generated epithelial spheroids containing differentiated airway epithelial cell types, suggesting that we had expanded normal airway basal cells from these tumors (Figure 1B; 4/4).

To further examine whether we had expanded cancer cells or normal epithelial cells, we subcutaneously injected  $1 \times 10^6$  culture-expanded cells in 100% growth factor-reduced Matrigel in immunocompromised (NSG) mice. After 3 months, no tumors developed in mice injected with tumor-derived epithelial cell cultures (0/3). In Matrigel retrieved from these mice, we found epithelial cells with the characteristics of differentiated normal human proximal airway epithelial cells (Figure 1C).

The preferential expansion of normal human airway basal cells in these conditions was subsequently confirmed using sequencing approaches. The primary patient tumors had undergone next-generation sequencing (NGS) of a targeted panel of SNVs in 15 lung cancer-associated genes and amplifications in a further 3 genes using the Illumina MiSeq system within the TRACERx study. Equivalent NGS of the ten patient-matched early passage cell

cultures confirmed that cancer mutations were not detected in nine of ten cultures ( $\leq 5$  passages; Figure 1D). Since p63+ cells can be found within LUAD tumors (Figure 1E), it is likely to be these cells that are expanded in culture. However, in one passage 2 cell culture that was not investigated in prior assays, we detected all three mutations that were found in the patient's tumor at high variant allele frequencies by MiSeq (81.3% mutant *KRAS*; Figure 1D) and these cells gave rise to a tumor on subcutaneous injection in an NSG mouse (Figure 2). Cells could be re-cultured in 3T3+Y following recovery of the xenograft tumor and Sanger sequencing demonstrated that these were *KRAS* mutant (Figure 2). Interestingly, Sanger sequencing of the parent cell culture just two passages later (i.e. passage 4) did not detect mutant *KRAS* (Figure 2), suggesting that normal epithelial cells rapidly out-grow cancer cells in this culture system when both are present.

Overall, these data show that 3T3+Y conditions supported tumor cell expansion for just 1 of 10 NSCLC tumors and that selection of cancer cells over normal epithelial cells is essential for cancer cell culture maintenance.

## Discussion

Our results suggest that a very small number of contaminating normal airway basal cells contained within LUAD tumors are sufficient to initiate cell cultures in these conditions, corroborating the recent findings of Sette et al.<sup>13</sup> and others<sup>11, 12</sup>. Even after careful dissection of tissue by a pathologist to obtain normal lung and tumor samples, bronchioles containing normal airway epithelium can be found in the NSCLC tumor microenvironment (Figure 1E).

It has been possible to establish cultures of tumor mutation-bearing cells in many NSCLC cases<sup>8, 9, 23</sup>, including from PDX tumors<sup>24</sup>, but the widespread utility of the 'conditional reprogramming' method still remains to be established, particularly in early stage disease and from tumor samples derived from surgical resection or autopsy. Studies that report normal epithelial cell expansion have tended to initiate culture from early-stage primary NSCLC tumors, whereas studies in which cancer cells have been expanded have initiated culture using biopsies or effusions from patients with late-stage, therapy-resistant disease<sup>7-9</sup> or from PDX models<sup>24</sup>, which may be more aggressive and/or more adaptable to cell culture. This would be consistent with traditional NSCLC cell culture methods and PDX models, where patient models are more readily established from late-stage, metastatic or therapy-resistant disease than from early-stage primary disease. As well as disease stage, tumor genotype could also be an important determinant of culture success in these conditions: previous studies focus on biopsies and plural effusions from *EGFR*-mutant/*ALK*-positive tumors, while 7 of our 10 LUAD tumors were driven by mutant *KRAS*.

Despite containing less tissue, biopsies or pleural effusion samples might also be advantageous for initiating tumor cultures by virtue of their lack of contaminating normal epithelium<sup>9</sup> as normal epithelial cells outgrow patient-matched cancer cells in controlled 3T3+Y experiments<sup>11</sup>. Normal cells also outcompete tumor-derived cells during cancer organoid derivation. Here, it has been possible to develop tumor-specific media to select tumor organoids over normal colonic<sup>25</sup> and hepatic<sup>26</sup> epithelial organoids. For example, intestinal epithelial cells are dependent on exogenous Wnt in culture but constitutively active Wnt signaling, due to genomic aberrations in genes such as *APC* and/or *CTNNB1*, often confers tumor cells with the ability to survive culture in Wnt-free culture media. Such

strategies might be relevant in NSCLC culture: for example, EGF could be withdrawn in cases where tumors have *EGFR*-activating mutations or nutlin-3 could be added to *P53*-mutant tumor cultures<sup>25</sup>. These selection strategies could be effective in cases where the tumor genotype is known at the time of surgery or in lung squamous cell carcinoma cell culture where the vast majority of tumors are *P53* mutant. While the current data suggest LUAD tumor cells are lost very early during culture in 3T3+Y, it remains possible that normal cells actively limit tumor cell expansion and selection might permit the growth of tumor cells.

Protocol optimization is clearly required to adapt recent progress in epithelial biology towards routine utility in cancer studies. Protocol differences exist between the aforementioned studies: key studies have used inactivated human dermal fibroblasts<sup>7, 8</sup> as feeder layers rather than the mouse embryonic fibroblasts often used in those that see normal cell expansion and it has also been possible to transition tumor cell cultures off feeder layers after six months of culture establishment<sup>8</sup>. We have previously demonstrated that adult human lung fibroblasts do not support long-term normal airway epithelial cell expansion in the same way as 3T3-J2 cells<sup>17</sup> so the use of adult and/or human feeder cells might favor tumor cell expansion. Relevant biological differences might also exist between mitotic inactivation using irradiation<sup>4, 9</sup> rather than mitomycin C, as used here. Alternatively, different tissue acquisition methods<sup>9</sup>, feeder cell-conditioned medium<sup>10, 27</sup>, low oxygen<sup>10</sup>, harsh tissue digestion protocols<sup>26</sup> and/or initially serum-starving cells<sup>6</sup> might help to select tumor cells by inducing differentiation or apoptosis in normal epithelial cells.

In summary, researchers should remain cautious in their use of NSCLC primary cell cultures, particularly from early-stage tumors; genomic analyses should confirm the tumor origin of individual cultures and, if present, the proportion of tumor cells should not be assumed to be consistent over passage<sup>23</sup>. While 3T3+Y culture conditions have clear applications in disease modelling in respiratory conditions with airway epithelial involvement, further work is required to realize its applications in modelling human cancer and in the development of personalized cancer therapies.

## Acknowledgements

The authors thank members of the TRACERx consortium for contributing to this study and Simon Broad and Prof. Fiona Watt (Kings College London, U.K.) for providing 3T3-J2 fibroblasts. TRACERx (Clinicaltrials.gov no: NCT01888601) is sponsored by University College London (UCL/12/0279) and has been approved by an independent Research Ethics Committee (13/LO/1546). TRACERx is funded by Cancer Research UK (CRUK; C11496/A17786) and is coordinated by CRUK and the UCL Cancer Trials Centre. R.E.H. is funded by the CRUK Lung Cancer Centre of Excellence, the Roy Castle Lung Cancer Foundation and a Sir Henry Wellcome Postdoctoral Fellowship from the Wellcome Trust (WT209199/Z/17). A.B.A. is funded by a CRUK Clinical PhD studentship. S.A.Q. is a CRUK Senior Fellow and is funded by a Cancer Research Institute Investigator Award and a CRUK Biotherapeutic Program Grant. C.S. is a Royal Society Napier Research Professor and is funded by CRUK, Stand Up 2 Cancer (SU2C), Rosetrees Trust, NovoNordisk Foundation (ID 16584), Prostate Cancer Foundation, Breast Cancer Research Foundation (BCRF), European Research Council (THESEUS) and Marie Curie Network PloidyNet. Support was also provided to C.S. by the National Institute for Health Research, the University College London Hospitals Biomedical Research Centre and the CRUK UCL Experimental Cancer Medicine Centre. S.M.J. is a Wellcome Trust Senior Fellow in Clinical Science (WT107963/Z/15/Z) and is supported by the Rosetrees Trust, the Roy Castle Lung Cancer Foundation, SU2C, the Welton Trust, the Garfield Weston Trust, the UCLH Charitable Foundation and the UCLH BRC. This work was supported by the Francis Crick Institute that receives its core funding from CRUK (FC001169, FC001202), the UK Medical Research Council (FC001169, FC001202) and the Wellcome Trust (FC001169, FC001202).

## Figure Legends

### Figure 1: Frequent expansion of normal human airway epithelial cells from non-small cell lung cancer (NSCLC) tumors in 3T3-J2 co-culture in the presence of Y-27632 (3T3+Y).

A) Immunofluorescence staining for pan-keratin, keratin 5, p63 and TTF-1 in a representative culture (n=3 patients; scale bar = 100  $\mu\text{m}$ ). B) *In vitro* tracheosphere assay. Hematoxylin and eosin (H&E) staining (top panel, scale bar = 1 mm; bottom left panel, scale bar = 50  $\mu\text{m}$ ) demonstrated airway differentiation capacity of cell cultures expanded from NSCLC tumors (<passage 5; representative images, n=4 patients). Immunofluorescence confirmed the presence of ACT+ ciliated cells and MUC5B+ mucosecretory cells (bottom right panel, scale bar = 50  $\mu\text{m}$ ). C) Subcutaneous injection tumorigenesis model. Injection of cultured cells (<passage 5) into NSG mice did not lead to tumor formation but H&E analysis showed the presence of epithelium resembling that found in human airways after three months (3/3 patients; top panel scale bar = 500  $\mu\text{m}$ ; middle panel scale bar = 50  $\mu\text{m}$ ). Immunofluorescence confirmed the presence of ACT+ ciliated cells and MUC5B+ mucosecretory cells (bottom panel, scale bar = 50  $\mu\text{m}$ ). D) Next-generation sequencing using the Illumina MiSeq platform did not detect mutations found in the tumor of origin in 9/10 NSCLC cell cultures. 'Multiple' indicates that more than one variant results from the specific mutation detected, i.e., it affects multiple isoforms at different positions. E) H&E analysis showed bronchiolar epithelium within a human lung adenocarcinoma tumor (left panel, scale bar = 1 mm; top right panel, scale bar = 100  $\mu\text{m}$ ). Immunofluorescence confirmed the presence of p63+ basal epithelial cells within these bronchioles (bottom right panel, scale bar = 50  $\mu\text{m}$ ).

### Figure 2: Expansion of primary human tumor cells from a *KRAS*-mutant lung adenocarcinoma in 3T3-J2 co-culture in the presence of Y-27632 (3T3+Y).

Cancer mutation-bearing cells were detected in 1 of our 10 early passage patient cultures (CRUK0557) by next-generation sequencing. Sanger sequencing of the same cell culture at later passage (P4; scale bar = 100  $\mu\text{m}$ ) revealed that the *KRAS* mutation was no longer detected (left panel). Injection of the early passage (P2) cell culture into an immune-compromised (NSG) mouse generated a tumor with mutant *KRAS* (center panel). A hematoxylin and eosin (H&E) stained section is shown (scale bar = 500  $\mu\text{m}$ ). A magnified

view of the black dotted box is shown below (scale bar = 100  $\mu$ m). Re-culture of cells from the cell culture-derived xenograft in 3T3+Y was possible (right panel; scale bar = 100  $\mu$ m) and mutant *KRAS* was again detected by Sanger sequencing.

## References

1. Chapman S, Liu X, Meyers C, Schlegel R, McBride AA. Human keratinocytes are efficiently immortalized by a Rho kinase inhibitor. *J Clin Invest* 2010;**120**: 2619-26.
2. Terunuma A, Limgala RP, Park CJ, Choudhary I, Vogel JC. Efficient procurement of epithelial stem cells from human tissue specimens using a Rho-associated protein kinase inhibitor Y-27632. *Tissue Eng Part A* 2010;**16**: 1363-8.
3. Rheinwald JG, Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 1975;**6**: 331-43.
4. Liu X, Krawczyk E, Supryniewicz FA, Palechor-Ceron N, Yuan H, Dakic A, Simic V, Zheng YL, Sripadhan P, Chen C, Lu J, Hou TW, et al. Conditional reprogramming and long-term expansion of normal and tumor cells from human biospecimens. *Nat Protoc* 2017;**12**: 439-51.
5. Liu X, Ory V, Chapman S, Yuan H, Albanese C, Kallakury B, Timofeeva OA, Nealon C, Dakic A, Simic V, Haddad BR, Rhim JS, et al. ROCK inhibitor and feeder cells induce the conditional reprogramming of epithelial cells. *Am J Pathol* 2012;**180**: 599-607.
6. Timofeeva OA, Palechor-Ceron N, Li G, Yuan H, Krawczyk E, Zhong X, Liu G, Upadhyay G, Dakic A, Yu S, Fang S, Choudhury S, et al. Conditionally reprogrammed normal and primary tumor prostate epithelial cells: a novel patient-derived cell model for studies of human prostate cancer. *Oncotarget* 2017;**8**: 22741-58.
7. Piotrowska Z, Niederst MJ, Karlovich CA, Wakelee HA, Neal JW, Mino-Kenudson M, Fulton L, Hata AN, Lockerman EL, Kalsy A, Digumarthy S, Muzikansky A, et al. Heterogeneity Underlies the Emergence of EGFR T790M Wild-Type Clones Following Treatment of T790M-Positive Cancers with a Third-Generation EGFR Inhibitor. *Cancer Discov* 2015;**5**: 713-22.
8. Crystal AS, Shaw AT, Sequist LV, Friboulet L, Niederst MJ, Lockerman EL, Frias RL, Gainor JF, Amzallag A, Greninger P, Lee D, Kalsy A, et al. Patient-derived models of acquired resistance can identify effective drug combinations for cancer. *Science* 2014;**346**: 1480-6.
9. Kodack DP, Farago AF, Dastur A, Held MA, Dardaei L, Friboulet L, von Flotow F, Damon LJ, Lee D, Parks M, Dicecca R, Greenberg M, et al. Primary Patient-Derived Cancer Cells and Their Potential for Personalized Cancer Patient Care. *Cell Rep* 2017;**21**: 3298-309.
10. Park KS, Raffeld M, Moon YW, Xi L, Bianco C, Pham T, Lee LC, Mitsudomi T, Yatabe Y, Okamoto I, Subramaniam D, Mok T, et al. CRIPTO1 expression in EGFR-mutant NSCLC elicits intrinsic EGFR-inhibitor resistance. *J Clin Invest* 2014;**124**: 3003-15.
11. Gao B, Huang C, Kernstine K, Pelekanou V, Kluger Y, Jiang T, Peters-Hall JR, Coquelin M, Girard L, Zhang W, Huffman K, Oliver D, et al. Non-malignant respiratory epithelial cells preferentially proliferate from resected non-small cell lung cancer specimens cultured under conditionally reprogrammed conditions. *Oncotarget* 2017;**8**: 11114-26.
12. Yu F, Lu Y, Tao L, Jiang YY, Lin DC, Wang L, Petersson F, Yoshiyama H, Koeffler PH, Goh BC, Loh KS. Non-malignant epithelial cells preferentially proliferate from nasopharyngeal carcinoma biopsy cultured under conditionally reprogrammed conditions. *Sci Rep* 2017;**7**: 17359.

13. Sette G, Salvati V, Giordani I, Pillozzi E, Quacquareni D, Duranti E, De Nicola F, Pallocca M, Fanciulli M, Falchi M, Pallini R, De Maria R, et al. Conditionally reprogrammed cells (CRC) methodology does not allow the in vitro expansion of patient-derived primary and metastatic lung cancer cells. *Int J Cancer* 2018.
14. Jamal-Hanjani M, Wilson GA, McGranahan N, Birkbak NJ, Watkins TBK, Veeriah S, Shafi S, Johnson DH, Mitter R, Rosenthal R, Salm M, Horswell S, et al. Tracking the Evolution of Non-Small-Cell Lung Cancer. *N Engl J Med* 2017;**376**: 2109-21.
15. Jamal-Hanjani M, Hackshaw A, Ngai Y, Shaw J, Dive C, Quezada S, Middleton G, de Bruin E, Le Quesne J, Shafi S, Falzon M, Horswell S, et al. Tracking genomic cancer evolution for precision medicine: the lung TRACERx study. *PLoS Biol* 2014;**12**: e1001906.
16. Hynds RE, Butler CR, Janes SM, Giangreco A. Expansion of Human Airway Basal Stem Cells and Their Differentiation as 3D Tracheospheres. *Methods Mol Biol* 2016.
17. Butler CR, Hynds RE, Gowers KH, Lee Ddo H, Brown JM, Crowley C, Teixeira VH, Smith CM, Urbani L, Hamilton NJ, Thakrar RM, Booth HL, et al. Rapid Expansion of Human Epithelial Stem Cells Suitable for Airway Tissue Engineering. *Am J Respir Crit Care Med* 2016;**194**: 156-68.
18. Pengelly RJ, Gibson J, Andreoletti G, Collins A, Mattocks CJ, Ennis S. A SNP profiling panel for sample tracking in whole-exome sequencing studies. *Genome Med* 2013;**5**: 89.
19. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, Genome Project Data Processing S. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 2009;**25**: 2078-9.
20. Koboldt DC, Zhang Q, Larson DE, Shen D, McLellan MD, Lin L, Miller CA, Mardis ER, Ding L, Wilson RK. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res* 2012;**22**: 568-76.
21. Cibulskis K, Lawrence MS, Carter SL, Sivachenko A, Jaffe D, Sougnez C, Gabriel S, Meyerson M, Lander ES, Getz G. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. *Nat Biotechnol* 2013;**31**: 213-9.
22. Fessart D, Begueret H, Delom F. Three-dimensional culture model to distinguish normal from malignant human bronchial epithelial cells. *Eur Respir J* 2013;**42**: 1345-56.
23. Correa BRS, Hu J, Penalva LOF, Schlegel R, Rimm DL, Galante PAF, Agarwal S. Patient-derived conditionally reprogrammed cells maintain intra-tumor genetic heterogeneity. *Sci Rep* 2018;**8**: 4097.
24. Borodovsky A, McQuiston TJ, Stetson D, Ahmed A, Whitston D, Zhang J, Grondine M, Lawson D, Challberg SS, Zinda M, Pollok BA, Dougherty BA, et al. Generation of stable PDX derived cell lines using conditional reprogramming. *BMC Molecular Cancer* 2017;**16**.
25. van de Wetering M, Francies HE, Francis JM, Bounova G, Iorio F, Pronk A, van Houdt W, van Gorp J, Taylor-Weiner A, Kester L, McLaren-Douglas A, Blokker J, et al. Prospective derivation of a living organoid biobank of colorectal cancer patients. *Cell* 2015;**161**: 933-45.
26. Broutier L, Mastrogiovanni G, Verstegen MM, Francies HE, Gavarro LM, Bradshaw CR, Allen GE, Arnes-Benito R, Sidorova O, Gaspersz MP, Georgakopoulos N, Koo BK, et al. Human primary liver cancer-derived organoid cultures for disease modeling and drug screening. *Nat Med* 2017.
27. Panaccione A, Chang MT, Carbone BE, Guo Y, Moskaluk CA, Virk RK, Chiriboga L, Prasad ML, Judson B, Mehra S, Yarbrough WG, Ivanov SV. NOTCH1 and SOX10 are Essential



for Proliferation and Radiation Resistance of Cancer Stem-Like Cells in Adenoid Cystic Carcinoma. *Clin Cancer Res* 2016;**22**: 2083-95.

Accepted Article

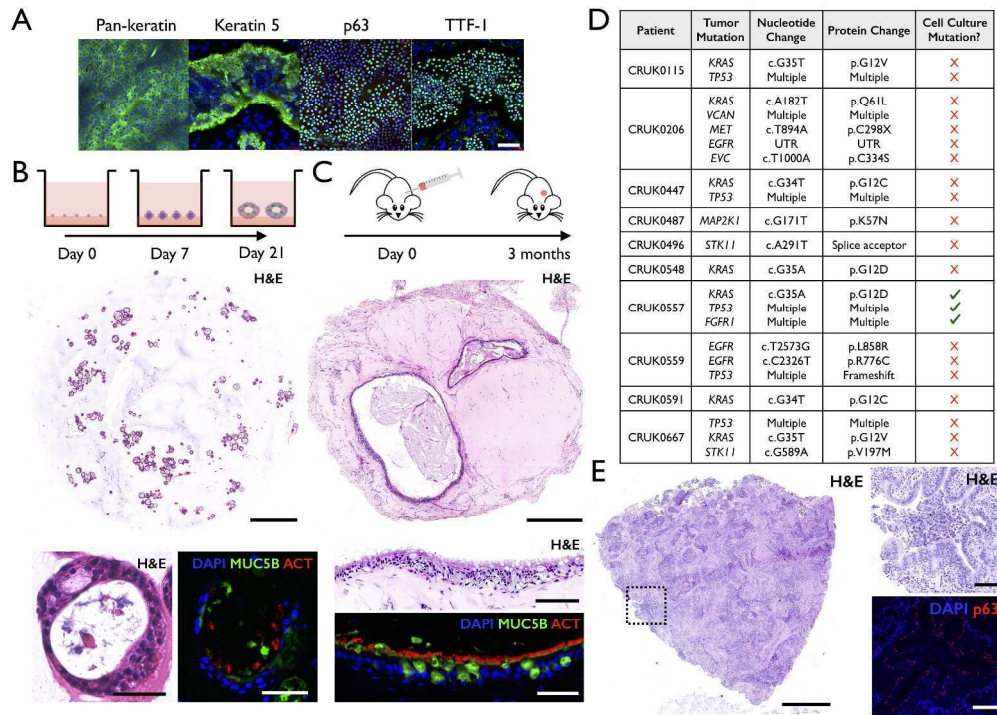


Figure 1: Frequent expansion of normal human airway epithelial cells from non-small cell lung cancer (NSCLC) tumors in 3T3-J2 co-culture in the presence of Y-27632 (3T3+Y).

320x230mm (300 x 300 DPI)

Accept

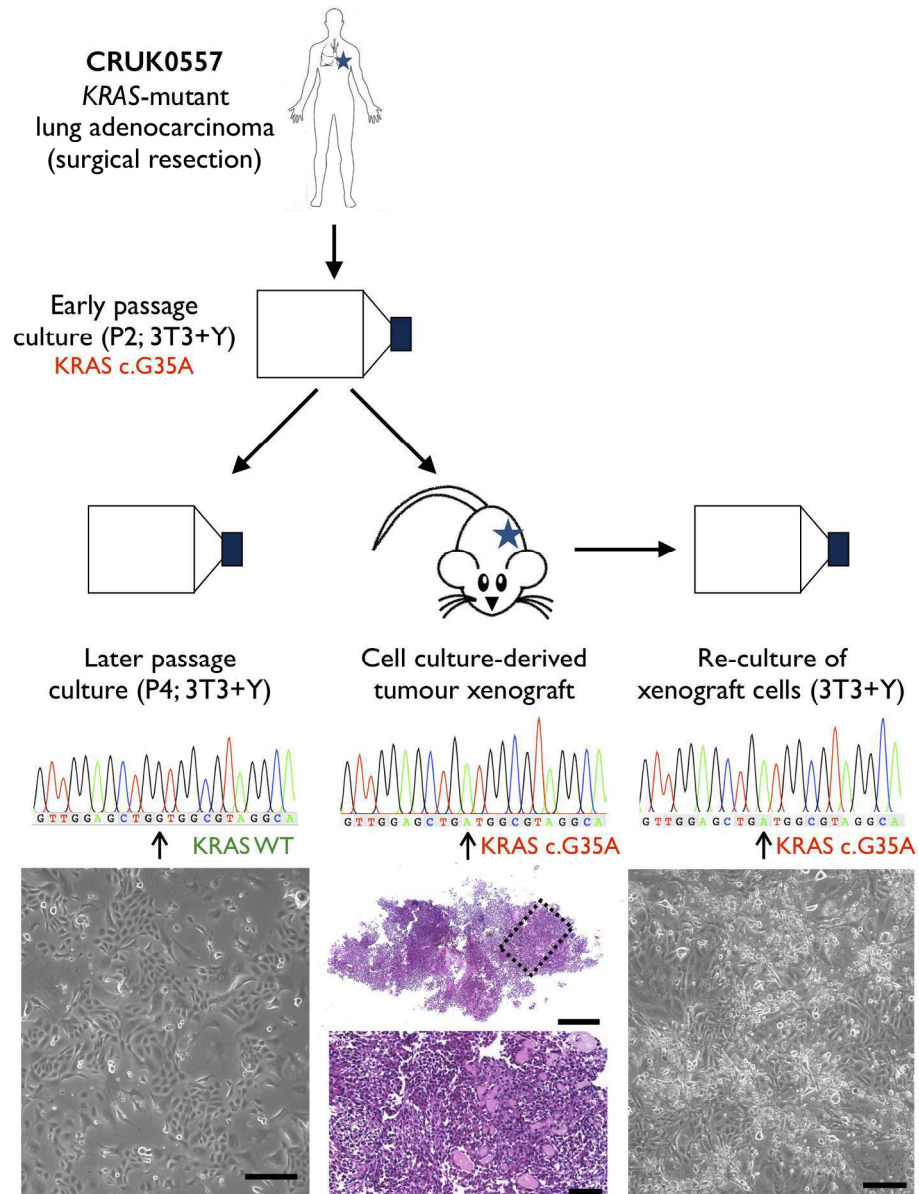


Figure 2: Expansion of primary human tumor cells from a KRAS-mutant lung adenocarcinoma in 3T3-J2 culture in the presence of Y-27632 (3T3+Y).

Cancer mutation-bearing cells were detected in 1 of our 10 early passage patient cultures (CRUK0557) by next-generation sequencing. Sanger sequencing of the same cell culture at later passage (P4; scale bar = 100  $\mu$ m) revealed that the KRAS mutation was no longer detected (left panel). Injection of the early passage (P2) cell culture into an immune-compromised (NSG) mouse generated a tumor with mutant KRAS (center panel). A hematoxylin and eosin (H&E) stained section is shown (scale bar = 500  $\mu$ m). A magnified view of the black dotted box is shown below (scale bar = 100  $\mu$ m). Re-culture of cells from the cell culture-derived xenograft in 3T3+Y was possible (right panel; scale bar = 100  $\mu$ m) and mutant KRAS was again detected by Sanger sequencing.

172x226mm (300 x 300 DPI)