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RESEARCH ARTICLE

How Much Do We Know About the Mouse Respiratory Epithelial Stem Cells?

Subtitle:
Characterization of a Doxycycline-Regulated Multi-Lineage Mouse Respiratory Epithelial Precursor Cell Line *In Vitro* And *In Vivo*

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ABSTRACT:
Background-Purpose of this study: Tissue-specific stem cell lines are useful tools for cell biology studies. Information on respiratory tissue cell lines is limited. A doxycycline-regulated epithelial precursor cell line was established from the lung tissue of a tTAxSV40 Tag double transgenic mouse. In this study, we have characterized this cell line *in vitro & in vivo*, and found to mimic a rare subpopulation of club- and pneumocyte type II-dual cells.
Methods: It was partially characterized using cell viability and death assays, H^3-thymidine incorporation assay, chloride efflux assay, Western blotting of proteins secreted, RT-PCR assays for RNA isolated. In addition, immune-deficient SCID mice were used as hosts for implantation of this precursor cell line, and feed with/without doxycycline containing water. Immunofluorescent typing using different antibodies were used to characterize the implanted lung.
Results: This cell line was found to mimic a rare subpopulation of club- and pneumocyte type II- dual cells with multiple phenotypes. Cell growth was doxycycline-regulated and observed only when

doxycycline was omitted from the medium or present at concentrations up to 1 µg/ml, higher concentrations were inhibitory. ACT+ ciliated cells were found upon implantation into immune-deficient mice, in addition. Cell growth was doxycycline-regulated *in vitro*. When transplanted subcutaneously into immune-deficient mice, these cells migrated to the lung to form organized chimeric structures of donor and host origins, with club cells in the terminal bronchioles, ACT^+ ciliated cells along the epithelial lining, and pneumocyte type II-cells in the alveolar interstices. No such homing of donor cells to the lung was observed when the implanted mice were fed doxycycline-containing water.

Discussions-Conclusions: This lung stem cell line might be able to provide us with an insight into the differentiation pathway of lung epithelial cells as well as with some understanding of the nature of air trophic-pulmonary epithelial cells. The results of this study underline the possibility of a future application for somatic (stem / precursor) cells in tissue replacement and tissue engineering of the damaged lung. Its ability to secrete and deliver soluble protein, might be a potential novel way for drug delivery. In addition, stem cells are thought to proliferate and differentiate in response to a deficiency or as a result of injury. Successful migration to the target organ and subsequent maturation of these precursors could be attributed to a requirement of lung stem cells to search for an aerated environment. Our findings challenge some current concepts of stem cell biology.-This lung stem cell line may become a rich source of cells for tissue engineering and cell-based therapy for lung injury. The route and protocol established for cell introduction into the lung may provide a novel alternative to delivery of soluble protein substances through the airways. This lung stem cell line might also be modified to provide models for screening drugs against respiratory infection.

Keywords: Tet-off expression system, Lung stem cells, Lung disease, Lung viral infection, Drug screening and delivery.

Abbreviations

ACT: acetylated αtubulin
CC10: club cell 10kDa protein, or
CCSP: club cell specific protein
CFTR: cystic fibrosis transmembrane conductance regulator
CGRP: calcitonin gene related peptide—ck cytokeratin
Doxy: doxycycline
FITC: Fluorescenlsothiocyanat
hCNTF: human Ciliary NeuroTrophic Factor
hESC: human Embryonic Stem Cells
hiPSC: human induced Pluripotent Stem Cells
mAb´s : monoclonal antibodies
L14: a doxycycline regulated lung cell line
MHC: major histocompatibility antigen
PNEC: pulmonary neuroendocrine cell
SpA, SpB, (pro)SpC, SpD,: surfactant protein A, B, (proprotein)C, D
SCID-NOD mice: severe combined immune-deficient-non-obese diabetic mice
RT: room temperature
RT-PCR: reverse transcription-polymerase chain reaction
SpA, SpB, (pro)SpC, SpD: surfactant protein A, B, (proprotein)C, D
SPQ: 6-methoxy-N-(3-sulfopropyl)-quinolinium
tTA: tetracycline/ doxycycline-regulatable transactivator
tetophCMV promoter: tet off human cytomegalovirus promoter
phCMV-tTA x tetophCMV-SV40 Tag: promoter hCMV-tTA x tet off promoter hCMV-SV40 Tag
TR: Texas Red

Introduction

Somatic stem cell therapy offers the promise of life-long replacement of defective tissue, since it relies on the unique ability of stem cells to renew themselves and provide functional progeny with which to replace lost tissue. However, it is a challenge to supply sufficient amounts of tissue-specific stem cells for this purpose. With the exception of rodent neuronal stem cells, most adult primary stem cells are difficult to expand *in vitro*. There are several reports claiming that it is possible to culture adult primary stem cells for several passages, including alveolar type II (AT2) in human[1] and mouse[2-4] using trachea cells and not lung tissue.

Genetically modified stem-like cells with the ability to conditionally expand are a useful tool for stem cell biology studies.-A breakthrough which rendered this possible is to conditionally expand transgenic stem/precursor cells by means of a tetracycline (or its derivative, doxycycline = Doxy)-dependent/regulated expression of SV40 Tantigen[5-9]. SV40 Tag can transform cells in the absence of other proteins which promote cell proliferation. This is due to its multiple effects on cell replication, including inactivation of the growth-suppressing proteins p53 and Rb (retinoblastoma protein), stimulation of gene transcription, and DNA unwinding[9].

Most studies of the tracheo-bronchial epithelium use human tissue, and others have dealt with tissues from rhesus monkeys, sheep, rabbits, or rats. In recent years, there is a progress reporting the study of mouse respiratory epithelium and the origin of mouse alveolar cells[10-28, 29-31]. We have established a mouse epithelial precursor cell line from the lung tissue of a tTA x SV40 Tag double transgenic mouse[9, 29, 30]. In this study, we further characterize this cell line, referred to as L14, whose growth depends on SV40 T antigen-driven pathways and is under control of a doxycycline-regulated promoter. The L14 line possesses dual characteristics of club- and pneumocyte type II-cell lineages and may be derived from the rare cuboidal cell population at the junction of the bronchial tree and the alveoli[-15, 34].

Somatic stem cell offers the promise of life-long replacement of damaged tissue. Here we report the partial characterization of L14, a cell line isolated from lung tissue of tTAxSV40Tag double transgenic mice, whose growth depends on SV40 T antigen-driven pathways and is under the control of a doxycycline-regulated promoter. The L14 line possesses trio-characteristics of club-, pneumocyte type II-like precursor and ciliated airway epithelial precursors. These cells may be derived from the rare cuboidal cell population at the junction of the bronchial tree and the alveoli[51, 52].

We further examine the fate of L14 cells *in vivo* after implantation into adult immune-deficient mice and nude mice. The donor cells migrated out the implant site were trophic to host lung and formed chimeric lung. These results suggest possible future applications for somatic (stem/precursor) cells in tissue replacement and tissue engineering of the damaged lung. The ability of L14 cells to secrete soluble protein also suggests novel ways to deliver soluble molecules to the lung.

Materials and Methods

CULTURE CONDITIONS

Cells were grown in RPMI-1640 medium (Invitrogen, Karlsruhe) supplemented with 10% heat-inactivated, preselected fetal calf serum (Roche-Boehringer, Mannheim). The cultures were kept at 37ºC in a humidified incubator with air and 5% CO_2. They were passaged weekly following

trypsinization (Invitrogen). Whenever needed, freshly prepared doxycycline at 1-50 µg/ml was included in the culture medium.

H^3- THYMIDINE INCORPORATION ASSAY

Proliferation of L14 cells was assessed by culturing $1x10^3$ cells for 4 days in triplicate wells of 96 well microtiter plates (Costar, Cambridge, MA) in 100 µl of RPMI 1640 complete medium with 10% FCS. The [H^3] deoxythymidine (dThd) was added from day 3 to day 4. Incorporation of [H^3] dThd was measured following a pulse of 16h. Data are expressed as mean of triplicate cultures +/- standard deviation (S.D.).

CELL VIABILITY AND DEATH ASSAYS

Cell viability at various times after culture in the absence or presence of doxycycline was monitored by trypan blue exclusion. Cell death was assessed, using (i) the cytoplasmic histone-associated DNA fragment release assay which measures apoptotic cell death, and (ii) the lactate dehydrogenase (LDH) assay for determining necrotic cell death. Both assays were performed using kits, according to the manufacturer's instructions (Roche-Boehringer, Mannheim). Cells were cultured in 96-well plaques in 10% FCS-RPMI medium with supplements, without or with doxycycline (10 or 50 µg/ml). 72 hours later, plates were centrifuged, and the culture supernatants collected onto a microtiter plate for the LDH release assay, while the cells were lysed and the lysates used for the cytoplasmic histone-associated DNA fragment release assay.

The amount of LDH in the culture supernatants was quantified, following the protocol specified by the manufacturer. A catalyst (diaphorase/NAD^+ mixture) and a dye solution (tetrazolium salt INT) were added to the supernatants and the O.D. of the formed formazan dye was read at 490 nm. Photometric quantification of cytoplasmic histone-associated DNA fragments was realized by ELISA, using anti-histone and anti-DNA fragment antibodies, according to the manufacturer's instructions.

MEASUREMENT OF CHLORIDE EFFLUX

Chloride efflux was assessed using SPQ fluorescent dye (=6-methoxy-N-(3-sulfopropyl)-quinolinium; Sigma, Deisenhofen) as previously described[46]. Briefly, L14 cells were grown-and then loaded with 3.5 µM SPQ for 10 min at 37°C in a hypotonic chloride buffer (130 µM NaCl, 2.4 µM K_2HPO_4, 10 µM D-glucose, 1 µM $CaSO_4$, 1 µM $MgSO_4$, and 10 µM HEPES, pH 7.4). After washing, cells were placed on an inverted microscope (Zeiss IM35) and incubated at 37°C in a quenching buffer (NaCl was replaced by 103 µM NaNO3) before stimulation with 25 µM forskolin (Sigma, Deisenhofen). The chloride secretion of 40 cells was estimated by measuring SPQ fluorescence variations (Ex: 344/Em: 433). Mean variation in SPQ fluorescence after cAMP stimulation was plotted against time over the 15min period. Data are presented as the relative fluorescence = Ft/F0 x 100%, in which FT is the fluorescence intensity at time t after stimulation of cells with forskolin and F0 is the fluorescence intensity at time 0 before the stimulation.

WESTERN BLOTTING OF PROTEINS SECRETED BY L14 CELLS

Proteins secreted from L14 cells cultured under serum-free conditions were separated on an SDS-17% polyacrylamide gel and blotted onto a 0.45 µM nitrocellulose membrane (Schleicher and Schuell, Dassel). Mouse lung extract, which included lavage, freezing thawing extract and homogenate, served as positive control. Proteins were quantified with Coomassie Brilliant Blue (Biorad, Richmond, CA). The blots were probed with mAb's: CC10: Rabbit (R) anti-CC10 (1:2000 dilution, gift of G. Singh[35, 36]), and AP (= alkaline phosphatase)-sheep (S) anti-RIgG (1:1000, Sigma); SpA: R anti-SpA (1:1000, gift of D. Phelps[37]) and AP-S anti-RigG; SpB: m anti-SpB (1:1000, gift of R. Schmidt[37]), and AP G anti-mIgG (1:1000, Sigma); proSpC:R anti-human proSpC (1:25,000, gift of J. Whitsett[40-42]) and AP-S anti-RIgG; sSpC: R anti-human SpC (1:1000, gift of R. Schmidt[38, 43-45]), and AP-S anti-RIgG; SpD: R anti-SpD (1:2500, Chemicon, Temecula, CA), and AP-S anti-RIgG. After incubation with alkaline phosphatase (AP) conjugated-sheep (S) anti-rabbit (R) IgG (Sigma, Deisenhofen), or AP-goat (G) anti-mouse (m) IgG (Sigma) bands were visualized by the BCIP (= 5'-bromo-4-chloro-3-indolylphosphat, GERBU, Gaiberg) and NBT (= nitroblue-tetrazolium, Sigma) substrates for AP. No nonspecific reactions were observed when the blots were incubated with normal rabbit serum and AP conjugated mouse anti-RIg.

RNA ISOLATION, RT-PCR ASSAYS, AND PRIMERS DESIGNED FOR CFTR AND OTHER GENES

Expressed mRNAs were detected as follows: Total RNA was isolated using a RNA isolation kit (Qiagen, Hilden). Cells were harvested and re-suspended in 500 µl of the lysis buffer according to the manufacturer´ protocol. RNA was reverse-transcribed (RT-) using random hexamer primers and superscript reverse transcriptase (Gibco, Karlsruhe). Products for reverse transcription reactions were denatured for 5 min at 95°C and one-tenth was subjected to amplification by PCR.

Primers used in this study are listed in Table 1. For example, the following primers for mouse CFTR gene[34] are given in a forward/reverse, 5'-3' orientation (listed in Table 1, No. 18). PCR using these primers yielded products corresponding to the 323bp of the CFTR gene, indicated in the figure. PCR conditions were as follows. To 1 µl DNA (one tenth of the product) were added:1 µl of 10x reaction buffer (Promega), 2.5 µl each of primers, 1 µl of dNTP mix (2.5 mM), 1 µl of $MgCl_2$ (2.5mM, Promega), 0.25 µl of Tag polymerase (4 units/µl TaKaRa Tag, BioWhittaker), then H_20 up to 10 µl per reaction. PCR was performed according to the following schedule: 94°C for 25 sec, 58°C for 25 sec, 72°C for 30 sec, for a total of 30 cycles in a Biometra Thermocycler (Biometra, Goettingen). After the PCR reaction, DNA amplified from the transgenic cells was analyzed using 8% polyacrylamide gel electrophoresis. The same condition and method were used to assay the mRNA expression of other genes.

Table 1. Primers used for analysis of transgenic lung cell line (L14) identified by RT-PCR method, which were harvested and examined one year after continuous culture.

Nr. Name of gene	Specificity	Primers (forward, backward)	Primer No.
1. SV40	SV40Tag	TCAGGGGGAGGTGTGGGAGG ACGCAGTGAGTTTTTGTTAG	C
2. ck 18	epithelial	TTGTCACCACCAAGTCTGCC GTTCCCTCCTTCTCTTGCCTC	E
3. ck 10	epithelial	CGCAAGGATGCTGAAGAGTGGTTC TGGTACTCGGCGTTCTGGCACTCGG	2C
4. ck 8	epithelial (embryonal)	CCGGAGCACCTTGGCCACC CCTGGTTCGCACTTGAGTGTC	2B
5. ck 5	epithelial	CCGGAGCACCTTGGCCACC CCTGGTTCGCACTTGAGTGTC	2A
6. involucrin	(skin) epithelial	TGGAAAGCAGCAGTCAC CGTGTTTCTTCCTTTCCAGTTG	D
7. alpha-tubulin	cytoskeleton	TCTCCATCCATGTTGGCCAG TCAACCACAGCAGTGGAAAC	2D
8. beta-tubulin	cytoskeleton	GAGGTGGATGAGCAGATGCT CTACGCCTCCTCTTCTGCC	B
9. Actin	pan	GTTTGAGACCTTCAACACCCC GAGGTCTTTACGGATGTCAACG	A
10. beta-integrin	epithelial	TCCTACTGGTCCCGACATCAT GAAACTCAGAGACCAGGCTTTA	P
11. FGFR1	fibroblast	GAAGATGATGACGACGACGA TGCCTGAAGGATGGGTCGGT	2E
12. FGFR2	fibroblast	TTGGTCACCATGGCAACCTT TTGCAGGCAGTCCAGCTTGGA	2F
13. FGFR4	fibroblast	TTGAGGCCTCTGAGGAAATG AAGGCATGTGTATGTGAAG	2G
14. tTA*	transgenic	GTTGCGTATTGGAAGATCAAG CGTCAATTCCAAGGGCATCG	2H
15. CC10	airway epithelial (club cells)	TCATGCTGTCCATCTGCTGC ATCTTGCTTACACAGAGGACTTG	2I
16. CYP2F2	airway epithelial (club cells)	ATGACCACACACAACCTGCTC CAGCACAGTCGACGTCCAG	2J
17. CGRP	airway epithelial (PNEC)	CCTTTGAGGTCAATCTTGGA TGGGATTGGTGGTTTGTCTC	2L
18. CFTR	airway epithelial	CTT GTG GGA AAT CCT GTG CT TCT CCA AGA ACT GTG TTG TC	2M
19. SpA	lung epithelial	CTGGAGGAATGCCAGGTCTT ACACATTTCTCTTTGCCCCG	I
20. SpB	lung epithelial	ATT ACT CGG CAG GTC CCA GGA GAGGTAGCTCTCCACACAGGGT	G
21. SpC	lung epithelial (Alveolar II)	GACATGAGTAGCAAGAAGTCCTG ATCGGACTCGGAACCAGTATC	H
22. SpD	lung epithelial	CTGAGGCAGCAGATGGAGG	J

23. Notch 1	**many**	**CTCTCCACAAGCCTTATCATT GCCACTTGAATGTGGCAGC**	**K**
24. Delta 1	**many**	**GAGGAGGAGTAACTGTGCTGTG CAGCGCTACATGTGTGAGTGC GGAATCTCCCCACCCCTAAG**	**L**

Legend of Table 1: Primers used for analysis of transgenic lung cell line (L14) identified by RT-PCR assay. Cells were harvested and examined one year after continuous culture. A set of primers coding for candidate genes proposed to be expressed in lung epithelial cell – tissue are applied to perform these RT PCR assays.
* positive with luciferase assay after skin and lung cell lines transfection experiment with luciferase indicator gene. **CFTR** = Cystic Fibrosis Transmembrane conductance Regulator; GeneBank accession no.: M60493, Yorifuji, T. et al 1991[90]. **SpB** GeneBank accession no.: M38314, Glasser, S.W. et al 1990[91]. **CGRP** = Calcitonin Gene-Related Peptide. **PNEC** = Pulmonary NeuroEndocrine Cells. **CYP2F2** = Cytochrome P-450 2F2

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FLOW CYTOMETRY ANALYSIS AND SPECIFICITY OF MABS USED FOR FACS ANALYSIS

Cell phenotype was determined by immunofluorescent labeling and flow cytometry analysis [53, 54]. For cytofluorometric analyses, cells were stained with monoclonal antibodies (=mAb´s) of many specificities and revealed with FITC-labelled secondary reagents. Analyses were performed on FACScan, using Cell Quest software. In detail, cells were first fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin at RT for 30 min (=minutes) each for intracellular staining. Cells were washed with PBS twice in between. Then, 10% FCS in PBS was used to block non-specific binding to cells at RT for 30 min. Cells were then stained with the mAb's diluted in PBS+10% FCS for 30 min at RT (=room temperature). Unless specifically indicated, antibodies used were diluted to 1:100. mAb's used for FACS are, Notch-1: Goat (G) IgG anti-Notch-1 (Santa Cruz Biotech, Santa Cruz, CA), biotin-donkey (D) anti-G (Santa Cruz Biotech), and FITC-streptavidin (Amersham, Amersham); Jagged-1: GIgG anti-Jagged-1 (R&D, Wiesbaden, gift of J. Dando), and biotin-D anti-GIgG and FITC-streptavidin; Delta-1: GIgG anti-Delta-1 (Santa Cruz Biotech), and biotin-D anti-GIgG and FITC-streptavidin (the profile was similar to anti-Jagged, data not shown); PNA-FITC: FITC-peanut agglutinin (=PNA) (1:250 dilution, Vector, Burlingame, CA); beta−1 integrin: rat (r) IgG anti-m beta-1 integrin (Pharmingen, San Diego, CA), and FITC-m anti-rIgG (Pharmingen).

FLOW CYTOMETRY ANALYSIS AND IMMUNOPHENOTYPING BY FLUORESCENCE

Expression of antigens in cells and tissue sections was demonstrated by immuno-fluorescent labeling and visualization with conventional and confocal microscopy[53, 54]. Immunofluorescence was evaluated using a Leica immunofluorescent microscopy equipped with a 100x objective, and coupled with a digital camera (SPOT, RT Slider Diagnostics, Sterling Heights, MI). Confocal laser scan microscopy was done on a Leica DM IRBE microscope equipped with a 63x Plan Apochromate objective, using the FITC- and TRITC settings. Eight pictures were accumulated at a pinhole size of 1.5. For immunostaining, cells were seeded onto coverslips and cultured for two days. They were fixed with 4% paraformaldehyde, washed with PBS and incubated for one hour with PBS+2% FCS at RT to block nonspecific binding sites. Staining with the first antibody, followed by labelling with FITC- or Texas Red (TR)-labelled secondary antibody were then performed. Next, the cells were stained with DAPI for 1 min, washed, then embedded in Moviol containing 100 µg/ml DABCO (Vectashield, Vector, Burlingame, CA).

Unless specifically indicated, mAb's used in this study for immunotyping were 1:100 dilution. The mAb's used for immunotyping the epitope specificities of L14 cells were: ck5: mIgG1 anti-cytokeratin 5 (Sigma, Deisenhofen) and FITC-G anti-mIgG (Jackson, West Grove, PA); ck9: mIgG1 anti-cytokeratin 9 (Sigma) and FITC-G anti-mIgG; ck18: mIgG1anti-cytokeratin 18 (Sigma) and FITC-G anti-mIgG; ck19: mIgG2a anti-cytokeratin 19 (Sigma) and FITC-G anti-mIgG; Pan ck: mIgG1 anti-pan cytokeratin (Sigma) and FITC-G anti-mIgG; beta-tubulin: mIgG2b anti-beta−tubulin (Sigma) and FITC-G anti-mIgG; vimentin: mIgG1 anti-vimentin (Sigma) and FITC-G anti-mIgG; CC10: GIgG anti-club cell 10 kDa protein, or CCSP, club cell specific protein[59-61] (1:400 dilution, gift of G. Singh) and TR-R anti-G IgG (Jackson); CGRP: RIgG anti-Calcitonin Gene Related Peptide for PNEC (=pulmonary neuroendocrine cell) (1:8000 dilution, Sigma) and TR-m anti-RIgG; SpA: RigG anti-SpA (=surfactant protein A) (1:100 dilution, Wang et al, 2000[55], gift of D. Phelps); SpB: mIgG anti-SpB[56-58] (1:500 dilution, gift of R. Schmidt) and FITC-Sheep (S) anti-mIgG (Roche-Boehringer, Mannheim); proSpC: RIgG anti-human recombinant SpC proprotein[57] (1:1000, gift of J. Whitsett) and TR-m anti-RIgG; SpC: RIgG anti-human recombinant SpC, designated sSpC in this study (1:250 dilution[62], gift of R. Schmidt) and TR-m anti-RIgG; SpD: RIgG anti-SpD (1:250 dilution, Chemicon, Temecula, CA) and

TR-m anti-RIgG; GIgG CFTR (= cystic fibrosis transmembrane conductance regulator, 1:100 dilution, Santa Cruz Biotech), biotin-D anti-G and FITC-streptavidin; ACT: mIgG2b anti-acetylated alpha–tubulin (1:800 dilution, Sigma) and FITC SIgG anti-mIgG; Biotin-hCNTF: biotin anti-hCNTF (= human ciliary neurotrophic factor, 1:50 dilution, R&D) and FITC-Streptavidin (Amersham).

In addition, epithelial cells were identified with CD44v6; CD44v7; CD44v10 or anti-CD44v10 (1:50 dilution, gift of U. Günther[63]). All the antibodies were labelled with biotin-Rabbit (R) and revealed with FITC-Streptavidin. Lectins used to identify the cell type were: PNA-FITC: FITC-peanut agglutinin (1:250 dilution, Vector); UEA-biotin: biotin-Ulex Europaeus Agglutinin I (Vector) TR-Streptavidin; Isolectin B4-biotin: biotin-Griffonia simplicifolia lectin I Isolectin B4 for basal cells[64] (1: 500 dilution, Vector) and FITC-Streptavidin; Tomato lectin-biotin: biotin-Lycopersicon esculentum (Tomato) lectin for mouse pneumocyte type I cells[75] (1:500 dilution, Vector) and FITC-Streptavidin.

IMPLANTATION INTO NUDE AND SCID MICE AND IMMUNOHISTOLOGY

The transgenic L14 cell line was stably integrated with a plasmid construct encoding hCNTF driven by the early JC viral promoter. Clone 7a-L14 was identified and selected, using an ELISA assay[53]. Cells from this clone were seeded onto DED, prepared from adult breast skin obtained during reduction mammoplasty[65]. On the center of each piece of 1cm^2 DED (= de-epidermised dermis), 10^5 cells in 15-20 µl medium were seeded and grown at the air-liquid interface on a support, in 6-well cell culture inserts (8.0 µm pore size, Falcon, Becton Dickinson). The cells were allowed to grow submerged in RPMI medium with 10% FCS for 1 week before subcutaneous implantation into nude and SCID mice. Mice were given either normal drinking water or water containing doxycycline hydrochloride[54] (Sigma, 200 µg/ml). Four weeks after the implantation, DED, lung, and other organs were removed and immersed in 30% sucrose in PBS overnight and cryo-preserved. Cryosections were then prepared for immunofluorescence analysis with a panel of mAb´s. Balb/c nu/nu mice were from Harlan, Germany. S1 and S2 facilities are available in the Institute [Aktenzeichen (S2):32-GT/530 06.05.02G, UniGI1/93, (S1):32 - 53e621-GenA-UniGI 1/90]. For animal experiments with nude mice, the license number is 17a-19c20-15(1) at the Uni. of Giessen, Germany. C.B.17 scid/scid (SCID) mice were bred in the mouse facility of INSERM U506, F94807 Villejuif Cedex, France, in isolators supplied with sterile-filtered, temperature-controlled air. Cages, bedding and drinking water were autoclaved. Food was sterilized by X-ray irradiation. All experiments on SCID mice were performed under laminar flow hoods and the mice were exposed to 350 rad, low dose irradiations before the implantation. 7a-L14 lung cells seeded on DED were implanted into the subcutaneous region of the flanks of 6–8-week-old NOD-SCID mice anesthetized with Hypnomidate[66, 67] (Laboratoire Janssen, Boulogne-Billancourt, France).

Results

ISOLATION OF A CELL LINE FROM LUNG TISSUE OF TTAXSV40 T ANTIGEN DOUBLE TRANSGENIC MICE

Tissue-specific stem cells are normally extremely difficult to isolate in quantities sufficient for genetic modification and use in somatic gene therapy. We therefore decided to employ the SV40 Tag to amplify such a rare cell type. However, only conditional expression (upon induction by an external signal) of SV40 Tag is compatible with this application. Double-transgenic mice were produced to provide a potential source of various tissue-specific somatic stem cells. Transgenic mice carrying the SV40 Tag gene controlled by the tetophCMV promoter regulated by the doxycycline-regulatable trans-activator tTA, were crossed with mice in which the tTA trans-activator gene was cloned under the control of the hCMV promoter. Progeny should bear the tTA trans-activator and the SV40 Tag genes[9, 37]. The engineered tTA trans-activator is active only in the absence of doxycycline, and cannot bind to its synthetic tetophCMV promoter in the presence of doxycycline (a tet-off-system[40]). Genotyping for double-transgenic mice was performed using PCR for phCMV-tTA x tetophCMV-SV40 Tag. To determine whether cell lines of any tissue could be established from these transgenic mice, we cultured cells derived from 10 different tissues of 3 mice that had been typed as positive for tTA as well as SV40 Tag. Since SV40 Tag is expressed in these tissues in the absence of doxycycline, at least some of the cells should be rendered immortal. We eventually obtained several cell lines from skin, brain, and lung tissues[7], which have now been cultured continuously for more than 36 months. In this study, we report some properties of L14, a cell line isolated from lung tissue.

EFFECT OF DOXYCYCLINE ON THE PROLIFERATION AND VIABILITY OF L14 CELLS

Proliferation of L14 cells was downregulated when doxycycline was included in the culture medium (Figure 1). When doxycycline was omitted, or was present up to a concentration of 1 µg/ml, cells proliferated, as shown by ^{3}H-thymidine

incorporation (11-15 x 10^3 cpm/10^4 cells). Concentrations of doxycycline of 3 µg/ml and above completely inhibited DNA synthesis. However, no significant difference in cell viability was observed when the cells were cultured for 8 days in the absence or presence of 1-3 µg/ml doxycycline. Viability declined after 7 days only when 10 µg/ml of doxycycline was added (Figure 2).

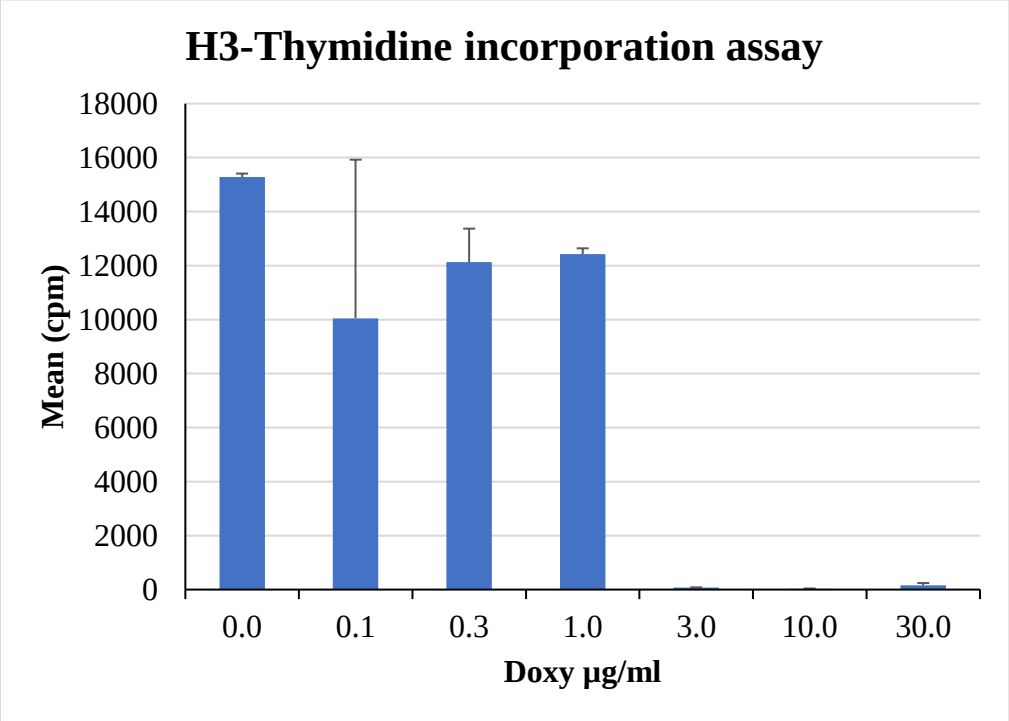

Figure 1. H^3-thymidine incorporation assay of L14 cells.
Cells (1x10^4/well) were cultured for 4 days with and without Doxy (doxycycline) at concentrations ranging from 0-30 µg/ml in the medium. H^3-thymidine was included in the medium from day 3 on for 16 hr. H^3-thymidine incorporation into cells of triplicated cultures is given in cpm (mean+/-S.D.).

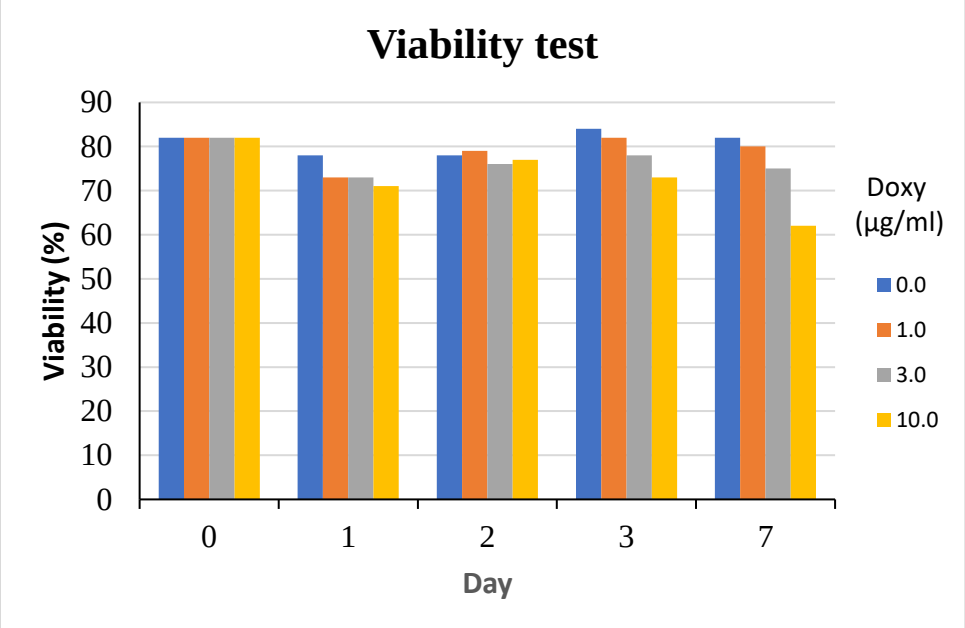

Figure 2. Viability test of L14 cells.

Cell viability of L14 cells was performed based on a trypan blue exclusion assay. Triplicate culture wells were each seeded with 1 x 10^4 cells in each well. Doxycycline (Doxy) of different concentrations (0, 1, 3, 10 µg/ml) was included in the culture medium. Cells were harvested on days 1, 2, 3, 7 after seeding, and (viable) cell number in each well were determined using a hematocrite chamber. The

viability of cells was calculated from an average of triplicate wells, and the percentage was determined by the formula: % viable cells = viable number of cells in well / total number of cells in well x 100%.

HIGH DOSES OF DOXYCYCLINE INDUCE APOPTOTIC CELL DEATH

In order to study further the effect of doxycycline on cells, the following two cell death assays were performed: (i) the cytoplasmic histone-associated DNA fragment release assay for measuring cell death caused by the apoptotic pathway, and (ii) the culture supernatant LDH assay for determining cell death caused by the necrotic pathway.

Culturing lung cells with doxycycline at a concentration of 10 µg/ml for 72 hr does not make these cells enter the apoptotic pathway (Figure 3 Panel A), consistent with the results of the cell viability assay (Figure 2, above). Culturing cells with doxycycline at a concentration of 50 µg/ml induces apoptosis and a 6-fold increase of cytoplasmic histone-DNA fragments. Cells become detached from the petri dish, and appear refractive and shrunken with an overall impression of being dead. To monitor necrosis, we measured the LDH released by cells cultured in the presence of different doxycycline concentrations. We found a twofold increase (statistically not significant) in the LDH activity in supernatants from cells cultured with 50 µg/ml compared to 0, or 10 µg/ml of doxycycline. Thus, doxycycline does not affect cells up to 10 µg/ml and only at 50 µg/ml causes apoptosis. No necrotic effect of doxycycline on this cell line was detected.

In many other cell types, TNFα is known to cause cell death by apoptosis and not by necrosis. We therefore tested the effect of TNFα on cultured L14 cells and monitored apoptosis and necrosis. TNFα was included in the culture medium at concentrations of 0, 10, 50 ng/ml for 24 hr, cells were then harvested and tested (Figure 3 Panel B). At 10 ng/ml, TNFα showed already a clearly apoptotic pattern on lung cells. Doxycycline and TNFα present together in the medium had an additive but not synergistic effect on L14 cells (Figure 3 Panel C). In all conditions, no sign of necrosis was detected, in contrast to another cell line isolated from the skin of these transgenic mice (data not included). Thus, doxycycline can also induce apoptosis of this cell line, if the concentration in the culture medium is 10 µg/ml and above. For the rest of this study, doxycycline is used at the concentration of 1 µg/ml in the culture medium.

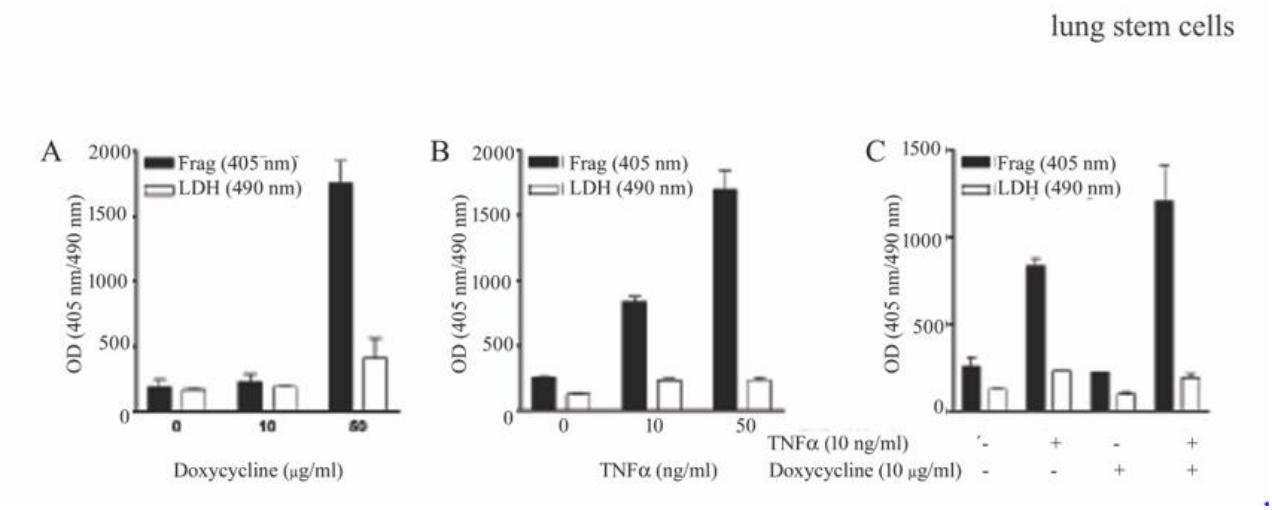

Figure 3. Apoptosis assay:
Panels A-C: Cell death assays to measure the effect of doxycycline on L14 cells.
A sandwich enzyme immuno-assay was used to quantify cytoplasmic histone-associated DNA fragments released due to cell death by the apoptotic pathway, at the end of culture. In addition, culture supernatant was collected, and the amount of LDH released due to cell death caused by the necrotic pathway was quantified colorimetrically. In Panel A, X-axis shows the amount of doxycycline, ranging from 0, 10 to 50 µg/ml, included in the cell culture (10^4 cells/well) for 72 hr. Y-axis shows the O.D. absorbance units measured at 405 nm for apoptosis, and at 490 nm for necrosis. Solid bars are the units quantifying the amount of DNA fragments released into the cytoplasm resulting from apoptosis. Open bars are the units quantifying the amount of LDH released into the culture supernatant resulting from necrotic events. In parallel experiments (Panel B), TNFα with the dose ranging from 0, 10 to 50 ng/ml, was included in the cell culture (10^4 cells/well) for 24 hr. Samples were harvested and assayed similarly. Solid bars are the units of cytoplasmic DNA fragments due to apoptosis. Open bars are the amount of LDH released into the culture supernatant due to necrosis. Data presented are the mean value of three (Panel A), two (Panel B) separate experiments, respectively. Panel C shows

the result of apoptosis induction by culturing cells with doxycycline and TNFα. Cells at the concentration of 10^4/culture in the 96 well plate were incubated with 10 μg/ml of doxycycline for 48 hr. TNFα at 10 ng/ml was then included in the same culture wells for an additional 24 hr. X-axis is the amount of TNFα and/or doxycycline included in the culture. Y-axis is the O.D. units measured at 405 nm for apoptosis (solid bars), and at 490 nm for necrosis (open bars). Data presented are the mean value of two separate experiments.

CYCLIC AMP-STIMULATED EFFLUX OF CHLORIDE IN L14 CELLS

The expression of functional CFTR protein in L14 cells was assessed by measuring the Cl^- secretion of cells after exposure to 25□μM forskolin, a cAMP analog. Chloride secretion was estimated in 40 cells through a 20X objective. The relative Cl^- fluorescence, measured from 0 to 16 min, is shown in Figure 4. SPQ-loaded cells exhibited a response to cAMP stimulation, as demonstrated by the 14% increase in the Cl^- fluorescence signal after the onset of the stimulation. The cAMP response is consistent with a CFTR protein expression in this cell line. The expression of CFTR is further demonstrated by the positive reaction using anti-CFTR antibodies and RT-PCR assays (Lane 18 in Figure 6 in the follow second section and Table 2). To our knowledge functional CFTR protein is expressed in primary mature ciliated epithelial cells of the airways[46] and in human bronchiolar epithelial cells[11].

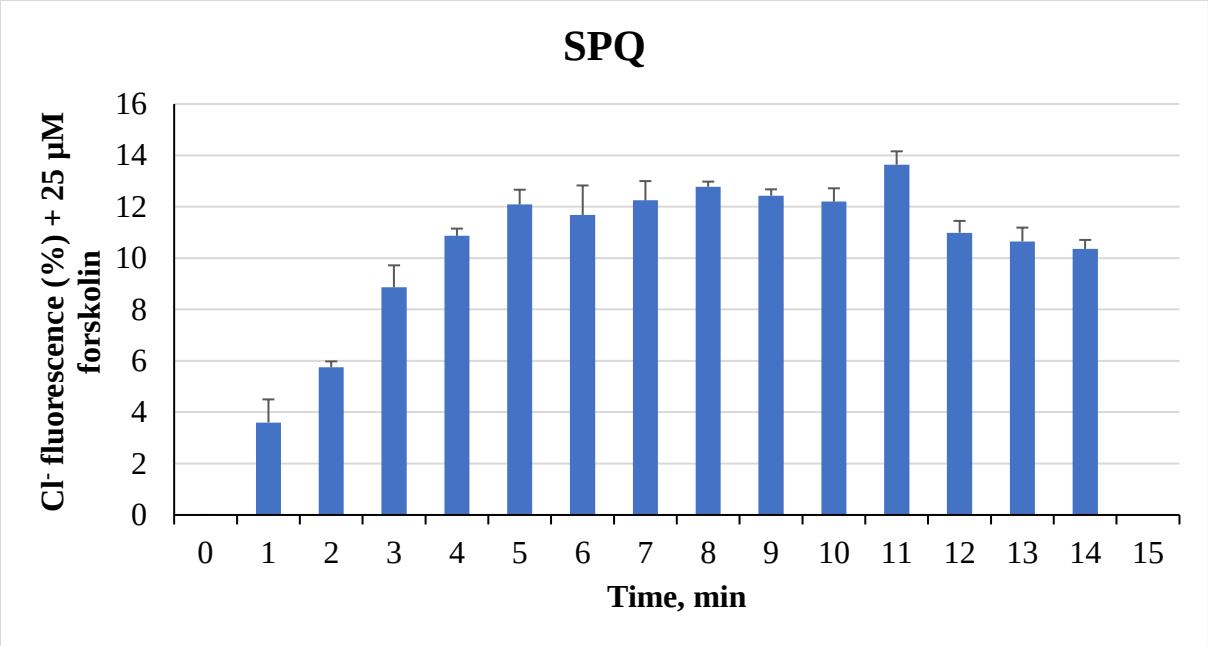

Figure 4. The c-AMP chloride efflux assay of L14 cells.
Changes in SPQ (= 6-methoxy-N-(3-sulfopropyl)-quinolinium) halide efflux for cells were evaluated. X-axis indicates the time, min = minutes. Y-axis indicates the percentage (%) of Cl^- fluorescence of cells, at the presence of 25 μM of forskolin. Data represent responses obtained from 3 experiments.

PROTEINS SECRETED BY L14 CELLS

L14 cells were grown under serum free condition, and the culture supernatant subjected to Western blot analysis using antibodies against CC10, SpA, SpB, proSpC, sSpC and SpD (Figure 5). Anti-CC10 antibodies detected bands of 5-6, 10, 16, 32 kDa and bands of higher MW in the culture supernatant and 10, 16, 32 kDa and larger bands from the lung extract, which served as positive control. In L14 culture supernatants, anti-SpA antibodies detected bands of 26-28 kDa and bands of higher MW from L14 cells culture supernatant. Anti-SpB antibodies detected bands of 9, 18, 28 kDa and bands of higher MW from L14 culture supernatant. Anti-human proSpC detected three bands estimated to be 14-16, 29-32 kDa and higher bands; and anti-human sSpC antibodies reacted with bands of 29-32 kDa and higher MW bands from L14 culture supernatant.

The antibodies used in this study were donated by colleagues who have characterized and published as cited in the M&M section and in References section. However, staining with anti-SpA antibody shows two additional higher MW bands, with anti-SpB antibody shows one more higher MW band. The reason for the detection of these higher MW bans is not clear. The speculation is: since this is a cell line, it might produce "proSpA", "proSpB".

Unlike human surfactant proteins SpC, no bands of 4, 8 kDa were detected in the L14 culture

supernatant using these two antibodies. Anti-SpD antibodies detected a major 43-kDa band in L14 culture supernatant. No bands could be detected when normal rabbit serum was used. CC10 is a hallmark for club cells[14], whereas SpA, B and D are reportedly secreted by various cell types including pneumocyte type II cells and club cells. Production and secretion of SpC seem to be restricted to pneumocyte type II cells and are hallmark of this cell type[47-49]; however, SpC promoter is active in lung and other tissues[48]. The mouse epitopes detected by anti-proSpC and anti-sSpC could be from the secreted SpC as well as proSpC of some apoptotic cells. Using anti-SpB, anti-SpC ELISA assays, we can quantify SpB, SpC secreted into the L14 cells culture supernatant. This L14 cell line seems to exhibit dual properties and secretes proteins characteristic of club cells, and surfactant proteins reportedly specific to pneumocyte type II cells.

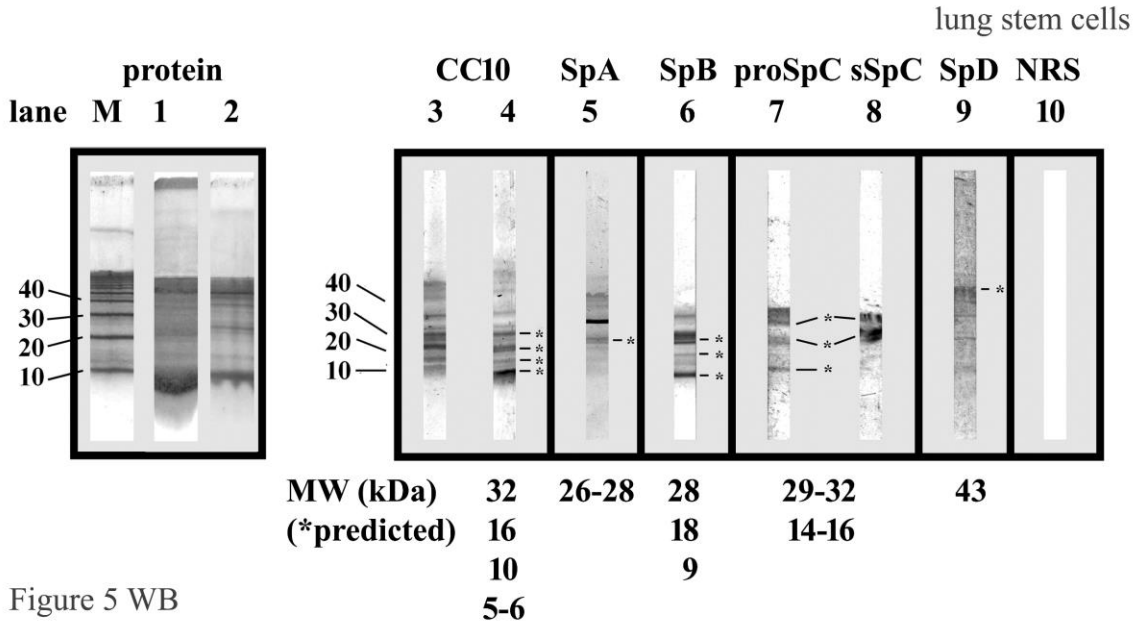

Figure 5. Western blot analysis of proteins secreted by L14 cells.
The cultured supernatant from L14 cells (1x10^5 cells) and lung extract were electrophoresed on a 17% SDS-PAGE gel and blotted onto nitrocellulose membrane. Lanes 1 and 2 of the gel were stained directly with Coomassie Brilliant blue to reveal the amount of proteins loaded. Lanes 1, 3: mouse lung extract, as positive controls; Lanes 2, 4, 5-10: L14 cells culture supernatant; M: protein markers. The blots were probed with mAb's: Lanes 3 and 4: R anti-CC10 and AP-S anti-RIgG; Lane 5: R anti-SpA and AP-S anti-RIgG; Lane 6: m anti-SpB and AP-G anti-mIgG; Lane 7: R anti-hu proSpC and AP-S anti-RIgG; Lane 8: R anti-hu sSpC and AP-S anti-RIgG; Lane 9: R anti-SpD and AP-S anti-RIgG; Lane 10: normal rabbit serum (NRS) and AP-S anti-RIgG. After incubation with alkaline phosphatase (AP) conjugated-sheep (S) anti-rabbit (R) IgG (Sigma, Deisenhofen), or AP-goat (G) anti-mouse (m) IgG (Sigma) bands were visualized by the BCIP (= 5'-bromo-4-chloro-3-indolylphosphat, GERBU, Gaiberg) and NBT (= nitrobluetetrazolium, Sigma) substrates for AP.

DETECTION OF MRNAS CODING FOR SPB, SPC AND OTHER PROTEINS IN L14 CELLS

The mRNAs expressed in this L14 cell line in the presence or absence of doxycycline one year after continuous culture were assayed by RT-PCR. A set of primers coding for 24 candidate genes proposed to be expressed in (lung) epithelial cell - tissue (Table 1) were used in these RT PCR assays. These mRNA expression patterns are shown in Figure 6, and the data are summarized in Table 2. Their relationship to mouse lung epithelial cell types isolated from freshly dissociated lung tissue is shown (Figure 6, Panel A, Table 2). In freshly dissociated lung tissue (in Panel A Lung Tissue), SV40 (Lane 1, SV), CC10 (Lane3, C10), Notch (Lane 23, NO) are not detected, while in L14 cell line they are positive (in Panel B L14, the corresponding Lanes).

We detected mRNA coding for SpB, (pro)SpC in the L14 cells (Lane 20, Lane 21 in Figure 6 and Table 2, consistent with the expression pattern at the protein level (see previous section).

At the presence of doxycycline, C18=ck18 (Lane 2 in Figure 6 and Table 2), C10=ck10 (Lane 3 in Figure 6 and Table 2), CY=CYP2F2 (Lane 16 in Figure 6 and Table 2), SD=SPD (Lane 22 in Figure 6 and Table 2), NO=Notch (Lane 23 in Figure 6 and Table 2), become negative-reduced, while C8=ck8 (Lane 4 in Figure 6 and Table 2), SB=SPB

(Lane 20 in Figure 6 and Table 2) showed positive/additional bands.

The mRNA expression pattern of SV40Tag in L14 cells without doxycycline is positive. In the presence of doxycycline, it becomes negative (Lane 1 in Figure 6 and Table 2), confirming that this L14 line is a tet-off cell line.

Thus, L14 cells have dual properties and secretes proteins characteristic of club cells, and surfactant proteins reportedly specific to pneumocyte type II cells.

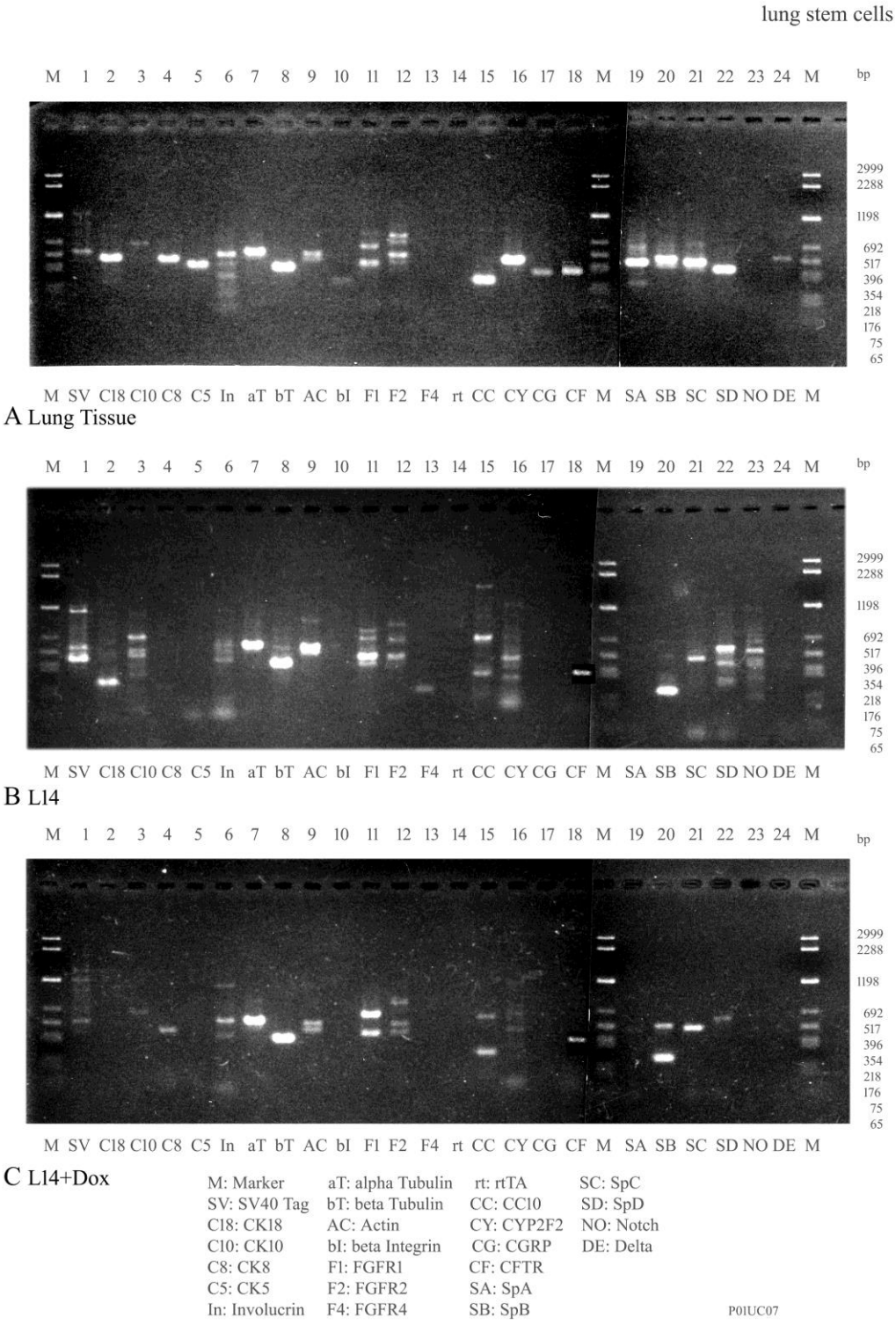

Figure 6 RT PCR

Figure 6. RT-PCR of CFTR mRNA.
RT-PCR products using primers coding for mouse 24 sets of genes, electrophoresed using 2% agarose gel, and stained with ethidium bromide. The size of RT-PCR products for each gene detected is summarized in

Table 2. The interpretation of the results is summarized in Table 4 and the results section. As shown below the panel, mRNA´s were derived from the indicated cells; Panel A: Lung Tissue of a NMRI mouse, Panel B: L14 lung cells, Panel L14 lung cells cultured at the presence of doxycycline (= Dox). M: Marker, SV: SV40 Tag, C18: CK18, C10: CK10, C8: CK8, C5: CK5, In: Involucrin, aT: alpha Tubulin, bT: beta Tubulin, AC: Actin, b1: beta Integrin, F1: FGFR1, F2: FGFR2, F4: FGFR4, rt: rtTA, CC: CC10, CY: CYP2F2, CG: CGRP, CF: CFTR, SA: SpA, SB: SpB, SC: SpC, SD: SpD, NO: Notch, DE: Delta.

Table 2. Detection of mRNAs expressed in L14 cells using RT PCR.

No.	Gene coded	size (bp) expected	(A) lung tissue	(B) L14	(C) L14+Dox	footnote	Primer #	specificity*
1.	SV40	440	-	+	-	*1	C	SV40Tag, transgenic
2.	ck18	469	+	-	-	*2	E	epithelial
3.	ck10	360	-	-	-	*3	2C	epithelial
4.	ck8	450	+	-	+		2B	epithelial, embryonal
5.	ck5	447	+	-	-		2A	epithelial
6.	Involucrin	500	+	+	+		D	epithelial, skin
7.	alpha-Tubulin	534	+	+	+		2D	cytoskeleton
8.	beta-Tubulin	364	+	+	+		B	cytoskeleton
9.	Actin	512	+	+	+		A	many
10.	beta-Integrin	242	+	-	-		P	epithelial
11.	FGFR1	395	+	+	+	*11	2E	fibroblast
12.	FGFR2	759	+	+	+	*12	2F	fibroblast
13.	FGFR4	595	-	-	-	*13	2G	fibroblast
14.	tTA	222	-	-/(+)	-		2H	transgenic
15.	CC10	240	+	+	+	*15	2I	epithelial, airway club cell
16.	CYP2F2	440	+	+	+	*16	2J	epithelial, airway club cell
17.	CGRP	320	+	-	-		2L	epithelial, airway PNEC
18.	CFTR	323	+	+	+		2M	epithelial, airway & other
19.	SPA	440	+	-	+ (weak)		I	epithelial, lung alveolar II & other
20.	SPB	525	+	+	+	*20	G	epithelial, lung alveolar II
21.	SPC	509	+	+	+		H	epithelial, lung alveolar II & other
22.	SPD	493	+	+	-	*22	J	epithelial, lung alveolar II & other
23.	Notch	700	-	-	-	*23	K	many

Table 2 footnote

* specificity/preferential expressed

***1.** negative and with artefact larger bands in (A) lung tissue and (C) L14+Dox, positive and with artefact larger bands in (B) L14

***2.** positive in (A) lung tissue, negative in (B) L14 & with artefact band of ca. 250 bp, negative in (C) L14+Dox

***3.** negative in all cases, with artefact larger bands in L14(B)

***11.** positive, with artefact larger bands

***12.** positive, with artefact larger bands

***13.** negative in all cases, with one artefact smaller band in (B) L14 => *11,12,13: same pattern as lung tissue

***15.** positive in all cases, but additional larger artefact band in (B) L14 and (C) L14+Dox

***16.** positive in (A) lung tissue, positive and with additional artefact bands in (B) L14, weakly positive with similar artefact bands in (C) L14+Dox

***20.** positive in (A) lung tissue, weak positive and with a strong band of ca. 220 bp (alternative sliced form?) in (B) L14, positive and with a similar strong band of ca. 220 bp in (C) L14+Dox

***22.** positive in (A) lung tissue, positive and with artefact one larger and one smaller bands in (B) L14, negative and with an artefact large band in (C) L14+Dox

***23.** negative in all cases, with artefact smaller bands in (B) L14

summary in text: - with epithelial markers, + with cytoskeletal markers.

Legend of Table 2. Detection of mRNAs Expressed in L14 Cells Using RT PCR. The mRNAs expressed at the presence or in the absence of doxycycline in this L14 cell line one-year after continuously culture are assayed using RT-PCF assay. A set of primers coding for 20 candidate genes proposed to be expressed in (lung) epithelial cell - tissue (Table 1) are applied to perform these RT PCR assays. The mRNA expression patterns shown in Figure 6 are summarized in this table and the results section.

PHENOTYPIC ANALYSIS OF L14 CELLS USING FACS AND IMMUNOFLUORESCENT MICROSCOPY

The labeling pattern of L14 cells, using PNA, or mAbs directed against Notch-1, Jagged, and β-1-Integrin, and is shown in Figure 7. When cultured in the absence of doxycycline, nearly all the cells (84%) stained positive for PNA, showed weak staining for Notch-1 (23%) and β-1-Integrin (31%), and were negative for Jagged-1 (Figure 7, Panel Aa). They were also negative for Delta-1 (data not shown). Culturing the cells in the presence of doxycycline for three days did not modify the expression of PNA, β1-Integrin, Jagged-1 and Delta-1 (Figure 7, Panel Ba). It is, however, worth noting that the cells became Notch-1 negative implying that the cell cycle status influences the expression of Notch-1 in these cells is. Table 3 summarizes the expression of various surface markers detected on L14 cells using immunofluorescence staining. Cells were negative for many of the mAbs screened, such as **mAb**´s against a series of cytokeratins: ck19, ck18, ck9, ck5, and pan ck. Thus, this cell line seems unlikely to be of airway basal cell origin[68-70]. The negative reaction with Griffonia simplicifolia lectin I (Isolectin B4) supports this statement. The cells are also negative for CGRP (a marker of PNE cells), tomato lectin (a marker of pneumocyte type I cells), and ACT (a marker of ciliated airway epithelial cells). Of the mAbs against CD44 variants (= v) 6, v7, and v10, L14 cells are CD44v10 positive only, a phenotype characteristic of epithelial cells[63]. L14 cells are β-tubulin+, PNA+, UEA+/w, and SV40Tag+. The positive reaction with anti-CC10 antibodies identifies this cell line as a candidate club cell line (Table 3, Figure 8, panels 1Ba) & References[59-61]. L14 cells are also positive for surfactants SpA, SpB, SpD, and, interestingly, for surfactants proSpC and SpC using two anti-human SpC antibodies (Figure 8, panel 2Ba), suggesting that it is a candidate pneumocyte type II cell line.

These markers are mainly located in the nucleus of this L14 cell line, as opposed to their cytoplasmic location in cells freshly isolated from adult mature mouse lung tissue, in which precursor-stem cells are a rare population. Further characterization of nucleus vs. cytoplasmic location of these markers is described in the next section.

When this cell line is stably transfected with the hCNTF gene, it expresses hCNTF protein (Figure 8, panels 1Aa and 2Aa). It indicates that this cell line is permissive for the transgenic expression of hCNTF. It serves as a transgenic marker to trace the fate of this cell line in the in vivo experiments (Figures 10, 11a, 11b, 12, 13). Thus, the L14 cell line possesses multi-lineage markers and could be a candidate club and pneumocyte type II cell line.

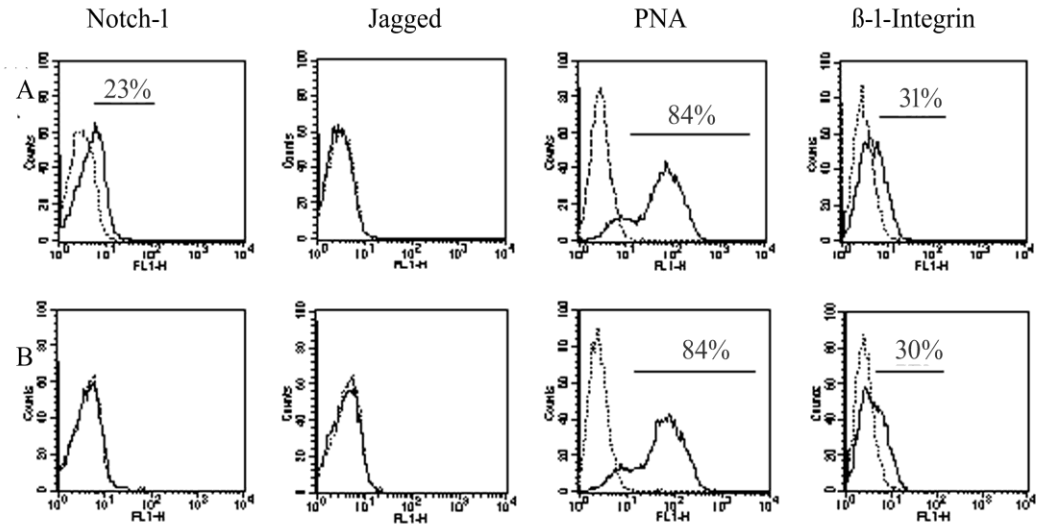

Figure 7 FACS

Figure 7. FACS analysis.

Immunostaining was performed after fixation of the cells with 4% paraformaldehyde and permeabilization with saponin. Cells were labelled with G anti-Notch1 or G anti-Jagged (or G anti-Delta-1) followed by donkey anti-G-biotin and Streptavidin-FITC; or with PNA-FITC, or with r anti β1-Integrin and anti-r IgG-FITC. Expression of Notch-1, Jagged, PNA and β-1-Integrin (unbroken lines) was assessed in L14 cells cultured in the absence (Panel A) or presence of doxycycline for 3 days (Panel B). Dotted lines represent negative controls (cells stained with the secondary FITC-immunoreagents or unlabelled cells for PNA expression).

Table 3. Immunofluorescent typing of L14

(m)Ab Name	(m)Ab Specificity	Expression pattern L14 cells	lung tissue
SV40Tag	SV40Tag, transgenic	+	-
ck 5	epithelial	-	n.d.
ck 9/18/19	epithelial	-/-/-	+/+/+
pan ck	pan cytokeratins	-	+
beta-tubulin	cytoskeleton	+	+
vimentin	mesenchymal	-	-
CC10	club cells	+	+
CGRP	airway (PNEC)	-	+
SpA	(lung) epithelial	+	+
SpB	(lung) epithelial	+	+
ProSpC/sSpC	pneumocyte type II	+/+	+/+
SpD	(lung) epithelial	+	+
CFTR	lung airway epithelial	+	+
ACT	ciliated airway epithelial	-	+
hCNTF	transgenic	+ (if transg.)	-
CD44 v6	B cells, some epithelial	-	n.d.
CD44 v7	many cell types	-	n.d.
CD44 v10	epithelial	+	+
PNA	many cell types	+	+
UEA	many cell types	- / +	+
Tomato lectin	pneumocyte type I	-	+
Isolectin B4	airway basal cells	-	+
beta1-Integrin	many cell types	+	+
Notch-1	many cell types	- / +	n.d.
Delta-1/Jagged	many cell types	- / -	n.d.

Legend of Table 3. Summary of Immunophenotyping of lung cells and L14 cells transgenic with hCNTF using a set of mAb´s. Cells were cultured on coverslip to 80% confluency, fixed with 4% paraformaldehyde, stained with mAb's or fluorescent dye coupled reagents, and evaluated using immunofluorescent microscope, as described in the M&M section. n.d.= not done.

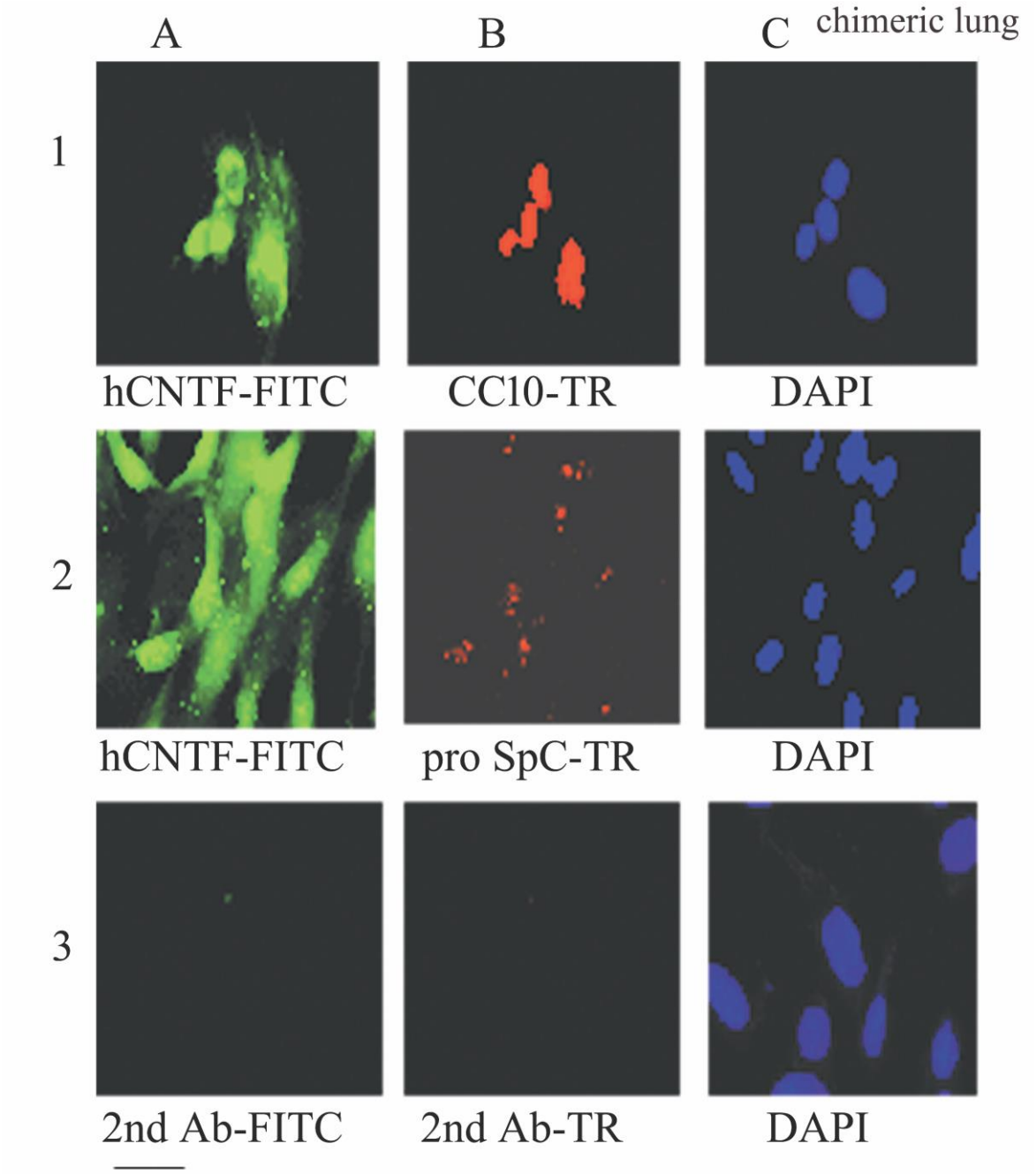

Figure 8 Immunophenotyping of L14 cells.
Cells were cultured on coverslip to 80% confluency, fixed with 4% paraformaldehyde, stained with mAb's or fluorescent dye coupled reagents, and evaluated using immunofluorescent microscope, as described in the M&M section. Immunofluorescence staining and digital image analysis of L14 Cells. Transgenic with hCNTF. Upper panel-1: hCNTF-FITC, CC10-TR, DAPI; middle panel-2: hCNTF-FITC, proSpC-TR, DAPI; lower panel-3: second Ab-FITC, second Ab-TR, DAPI. The staining and specificity of antibodies were described in M&M section. One bar = 30 μM.

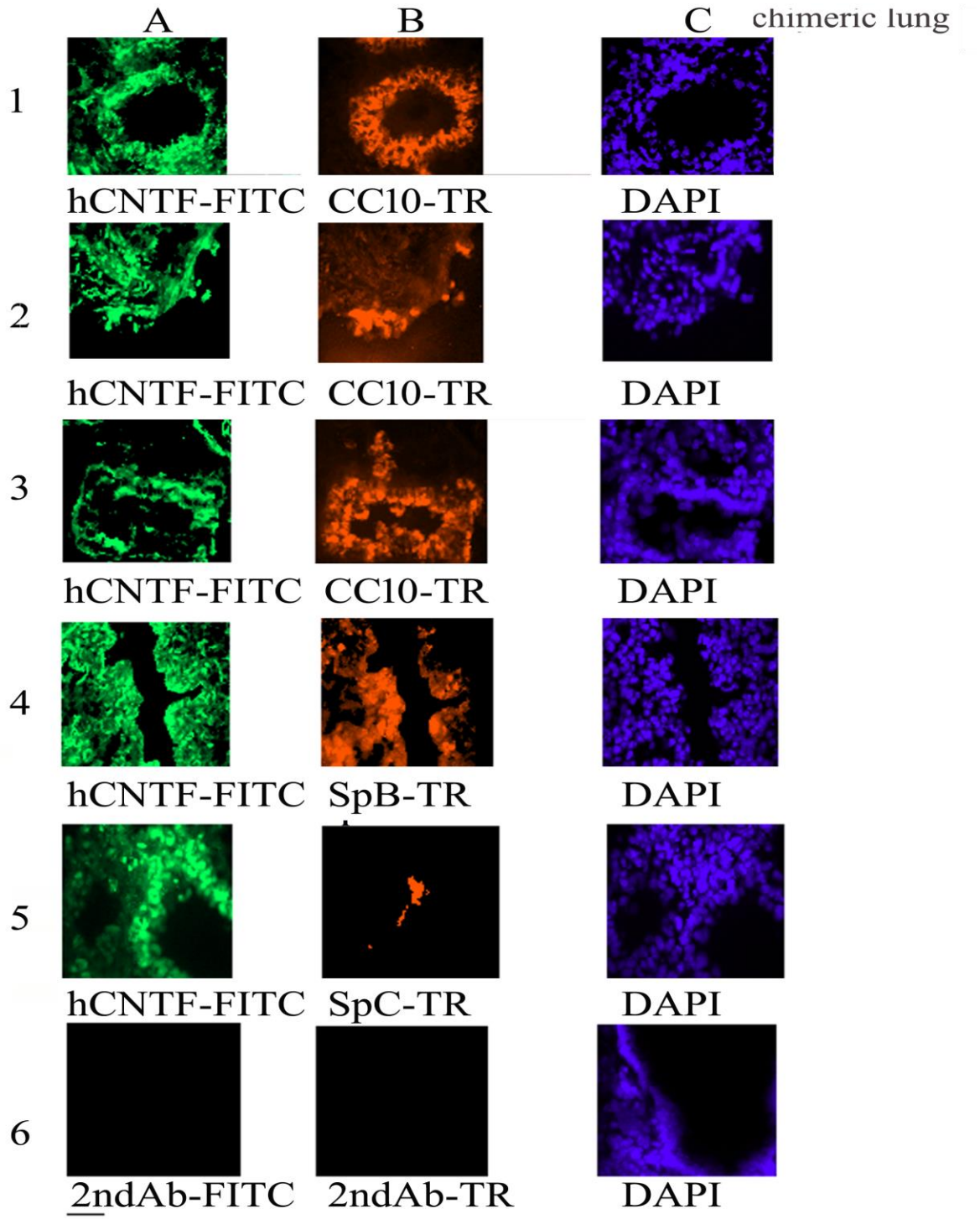

Figure 9 Lung CNTF+X

Figure 9. Immunofluorescent staining of host lung of immunodeficient mice after subcutaneous implantation of L14 cells.

L14-7a cells, seeded onto a DED and cultured, were implanted in immunodeficient mice subcutaneously. After 4 weeks, lung tissues were analyzed. Panels 1-3: hCNTF-FITC, CC10-TR, DAPI; Panel 4: hCNTF-FITC, SpB-TR, DAPI; Panel 5: hCNTF-FITC, sSpC-TR, DAPI; Panel 6: second Ab-FITC control, second Ab-TR control, DAPI. One bar = 30 μM.

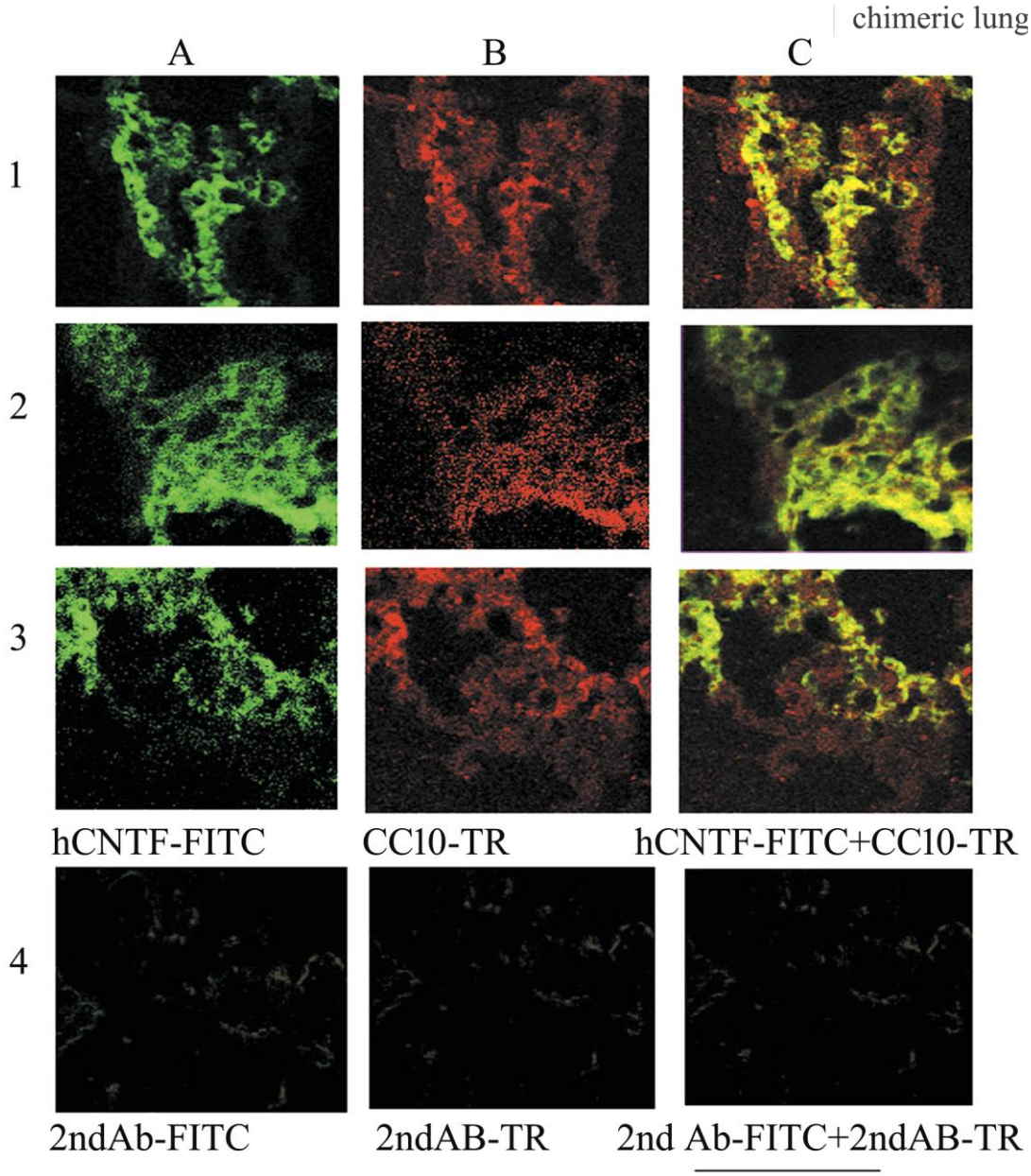

Figure 10 hCNTF+CC10 Elmshäuser et al

Figure 10. Immunofluorescent staining of host lung with anti-CC10 after subcutaneous implantation of L14 cells.
L14-7a cells were seeded onto a denuded derma matrix and cultured in a two-chamber culture system, then implanted in immune-deficient mice subcutaneously for 4 weeks. Analysis of fields at the terminal bronchiole is shown in upper 3 panels. Left Column: hCNTF-FITC; Middle Column: CC10-TR; Right Column, hCNTF-FITC & CC10-TR. Panel 4 shows the negative controls, Left Column: 2nd Ab-FITC; Middle Column: 2nd Ab-TR; Right Column, 2nd Ab-FITC & 2nd Ab-TR. 1 bar = 100 μM.

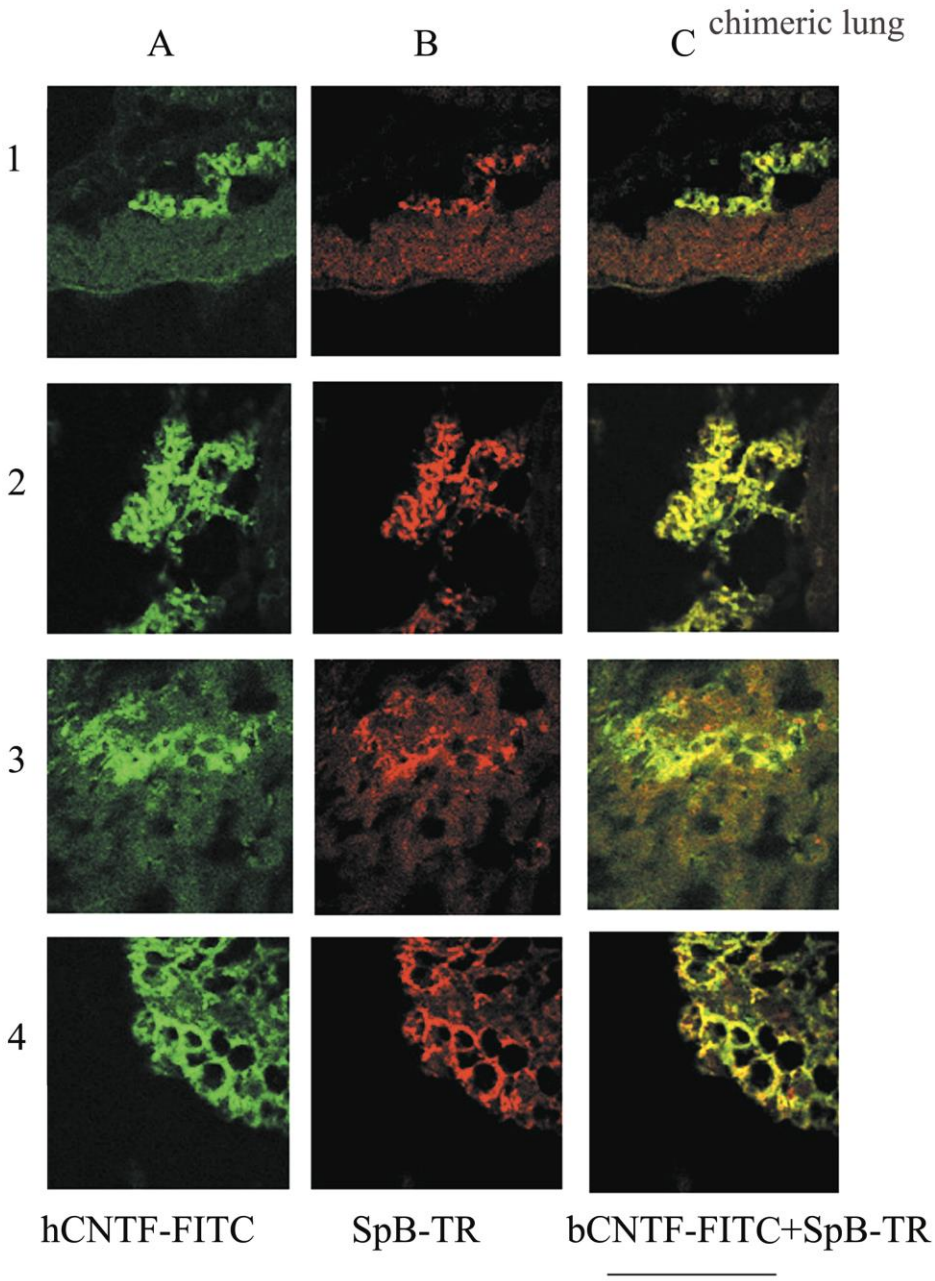

Figure 11a Lung SpB

Figure 11a. Immunofluorescent staining of host lung with anti-SpB after subcutaneous implantation of L14 cells.
L14-7a cells were seeded onto a denuded derma matrix and cultured in a two-chamber culture system, then implanted in immune-deficient mice subcutaneously for 4 weeks. Analysis of different fields of lung tissue at the alveolus neighboring to terminal bronchiole is shown in Panels. Column A: hCNTF-FITC; Column B: SpB-TR; Column C: hCNTF-FITC & SpB-TR. 1 bar = 100 μM.

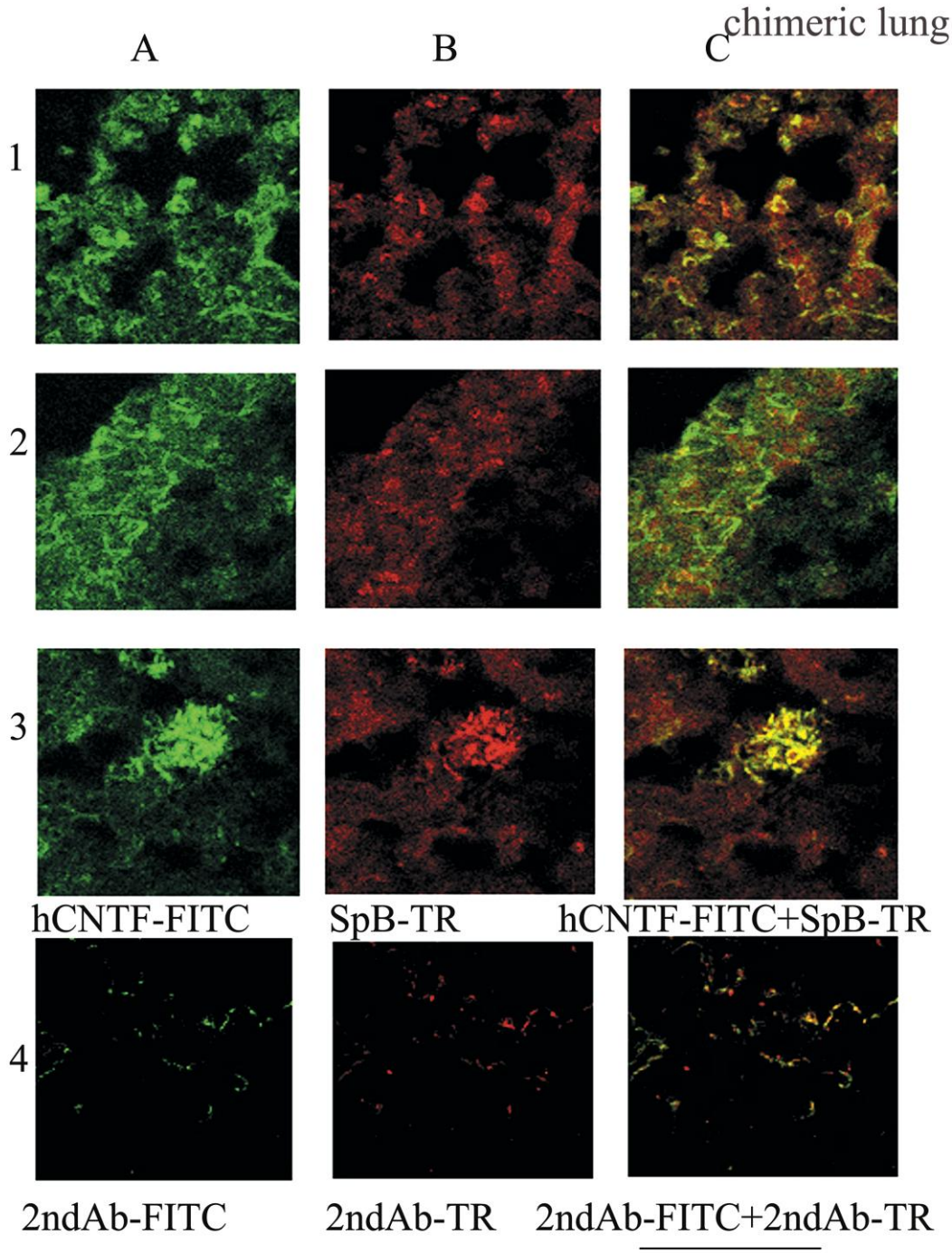

Figure 11b SpB

Figure 11b. Immunofluorescent staining of host lung alveoli with anti-SpB after subcutaneous implantation of L14 cells.

L14-7a cells were seeded onto a denuded derma matrix and cultured in a two-chamber culture system, then implanted in immune-deficient mice subcutaneously for 4 weeks. Analysis of fields of alveoli is shown in 3 Panels. Column A: hCNTF-FITC; Column B: SpB-TR; Column C, hCNTF-FITC & SpB-TR. In Panel 4 are the negative controls, Column A: 2nd Ab-FITC; Column B: 2nd Ab-TR; Column C: 2nd Ab-FITC & 2nd Ab-TR. 1 bar = 100 μM.

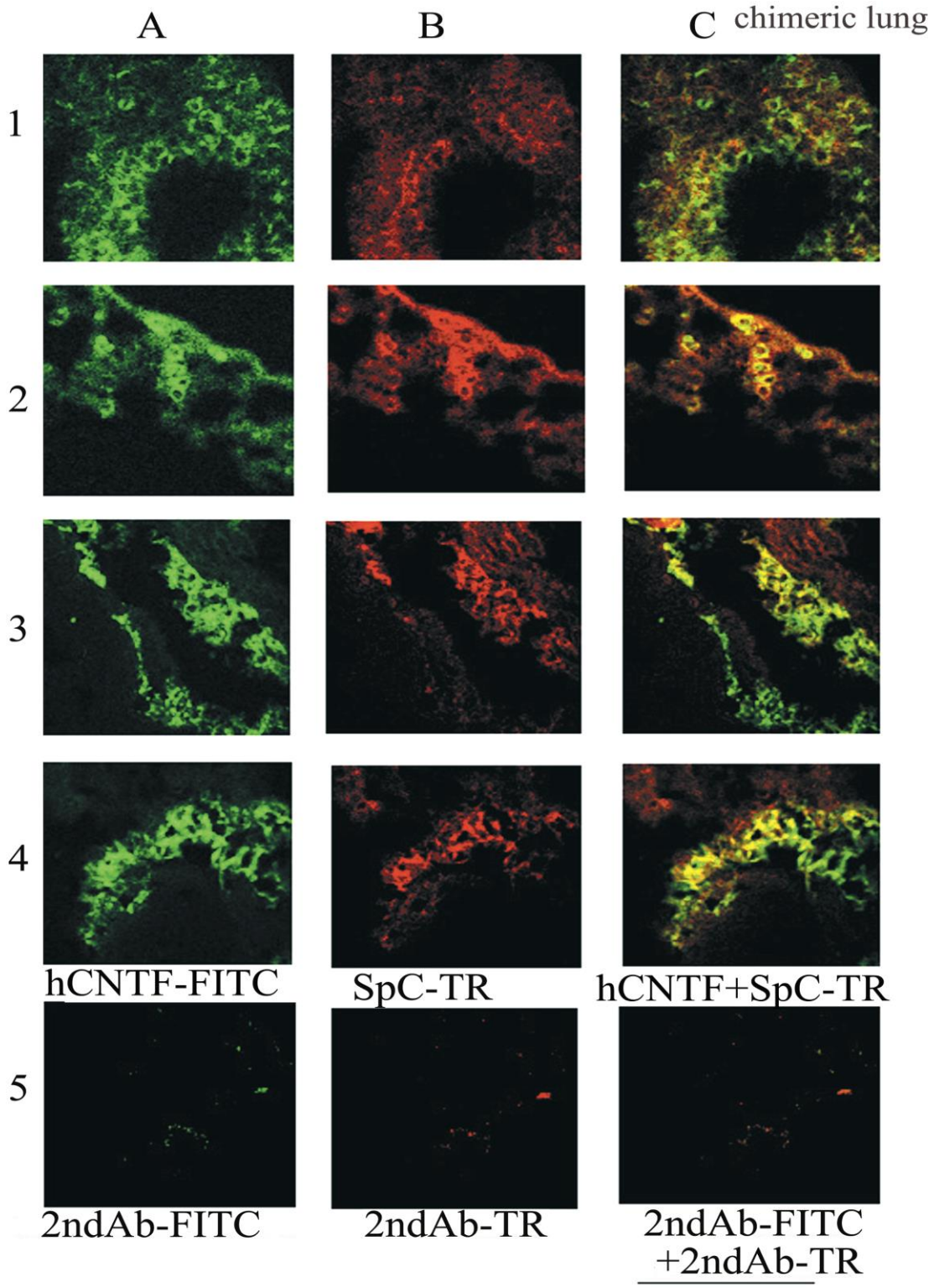

Figure 12 hCNTF-SpC

Figure 12. Immunofluorescent staining of host lung with anti-sSpC after subcutaneous implantation of L14 cells.

L14-7a cells were seeded onto a denuded derma matrix and cultured in a two-chamber culture system, then implanted in immune-deficient mice subcutaneously for 4 weeks. Analysis of fields of alveolus is shown in 4 Panels. Column A: hCNTF-FITC; Column B: sSpC-TR; Column C, hCNTF-FITC & sSpC-TR. In Panel 5 are the negative controls, Column A: 2nd Ab-FITC; Column B: 2nd Ab-TR; Column C: 2nd Ab-FITC & 2nd Ab-TR. 1 bar = 100 μM.

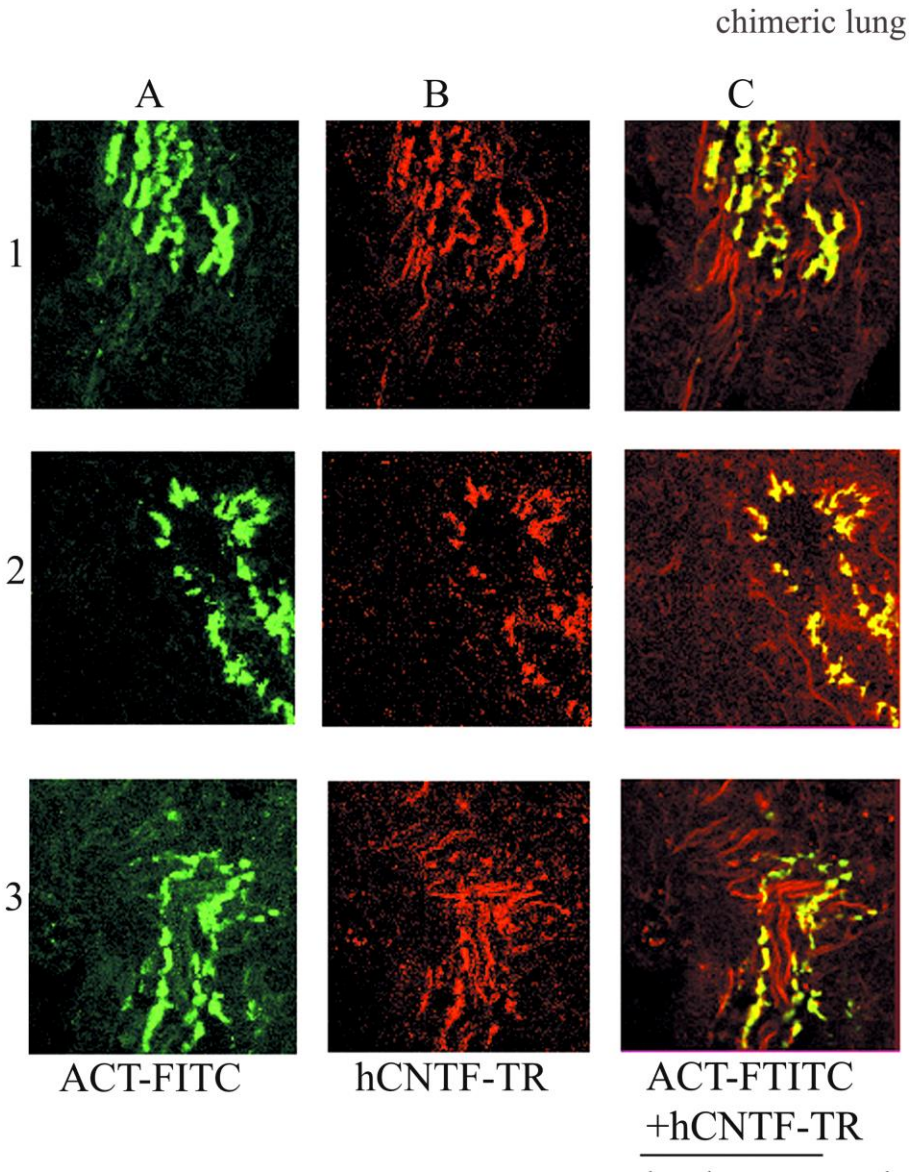

Figure 13, ACT

Figure13. Immunofluorescent staining of host lung with anti-ACT after subcutaneous implantation of L14 cells. L14-7a cells were seeded onto a denuded derma matrix and cultured in a two-chamber culture system then implanted in immune-deficient mice subcutaneously for 4 weeks. Analysis of lung tissues by immunofluorescent typing is shown. Three panels representative of three fields are shown. Column A: ACT-FITC; Column B: hCNTF-TR; Column C: ACT-FITC & hCNTF-TR. 1 bar = 100 μM.

CHIMERIC LUNG TISSUE WAS FOUND AFTER SUBCUTANEOUS IMPLANTATION OF L14 CELLS INTO IMMUNODEFICIENT MICE.

To test whether L14 cells could differentiate further *in vivo*, we performed implantation experiments using nude and SCID-NOD mice; the latter being routinely used for transplantation of human fetal airway[66, 67, 69]. DED matrixes were seeded with 7aL14, an L14-derived hCNTF+ transgenic clone (Figure 9), and cultured for one week in the two-chamber culture system, then implanted subcutaneously, into nude or SCID-NOD mice for four weeks (the same cell types were detected in the lung of both mice used as recipients). The lung tissue and cell-DED matrix were cryopreserved and examined using immunofluorescence microscopy. When the host mice were fed with doxycycline in the drinking water during cell-DED implantation, no cells were detected in the lungs of the 4 mice examined. In the absence of doxycycline, cells were found to grow in situ on the DED matrix (unpublished) and to migrate to the lung. The hCNTF+ transgenic cells integrated into host lung tissue at broncho-alveolar junctions, in areas stained with hCNTF+ CC10+bright, hCNTF+bright CC10-, hCNTF-CC10+bright (Figure 9, Figure 10). These

cell types could be non-club and club cells of donor (7aL14) origin and club cells of host origin. In addition, donor cells occupied the alveoli region and airway. In the alveoli, the staining patterns were SpB+ hCNTF+ (7aL14 origin), SpB+ hCNTF- (host origin) (Figure 9, panel 4a, Figure 11a & Figure 11b); SpC+ hCNTF+ (7aL14 origin), SpC+ hCNTF- (host origin) (Figure 9, panel 5a, Figure 12). In the lining epithelial, ciliated cells of ACT+ hCNTF+ (7aL14 origin), and ACT+ hCNTF- (host origin) were observed (Figure 13). A few cells of 7aL14 origin stained positively for pneumocyte type 1 lectin (not shown).

As mentioned above, expression of the marker CC10 protein in cultured L14 cells is mostly detected in the nucleus (Figure 8). After the subcutaneous implantation and migration into the lung, CC10 protein is detected in the cytoplasm of L14 cells that form normal bronchiolar structures (Figure 9, Panel 1a). while a large fraction of these proteins is localized in the nucleus of L14 cells that form unorganized lung structures (Figure 9, Panels 2a & 3a).

To our knowledge, no cell surface markers have been identified in these rare precursor-stem cells, which would allow their sorting and isolation in sufficient quantity from lung tissue of normal mice to study the nuclear vs cytoplasmic location of proteins such as CC10 inside these cells.

The specificity of the antibodies used in this study has been published by the authors who kindly supplied the reagents[51, 59-61]. Ward et al[61] observed the change in cytoplasmic-nuclear location of the SP protein expressed depending on the carcinogen induced oncogene expressed. In that study, the majority of large papillary adenomas and carcinomas in BALB/c mice exposed to ENU carcinogen and in untreated A strain mice contained SAP in the nuclei of many neoplastic cells but only in the cytoplasm of a few neoplastic cells. CC10 was found in normal club cells of bronchi and bronchioles but not in any hyperplastic or neoplastic lesion of any mouse studied.

In this study, the differences in nuclear vs cytoplasmic location of CC10 might have arisen during the maintenance of L14 cells in long-term culture (more than 36 months). Alternatively, it might be due to the expression of oncogene-SV40 Tag[61], or both.
To obtain a quantitative estimation of chimerism in the host lung tissue, we will perform serological analysis. For technical reasons, it is impossible to perform triple staining of hCNTF, CC10, and SpC for identifying the dual positive cell types located in the terminal bronchiole. However, in the area identified, no CC10+ cells could be found with alveolar morphology, and no SpC+ cells could be found in the airway.
Thus, the data suggest that L14 cells migrated out of the anaerobic subcutaneous DED matrix and seeded the pulmonary region, where they were able to form an organized chimeric broncho-alveolar junction and alveoli. They can be induced to express new markers such as ACT (ciliated cells) and change their phenotype from CC10+dull SpC+, to CC10+bright SpC- club cells at terminal bronchiole or to CC10-SpC+ pneumocyte type II cells in different lung regions. Whether the implanted DED was able to grow in situ depended on the size of the DED. If it is larger than 2 cm3, cells grew despite the inclusion of doxycycline in the drinking water.

Discussion and Conclusion

We have partially characterized a doxycycline regulated cell line isolated from lung tissue of transgenic mice expressing SV40Tag under the control of the tet-off system. Proliferation of these cells is controlled by doxycycline *in vitro* and is therefore primarily dependent on the activity of the SV40 Tag.

This cell line (L14) exhibits multi-lineage properties: it resembles club cells in expressing and secreting CC10 along with a functional CFTR protein, but it also produces and secretes SpA, SpB, SpD, and (pro)SpC, this latter being described as characteristic of pulmonary type II cells. It might be a rare population of cells reported to possess characteristics of both club and pneumocyte type II cells[37, 50]. However, other interpretation is also possible, for example, it can also be due to unknown genetic changes that result in different transcriptomes and proteomes. Also, whether it possesses functional CFTR protein or it produces and secretes SpC of similar sizes of (pro)SpC is not known, and the lineage relationship between this cell population and the L14 cell line remains an open question.

Further characterization of the L14 cell line *in vitro*, such as quantitative RT-PCR type assay, bulk or single cell RNA-Seq, karyotyping and DNA sequencing to validate their state, mycoplasma testing and short tandem repeats (STR) testing, are planned. In addition, *in vivo* characterization of the L14 cell line and some opportunities it might offer for further research are performed and discussed below.

SV40 TAG-DEPENDENT CELL PROLIFERATION AND MIGRATION OF L14 CELLS IS DOXYCYCLINE REGULATED

In this study, we have partially characterized a doxycycline regulated cell line isolated from lung tissue of transgenic mice expressing SV40Tag under the control of the tet-off system. This cell line (L14) exhibits multi-lineage properties. We consider the L14 cell line to be club- and pneumocyte type II-like precursors, and ATC+ ciliated airway epithelial precursors. The proliferative capacity of these CC10+SpC+ cells is controlled by doxycycline *in vitro* and *in vivo* and is therefore primarily dependent on the activity of the SV40 Tag. When the L14 cells themselves, or cells from an L14-derived hCNTF+ transgenic clone, were seeded onto DED and implanted subcutaneously into immune-deficient mice, they migrated to the broncho-alveolar regions of the lung. Next, they were integrated into well-organized chimeric structures in the alveoli and airway lining. The developmental potential of these cells includes the expression of new markers, such as ACT+ (CC10-SpC-) for ciliated cells in the airway and a phenotype change, from CC10+dull SpC+, to CC10+bright club cells at terminal bronchiole, or to CC10-SpC+ pneumocyte type II cells in alveoli of the lung.

DIFFERENCE OF L14 CELL LINE FROM OTHER PULMONARY CELL LINES

Other pulmonary cell lines with club cell[66, human] - or pneumocyte type II-like[71, mouse] properties, and more[86-89] have been reported. The L14 cell line described in this study differs from these cell lines in several aspects:

(i) hESC cells / hiPSC can differentiate in culture into lung cells, among many other tissues. However, no permanent human lung (stem) cell line from these sources can be established so far.

(ii) Proliferation of L14 is under the control of an external signal, i.e., doxycycline; L14 exhibits multi-lineage markers and functions of primary adult respiratory cell types which until now have not been observed in other cell lines, and L14 is the first cell line, to our knowledge, to be isolated from adult mice with club- and pneumocyte type II-like and ciliated airway epithelial cell characteristics. Most other cell lines mentioned are related to either club or pneumocyte type II cell type but not both nor all three or are adenocarcinomas.

(iii) The questions are for how long such primary cells can be cultured (without de-differentiation) and how many cells could be propagated in culture without de-differentiation. In detail, an organoid culture system with TGFβ/BMP inhibitors has been reported for culture human36a and mouse cells isolated from trachea (not lung) tissue[87-89] to generate airway epithelium with high efficiency through passage 12. The mouse trachea contains p63+/KRT5+ basal stem cells, which can self-renew and give rise luminal (KRT8+) secretory club cells and ciliated cells. Mucociliary differentiation remains robust through later passages, although the efficiency gradually declines. Secretory cell production appears to be more stable than ciliogenesis, as airway stem cells at P25 can still generate club cells. After P25–30 (depending on the donor), airway stem cells stop proliferating abruptly. It is also a question whether these cells can survive the freezing and thawing process, as a criteria of cell lines, which was not addressed in that organoid culture system. Another study has also shown that the cultured cells can be engrafted into mice and can be used in cell therapy applications[89]. However, the general observation is that primary (pulmonary) epithelial stem cells de-differentiate with time, even under optimal culture conditions, and these cells are not permanent cell lines.

A CHALLENGE TO THE CONCEPT OF NICHE REQUIREMENT

We have shown that L14 cells migrate out of the subcutaneous DED matrix, which is in the anaerobic environment, and seed into the lung to establish an organized chimeric structure along terminal bronchiole and alveolus. We hypothesize that this reflects the air-trophic nature of this cell line. How the cells migrate to lung and seed at broncho-alveolar junction remains unknown. It is a general belief that stem/progenitor cells proliferate in response to injury, trauma, or selection pressure and that the proliferation and differentiation of stem cells requires a specific microenvironment or niche[67, 68, 72, 84]. In both SCID-NOD and nude mouse systems, we found well-organized chimeric lung tissues with both host and implanted cells. Migration of the cells to the lung cannot be explained by the stimulus from injury or trauma of the respiratory system since the nude mice were not irradiated and their respiratory tracts were undamaged. Instead, we think that it is more likely to be the need to find an oxygen-rich environment that drives the respiratory epithelial cells to seek the air passages of the respiratory tissue. Other interpretations are possible, such as sensing of lung-specific matrix molecules, signaling molecules, or cells.

ALTERNATIVE ROUTE FOR LUNG CELL DELIVERY *IN VIVO*

The treatment of cystic fibrosis and other respiratory diseases has often favored drug

delivery using lipoplexis, aerosols, viral vectors containing transgenes, and soluble molecules introduced into the airway[73-75]. Surgical replacement of the entire lung has also been performed to treat severe cystic fibrosis, cancer and other lung diseases[75-76]. Respiratory epithelial stem cell experiments documented so far have involved either the extra-airway (subcutaneous) implantation of stem cells seeded on denuded trachea in immune-deficient mice[64, 66, 67, 69, 70, 77, 78] or the physical injury of the airway followed by its regeneration by endogenous stem cells[79-81]. To our knowledge, no other successful cell replacement experiment using the introduction of exogenous respiratory epithelial (stem) cells via the airway or any other routes have been reported. Subcutaneous implantation was found to be the optimal route through which to introduce L14 cells into mouse tracheo-bronchia system. It was preferred to delivery via aerosol into the airway, which has been the route of choice in gene/vector delivery because it bypasses the need to inflict life-threatening injury on the airway before implantation. Intravenous injection of L14 cells into SCID-NOD mice was unsuitable because the mortality rate was too high (3 out of 4 injected mice) and the cells failed to migrate and home to the host lungs. This was possibly due to failure of the blood circulation and heart function caused by aggregates of L14 cells, which tend to form clusters of mini-aggregates at 37°C a few minutes after trypsinization into a single cell suspension. However, 38/40 of nude/SCID-NOD mice with subcutaneous implants survived the four week-in vivo assay, making subcutaneous implantation the route of choice.

THE FREQUENCY OF MALIGNANCY DEVELOPMENT *IN VIVO*

We do not know the frequency of malignancy *in vivo*. However, the expression of SV40Tag in the cells does not lead to uncontrollable cell proliferation if doxycycline is applied. L14 cells underwent apoptosis in the presence of low levels of TNFα. This observation is important for predicting their fate in inflammatory surroundings, such as in injury sites, as it suggests that the host defense system may control the proliferation of L14 cells *in vivo* via inflammatory cytokine-induced cell death. We have attempted to isolate cells after implantation and some cells can be re-established from DED tissue after 4 weeks *in vivo* (unpublished). Similarly, we will try to isolate cells from host lung and re-establish cell lines in culture.

SOME PROSPECTS

In this study, we used a transgenic mouse cell line together with a neurotrophic factor, hCNTF, to trace cell fate *in vivo*. Whether hCNTF is secreted and participates in some functions of the host, and whether it is subjected to the regulatory machinery of the transactivator protein of tTA remain to be followed. The capacity of primary club-pneumocyte type II cells to similarly seek the natural lung environment also remains to be investigated, if sufficient primary (rare) cells can be purified for further migration experiments. Whether the L14 cells or 7aL14 transgenic cells alone could contribute to lung function remains to be investigated, and it would be interesting to test whether such cells can proliferate and differentiate fast enough to replace damaged lung tissue in animal models. Among candidate systems are club cell-specific naphthalene caused injury[51], or SpC/club cell-knockout mice[82, 83].

The L14 cell line provides a potentially useful tool for identifying and characterizing novel genes involved in cell division and the various stages of lung morphogenesis. The cell line could already be used for drug screening in culture. It might also be a rich source of cells for tissue engineering, and be applied in the design of protocols for delivery of soluble—molecules to the lung, resulting in the treatment of various diseases in the experimental models.

One implication/prediction derived from this study is that human lung fetal tissue could be transplanted subcutaneously into immune-deficient hosts such as AIDS- or elderly- patients who may also suffer from lung diseases, the implanted fetal lung precursor cells could be trophic to host alveolar-lung tissue to form chimeric alveolar-lung in the host for cell therapy.

Conflicts of Interest Statement / Disclosure Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation

Ethics-license statement for animal experiments

The studies involving genic work and animal experiments were reviewed and approved by institutes committee. S1 and S2 facilities are available in the Medical Microbiology and Biochemistry Institutes of UniGiessen [Aktenzeichen

(S2):32-GT/530 06.05.02G, UniGI1/93, (S1):32 - 53e621-GenA-UniGI 1/90].

For animal experiments with nude mice, the license number is 17a-19c20-15(1) at the Uni. of Giessen, Germany. The maintenance of NOD-SCID mice were performed in the mouse isolator facility of INSERM U506, F94807 Villejuif Cedex, France, under sterile condition, and the experiments followed the institutions guideline[66, 67]. The animal study was reviewed and approved by INSERM U506 ethics committee.

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Author contributions

O.T. performed the measurement of chloride efflux assay. O.T. did the graphic work. C.E., I.Z., with U.C. performed the cell culture, cell proliferation assay, western blot assay, cryo-section preparation and immunostaining, transgenic mice breeding and nude mice implantation experiments. D.A. with E.B. constructed plasmid vectors for transgenic cells expression, primer designs and RT PCR assays. B.P. and colleagues contributed to the SCID-NOD animal work. E.B. B.P. and O.T. contributed to discussions, graphic design and experimental planning. U.C. coordinated the project, planned the experiments, analyzed the data, and wrote the article.

Disclosure statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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