MECP2 is upregulated in alveolar type 2 cells when exposed to cigarette smoke extract.

By Mart Sietsma S3958655   7-6-2022

Abstract

Idiopathic Pulmonary Fibrosis is a chronic inflammatory disease which is associated with high levels of collagen accumulation in the lungs which causes lung tissue to stiffen up and thicken. There is not yet enough evidence to support a conclusive cause for the disease hence the term 'idiopathic'. What is known is that myofibroblasts, which are differentiated from fibroblasts, produce these excessive amounts of collagen when the lung tissue is damaged by for example cigarette smoke and it is known that cigarette also plays a role in altering expression levels of certain genes in alveolar lung cells(6). In a previous study by Biao Hu(2) *et al.*, the role of the MECP2 gene was elucidated to be playing an important regulatory role in this differentiation process. The MECP2 gene is expressed in the lung epithelial cells and regulates alpha smooth muscle actin which drives fibroblasts differentiation(2).

In this study, alveolar type 2 cells were tested for MECP2 expression under different levels of cigarette smoke exposure. A SA-β-gal activity staining was done to find out the proportion of cells that went senescent after the cigarette smoke exposure at different levels. Following up was a qPCR to measure the expression level of MECP2 in these cells. The data found in this study point towards the conclusion that expression of MECP2 is upregulated at higher concentrations of smoke exposure to a certain point where cells were dying off and could not express anything anymore.
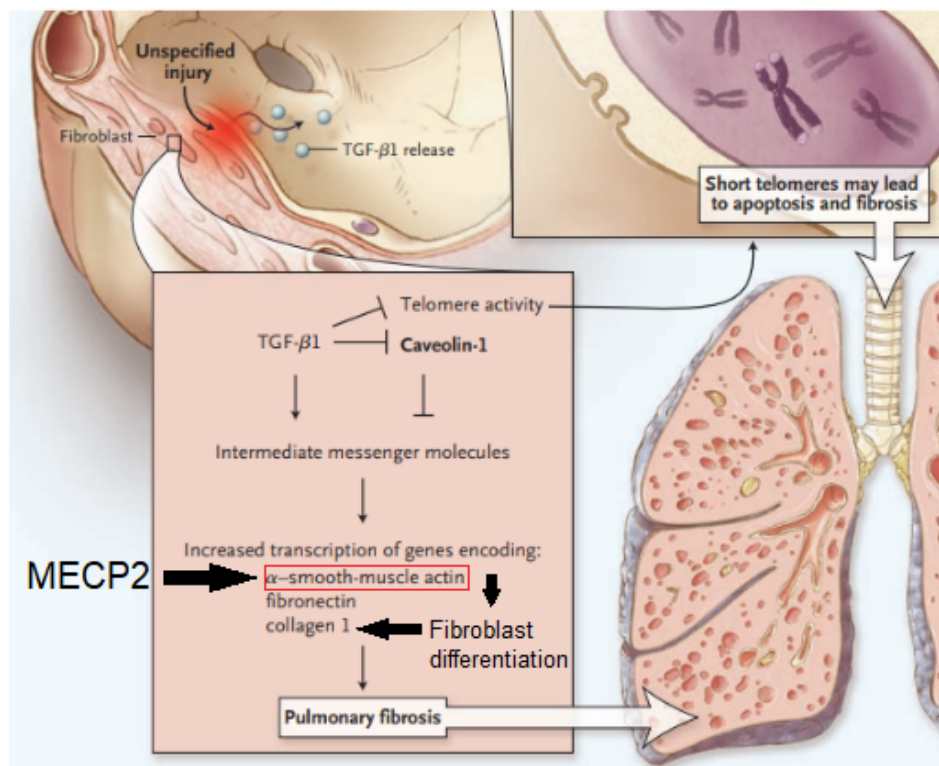
Introduction

Idiopathic pulmonary fibrosis (IPF) is a serious condition which in the Netherlands alone is estimated to be afflicting around 15.000 people of which 10% has been diagnosed according to the ROCHE health institute(1). Every year, an extra 500 people are being diagnosed with IPF and there is currently little to no treatment available. The exact cause of the disease remains unknown hence the term 'idiopathic' but it is believed that certain environmental factors and exposures increases the risk such as smoking or inhaling other damaging substances(4). The disease is characterized by the stiffening and thickening of lung tissue because of its effect on the formation of scar tissue. It has been hard to diagnose people with IPF since many of the symptoms of IPF are very similar to those of other lung diseases. People who suffer from this disease usually are very short of breath and develop a dry cough. The age of the patients who develop IPF lies around 50 or so years and there are also a lot of other smaller symptoms which are commonly present in other diseases as well such as tiredness or hypertension(3). The only real solution for patients with IPF right now is a lung transplant which comes with enough complications as it stands and is not a reliably present treatment option due to large waiting lists and limited availability of donor lungs.

Even though the cause of IPF is not exactly known, there are elucidated mechanisms that have been found which might contribute to the pathology of IPF. In IPF epithelial lung cells play a very important role by expressing certain factors or genes which seem to drive this disease(7). Most of the dysregulation seen in epithelial lung cells to express these genes and factors come from damage to these cells on a long term basis(6). A thought leading cause of IPF is the increased amount of cell senescence in the lung epithelial cells which activates damage sensing signaling pathways since they are no longer able to proliferate but remain metabolically active(8). Eventually this will lead to continuous damage repair and accumulation of collagen by myofibroblasts being activated by these senescent cells(2).

For this research paper, we investigated alveolar type 2 cells as it is believed that these cells and more importantly the dysfunctioning of these cells plays an important role in the pathogenesis of IPF. When the lung epithelial cells are constantly under pressure from damaging agents such as cigarette smoke the ability of the lungs to repair themself becomes altered which involves an excessive and abnormal amount of collagen accumulation. This collagen accumulation is the result of fibroblasts differentiating into myofibroblasts which secrete the collagen. In a previous study by Biao Hu(2) et al. they found that the MECP2 gene plays an important regulatory role in fibroblast differentiation in a way that when MECP2 was suppressed the alveolar wall thickness, inflammatory cell invasion and collagen accumulation is significantly decreased. This led to us being interested in the expression of this MECP2 gene when it is treated with different levels of smoke.

Therefore the aim of this study was to find out how MECP2 was expressed in alveolar type 2 cells after different levels of cigarette smoke exposure.

Materials & Methods

Dataset filtering & gene selection for ou research

To select our gene of interest for our research, we analyzed a gene expression dataset from the Lung Tissue Research Consortium - also described by Tan et al. (2016) - featuring 134 patients with IPF, 48 ILD patients without IPF and 108 controls on specific parameters. More details on this dataset are also available in the Gene Expression Omnibus database (accession number GSE47460). For this dataset, IPF lung fibroblasts were obtained from patients' lungs at explant or autopsy. Lung fibroblasts of the control group were obtained from donor lungs that appeared to have no injury by histology, but that were rejected for lung transplant.

We used Rstudio version 4.2.0, PBC to filter the LTRC dataset on specific parameters. Data of De Vries et al. (2018) was used to make a selection of genes that are associated with change with age, resulting in one list of genes increasing with age and another list of genes decreasing with age. The lists were adjusted for the new number of subjects. A selection of IPF patients was created from this list. Age and gender were added as variables to exclude influence of age and gender. Both lists of genes 'increasing with age' and 'decreasing with age' were analyzed on variables age, gender and IPF, resulting in two lists with numeric estimates of genes 'increased in IPF' and genes 'decreased in IPF'. Both lists were adjusted for multiple testing by applying FDR testing (Benjamini-Hochberg). Another subanalysis was run to find genes increasing with smoking in IPF by running an analysis on the 'increased in IPF' list with variables age, gender, IPF and smoking. MECP2 -a gene with high expression increase in smoking that is expressed in alveolar type I and type II cells (expression levels >0.10) according to the 'Gene explorer' function of the online IPF database of Kaminski and Rosas (2022)- was finally selected for the lab portion of our experiment.

Primer design
The primer was based on our gene of interest which we found on the NCBI database. Because we planned to run a qPCR, the primer ideally had to be around 70-200 base pairs long with an annealing temperature around 60 degrees. The primer melting temperature should then be around 62-63 degrees as the annealing temperature of the PCR reaction is roughly 3-5 degrees lower than the primer pair. The minimum temperature was set at 59 degrees, optimal temperature at 62 and maximum temperature at 65. Furthermore, we included introns and no preference for the exon junction span. The output of the primer blast gave us the following primer which complied to the aforementioned criteria: Homo sapiens methyl-CpG binding protein 2 (MECP2), transcript variant 8, mRNA. This primer was selected to help measure the expression of MECP2 in qPCR. We then ordered our primer from the company Invitrogen.

Cell culturing
The Cells that were used for the experiment were cultured via two plates that had been seeded with A549 alveolar lung cells, which were cultured according to the general culture protocol. One plate was prepared with a 10,000 cells/mL concentration and another with a 50,000 cells/ml concentration.

CSE stimulation
Dilutions of CSE from University of Kentucky cigarettes of 0%, 5%, 10%, and 20% were prepared with the goal of exposing our cells to different concentrations of CSE extract. The smoke extract dilutions were pipetted onto the cells of both the 10.000 cells plate and 50.000 cells plate. The 0% CSE served as a control. See appendix B for details.

Senescence associated-β-gal activity staining
In order to identify senescent cells due to the smoke extract exposure, the 10.000 cells per well plates were prepared for β-gal activity staining. The SA-β-gal makes it possible to observe prescenecent and senescent cells under the microscope based on an increased level of lysosomal β-galactosidase. The cells were fixated with a Fixation solution (see protocol in appendix C), and subsequently treated with a Staining solution (see protocol in appendix C). After incubating the cells overnight and following the rest of the protocol (see appendix C), the cells were observed under the microscope and counted.

RNA harvesting and isolation
For eventually creating the cDNA needed for the qPCR, an RNA isolation was performed. The 50.000 cells/mL samples were used to isolate the RNA in accordance with the TRIzol sigma protocol (appendix D). After acquiring the final pellet, a nanodrop was used to read out the RNA yields of the samples.

cDNA synthesis
After the RNA isolation, the RNA samples were used to create the cDNA necessary for the qPCR which was done following the steps of the cDNA synthesis protocol (appendix E).

qPCR
The goal was to measure the mRNA expression using qPCR and find out whether our target gene was being up- or downregulated after the varying quantities of smoke treatment. The qPCR was done in accordance with the provided qPCR protocol (see appendix F).
After preparing the samples for the qPCR, they were run through the ViiA7 machine to start cycling. See appendix F for the protocol.

Results

β-gal staining

The data obtained in the β-gal staining was sourced from the A549 alveolar lung cells when they were induced with β-galactosidase to produce X-gal which colors blue.
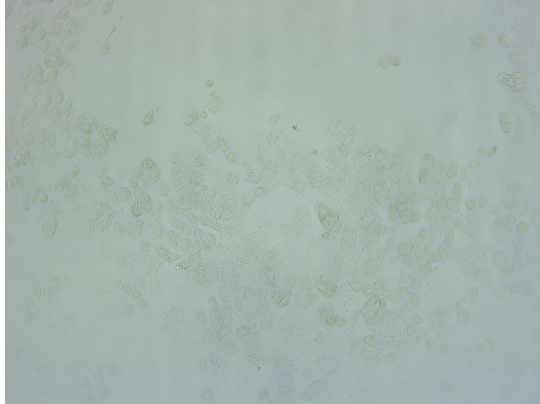

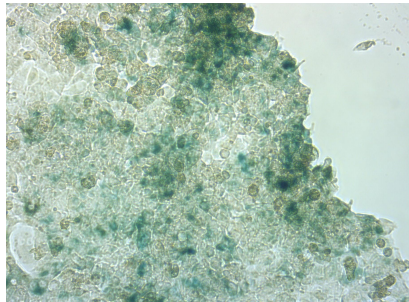*Figure V: Example of mostly healthy cells at 5% CSE exposure*

Figure t: Example of observed senescent cells at 10% CSE exposure

It was observed that the total number of cells had decreased drastically at 20% CSE exposure. In 20% CSE exposure, the absolute number of senescent cells is low, though senescence is quite high proportionally (37,3%). Senescence is especially high at 10% CSE, both in absolute number of cells as well as proportionally to the total number of cells.

When pictures were taken before and after the cigarette smoke stimulation only slight morphological changes could be observed with the cells being more clumped and appearing to be less diverse in shape(appendix G).
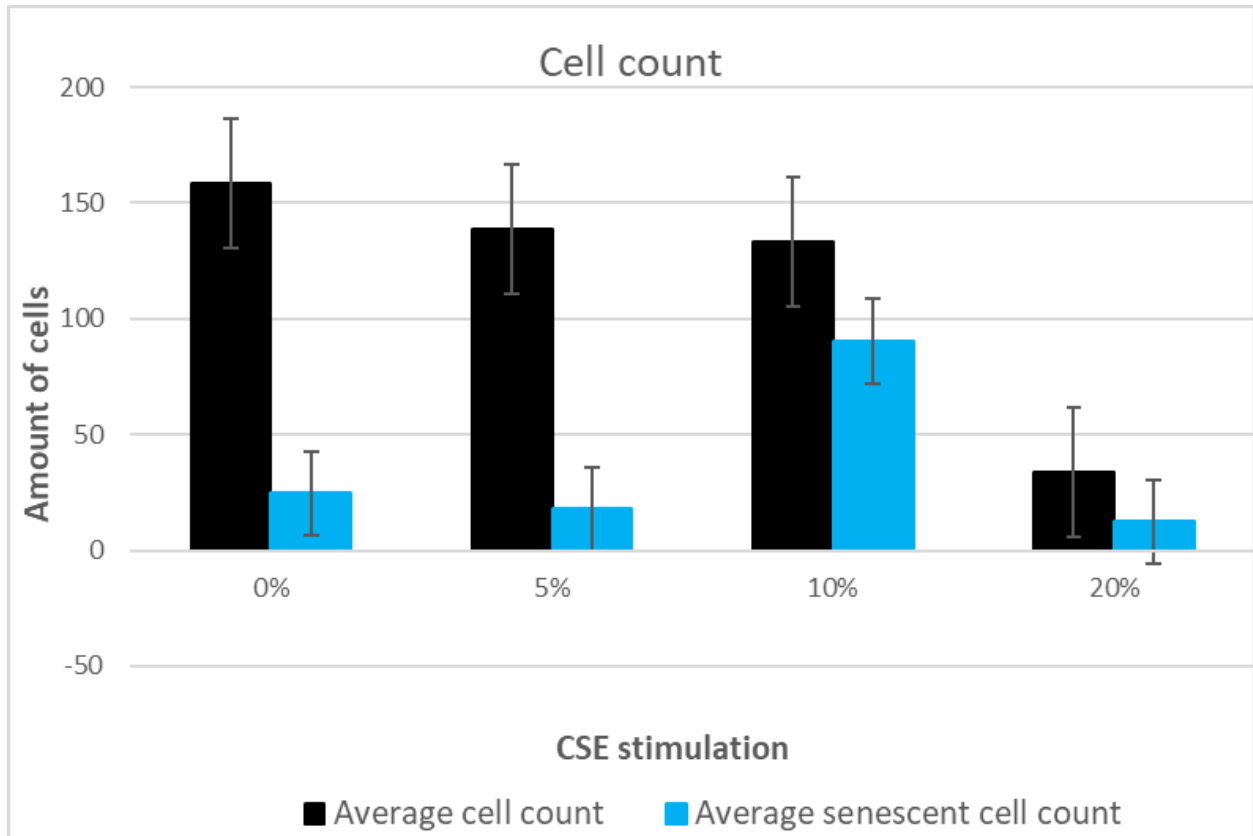
Figure C: Graphical results of cell counting and β-gal staining, it presents the cell count and average percentages of blue staining observed in our cell wells under bright-field microscopy. Blue staining indicates cell senescence. The 0 CSE serves as a control, and 5, 10 and 20 indicate different quantities of smoke induced to the cells.

The ANOVA-test (P-value <0.05) that was performed on our β-gal activity staining resulted in a P-value of 0.35. 0.35 > 0.05, therefore, we cannot say that there is a significant difference in the amount of senescent-stained cells between the CSE 0%, 5%, 10% and 20% groups.

Nanodrop data

Table D presents the RNA concentrations in our samples after RNA isolation and RNA purity. cDNA synthesis sample concentrations were adjusted accordingly. A260/230 values were quite low for sample CM0 and CM5 (<1.7), but the qPCR proceeded either way.

Parameters for the nanodrop:

        Method   Nucleic Acids Nucleic Acid Factor 40.00
        Type   RNA Background Correction 320 nm
        Mode   NanoVolume Air Bubble Recognition Off
        Volume(ul) 1-2 Manual Dilution

Table D: Nanodrop data results for samples

	Name	Conc.	A230	A260	A280	A320	A260/280	A260/230
1	CM0	48.920 ng/ul	0.770	1.231	0.795	0.008	1.554	1.605
2	CM5	60.120 ng/ul	2.255	1.504	0.966	0.001	1.558	0.667
3	CM10	109.08 ng/ul	1.091	2.754	1.852	0.027	1.494	2.563
4	CM20	9.640 ng/ul	0.116	0.262	0.187	0.021	1.452	2.537

qPCR

Figure F presents the melt curve plot for the qPCR of our six MeCP2 primers. The plot has two peaks, with the latter one being significantly higher than the other. The large peak indicates the amplification of our primer. The smaller peak indicates the amplification of a second product, which is not desired in this experiment.
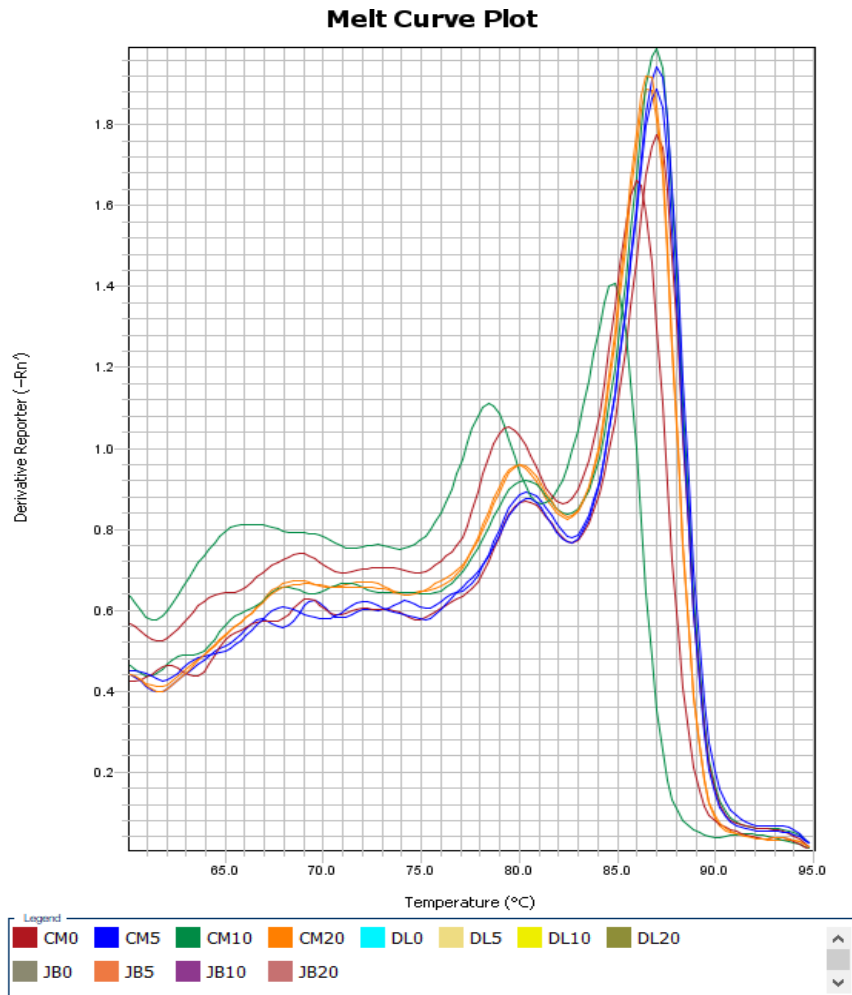

Figure F: Melt curve plot of MeCP2 primer obtained from QuantStudio showing the results from the duplicate groups as well as our own.
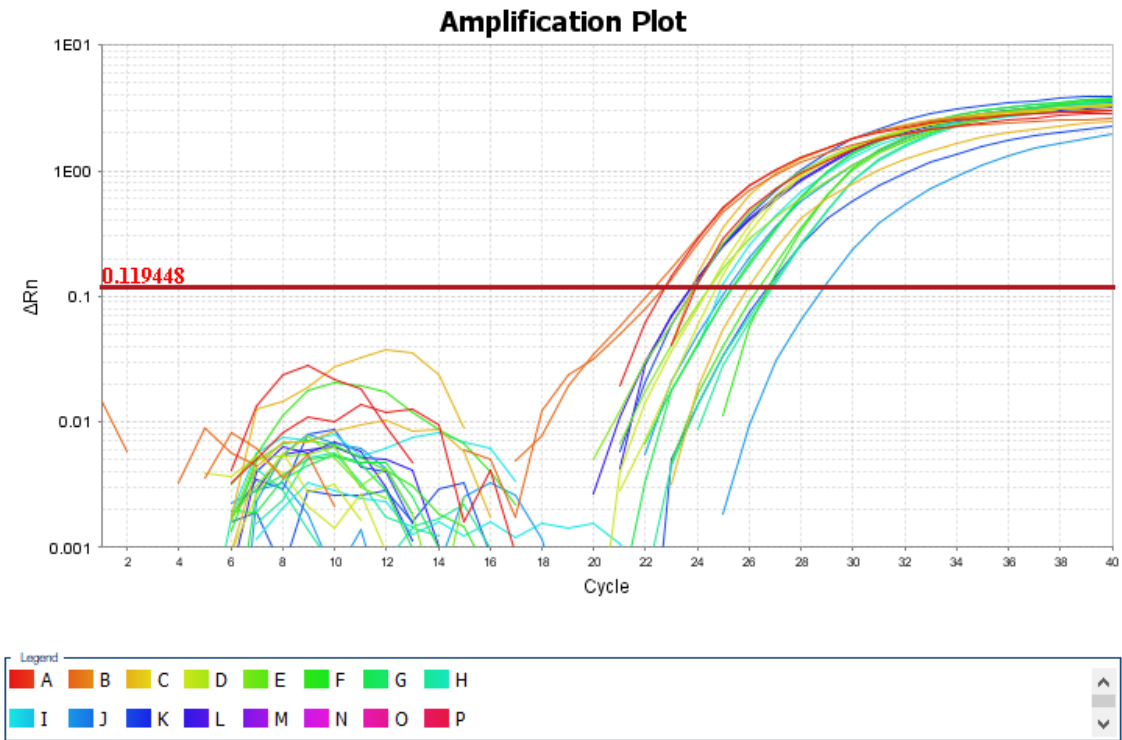
Figure G: Amplification plot from our MeCP2 primers in QuantStudio

Figure G presents the Amplification plot from our MECP2 primers. Most Ct values range between 22-27, which indicates that MeCP2 was present in our samples.
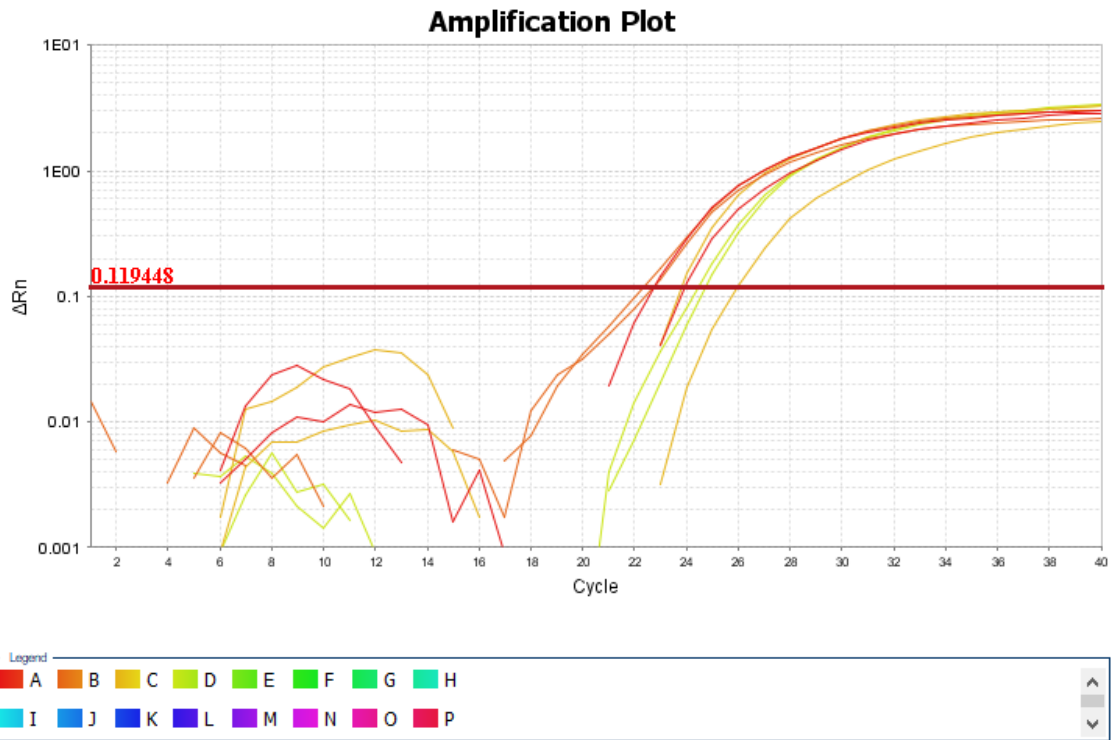
Figure K: *Amplification plot from our MeCP2 pipetting samples in QuantStudio*

Figure K presents the MeCP2 primer samples that our research duo has pipetted specifically. Here, Ct values range between 21-25.

Figure x and y present the Ct values of the housekeeping genes through different CSE concentrations. These Ct values of the housekeeping genes served as an example for 'base expression'. The graphs show that housekeeping genes were present in large quantities throughout all CSE treatment concentrations.
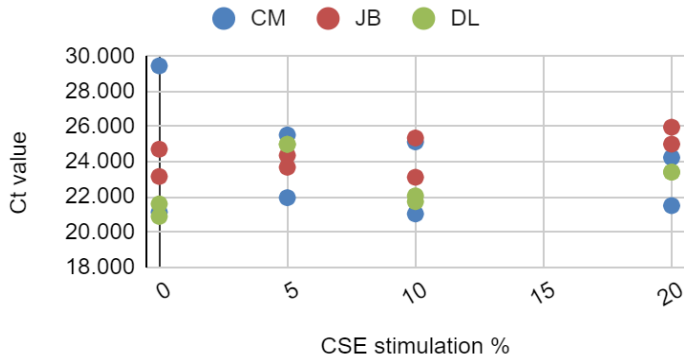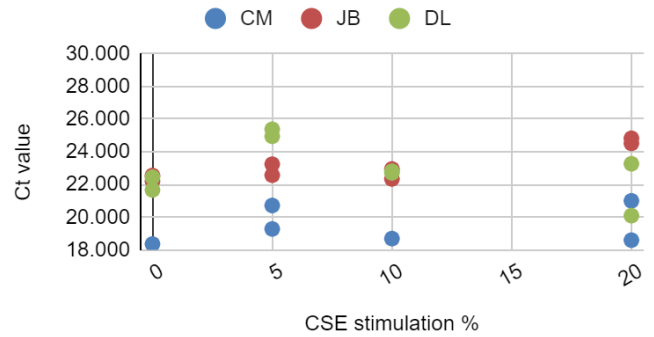
B2M

RSP9

Figure X

Figure Y

When we did an ANOVA test on the significance of the housekeeping genes we found that there was not a significant difference in expression of the housekeeping genes with P>0.05.

Derived from the amplification plot data, Figure D panel A Below presents the calculated fold change of the MeCP2 gene compared to the housekeeping genes B2M and RSP9 as a result of different CSE stimulations. Figure D panel B presents the average of these duplicates. Noticeable is that especially for 5 and 10% CSE stimulation, there seems to be a fold change increase of MeCP2.
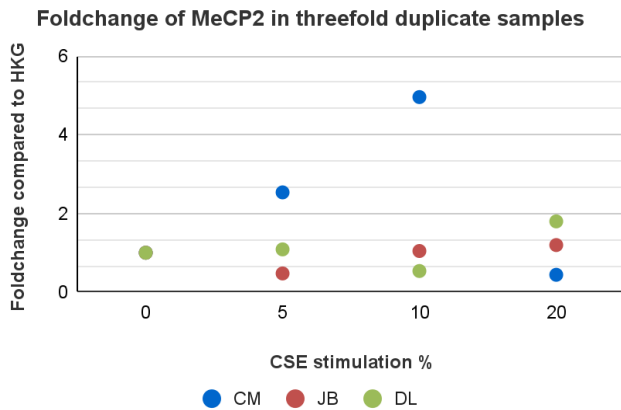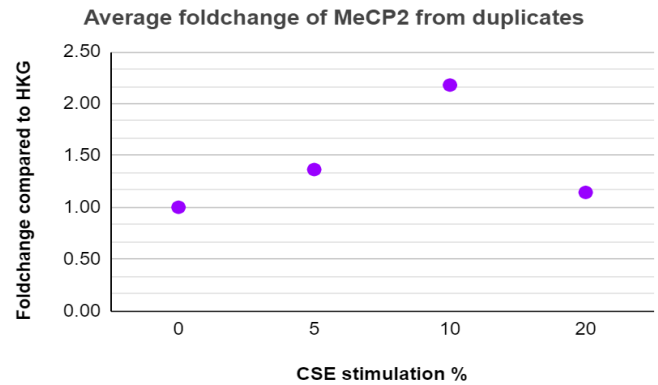

Figure D Panel A

Figure D Panel B

Figure D panel A: Fold Change of MeCP2 calculated from qPCR results The different colors in the graph represent the different duplicates of the same experiment as genes were tested threefold   *Figure D panel B: Average Fold Change of MeCP2*

ANOVA						
ce of Vari	SS	df	MS	F	P-value	F crit
Between	2,4975	3	0,83250868	0,44729677	0,72598196	4,06618055
Within G	14,89	8	1,86119983			
Total	17,387	11				

When looking at the data we can see that there is an increase of fold change at the 5-10% CSE exposure levels but when we did a single factor ANOVA test the change was not significant with P-value set at 0,05.

Discussion

This study which focused on the MECP2 gene expression in alveolar type 2 cells highlights the reaction of these cells to cigarette smoke exposure via a β-gal activity assay and a qPCR.

MECP2 gene expression has been found to play an important role in differentiation of fibroblasts into myofibroblasts(2). In this study, we have investigated the expression levels of this MECP2 gene under different levels of cigarette smoke exposure in A549 alveolar type 2 cell line. We were able to measure the expression levels of MECP2 using a qPCR on alveolar type 2 cells which underwent CSE stimulation. When pictures were taken before and after the cigarette smoke stimulation only slight morphological changes could be observed with the cells being more clumped and appearing to be less diverse in shape(appendix G).

We performed a SA-β-gal activity staining to measure the quantity of cells and then the number of senescent cells among them. What was immediately apparent from this assay was that the total number of cells was far lower in the 20% cigarette smoke dilution group which could be explained by an increased number of cells dying at those levels of smoke exposure. This resulted in the initial number of senescent cells being lower as well but proportionally it was still significant in comparison to the control group. The overall trend showed that with increasing cigarette smoke concentrations the amount of senescent cells increases as well. Only at 5% cigarette smoke exposure is the relative percentage of senescent cells lower than the control group and we think this could be explained by a human error margin in the counting of the cells. The photos obtained were based on where we saw the highest number of cells and the counting had to be done by hand. We do believe that the results are accurate enough but we speculate that it might also have something to do with the cells simply needing a higher concentration of smoke extract to show any significant amount of senescence appearing among them. The highest percentage of senescent cells is found to be at 10% smoke stimulation, where the total amount of cells is still high and senescence is found in about ⅔ of all the cells.
In account of this big leap from 5% to 10% stimulation it would have been interesting to see results in more gradual steps as to get a better understanding of where the absolute cut-off point would be for this significant increase. This would show us more clearly how MECP2 is expressed in a more detailed manner.

Next was to run a qPCR for our samples to measure the expression of the MECP2 gene. We saw in the nanodrop data that the A260/230 values were quite low for sample CM0 and CM5 (<1.7), but that should not influence the outcome drastically. In figure F, the melting curve plot is shown from the qPCR which shows 1 small and 1 large peak. The small peak can be explained by the fact that our primer was relatively similar to another primer which was 50 base pairs longer. For this experiment we had no extra time to optimize the qPCR to remove this extra peak by doing extra runs and altering the parameters to acquire a more clean melting curve. The extra peak is not significant enough to affect the amplification plots. For the amplification plots our CT-values generally were around 21-27 and the duplos were pretty close to each other which tells us that there were not any big mistakes made during the pipetting of the samples. We also ran two housekeeping genes with our gene of interest to measure them against each

other and use them as a baseline. The fold change in the expression levels of MECP2 are represented in figures D and E which shows us comparable results to our SA-β-gal activity staining where we see a high peak at 10% and a decrease at 20%. The decrease at 20% smoke exposure could be explained by the relatively low amount of cells seen in the SA-β-gal activity staining with a high proportion of senescence which could have an impact on the ability of the cells to express MECP2. What is interesting to see is that there is a higher fold change occurring at the 5% level in figures D and E. In general the MECP2 expression levels were not significant according to our ANOVA test but the huge spike at 10% CSE does show a huge spike and we speculate that the increase in MECP2 expression does have some relation to the amount of cigarette smoke extract since we also saw more senescence in cells with the same percentage of exposure which triggers the damage response from the epithelial cells to express signaling molecules and perhaps also MECP2. It would certainly be in line with the paper by Biao Hu(2) et al. which argues that a decreased expression of MECP2 also reduces the induction of senescence in cells.

The findings in this study support our hypothesis that MECP2 expression in alveolar type 2 cells is affected by cigarette smoke exposure and in this case we saw from the fold change plots in figures D and E that MECP2 is upregulated. Because of time limitations we could not do as many trials as we wanted to but the general outcomes of our assays all point in the same direction nonetheless even though the statistical analysis did not show significance. A follow up research which we thought would be interesting is finding out more about the relationship between MECP2 expression and senescence in cells which plays a major role in the pathology of IPF. One of the biggest problems of IPF is not being able to diagnose people properly and perhaps by using MECP2 as a biomarker could prove to be interesting in future research.
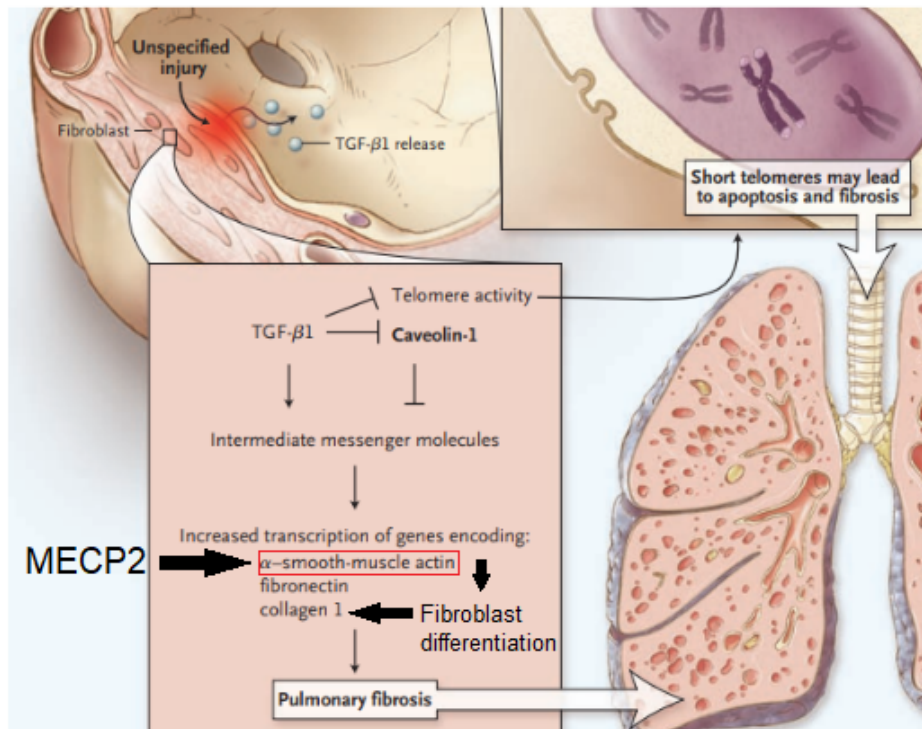
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Appendices

Appendix A

Protocol: General culture protocol   **EXPIRE-CEL-01 Update 2016 JN**

1. Title: General culture protocol: culturing strains of epithelial cell lines

2. Goal: Culturing epithelial cells for research aims.

3. Introduction / Background information

4. Materials

Reagens / media	Composition of the solutions	Storage	Supplier	Art no.	Danger symbols
FCS	Hospital batch Complement inactivated	-20°C Nr 11			None
Trypsin / EDTA (TEP)	97.5ml PBS + 2ml 10x Trypsin + 0.5ml EDTA	-20°C Nr 11	Homemade		
Trypsin 10x		-20°C MLII	MP Biochemicals	0916893	None
EDTA	100mM in PBS	-20°C MLII	MERCK	108418	H332, H373 R20 / R48/20
HBSS	Hank's Balanced Salt Solution, without Ca and Mg	4°C CR	LONZA	BE10-543F	None
BSA cell culture tested	Albumine fractie V 10 mg/ml PBS, 022µm filter	4°C prim	Sigma	A2058-1G	None
PureCol^{TM}	3 mg/ml collagen solution	4°C prim	CellSystems	5005-B 100ml	None
Fibronectin	0.1%, from bovine plasma	4°C prim	Sigma	F-1141-5MG	None
P / S	10.000U/ml Penicilin 10.000µg/ml Streptomycin	-20°C Nr 11	GIBCO	15140-122, 100ml	H317 H334 H361
UltraGlutamin	200mM	RT	LONZA	BE17-605E	None
BEBM	Bronchial Epithelial Cell Basal Medium	4°C CR	LONZA	CC-3171	None
BEGM	Single Quots (bullit kit)	-20°C Nr 11	LONZA	CC-4175	None
EMEM	Minimum Essential Medium Eagle with L-Glutamine	4°C CR	LONZA	BE12-611F	None
RPMI 1640	With L-Glutamine with 25mM Hepes	4°C CR	LONZA	BE12-115F	None

CR = cold room **prim** = primary culture room **RT** = room temperature

5. Equipment/ Configuration

Safety cabinet (down-flow cabinet) with a closed suction system. CO2 incubator Centrifuge Inverted microscope

6. Methods

Work according to the laboratory safety regulations.
When appropriate work in a fume hood or flow cabinet.
Every action / observation / deviation must be written down in the laboratory notebook.

Subculture the cells at ca. 90% confluence, twice a week.
Remove the medium and wash the flasks with the cells 2x with pre-warmed HBSS medium or PBS (for 16HBE 5 minutes per wash)
Remove the medium and add trypsin / EDTA (1ml for 12,5cm2 and 25 cm2 flasks and 3,0 ml for 75cm2 flasks)
Incubate in the CO2 incubator for ca. 5 till 10 minutes.
Check with the inverted microscope if the cells are completely detached.
Inactivate the trypsin / EDTA by adding an equal volume of FCS.
Harvest the cell suspension in a 15 ml or 50 ml Falcon tube and centrifuge 5 minutes at 500g at RT.
Resuspend the pellet in a known volume, count the cells with the Coulter Counter and seed in a ratio (see table) or seed for your experiment.
The culture was diluted in such a way that there were $1*10^6$ living cells per ml. Afterwards, two dilutions were made: a 50µl A549 culture and 10µl A549 culture. Two 24-well plates were seeded with the A549 cells, one plate with the 50µl dilution and another with the 10µl dilution. For each plate, 8 wells were filled with cell culture.
The plates were then incubated at 37°C for approx. 20 hours. After 20 hours, the cells were washed with growth medium and serum deprived and stored for another 4 hours in the incubator at 37°C before the CSE stimulation.
Throughout several steps, the presence of cells was checked under the microscope.

Cells	Medium*	Ratio	Coating	Restriction
16HBE	EMEM	1:5 - 1:10	Purecol overnight	ML-I for culture, ML-II when transfecting or siRNA
Beas 2B	RPMI	1:10	Purecol overnight	ML-I
A549	RPMI	1:10 - 1:40	None	None

*Add to the media 10% decomplemented FCS 1% Pen / Strep 1% Ultra Glutamine

7. Processing of the results and reporting

Processing of the results and reporting depends on directives, agreed at the beginning of the concerning project.

Appendix B – CSE stimulation protocol

Preparation of plates (4 hours before exposing to CSE):
   - serum deprive plates
   - Incubate plates for 4 hours at 37℃

Making Cigarette Smoke Extract (CSE). **EXPIRE-CEL-08** Update 2016 AF
1. Title: Making Cigarette Smoke Extract (CSE).
2. Goal: Making Cigarette Smoke Extract (CSE).
3. Introduction / Background information

4. Materials

Reagens/Media	Composition of the solutions	Storage	Supplier	Art no.	Danger symbols
Culture medium e.g. RPMI		RE			
Research Cigarettes		RE/RT	University of Kentucky		

RT = RoomTemperature (18-25°C) CC=Chemical Cabinet

5. Equipment/ Configuration

Peristaltic pump Watson & Marlow 603S (Inventnumber: 95-000-3251)
Extended with 3 pumpheads.
Research cigarettes (University of Kentucky)

6. Methods
Work according to general Lab safety regulations, write down every deviation in your laboratory notebook.

Creation of 100% CSE (approx. 3 hours after incubating plates):
  ☐   Cut off the filter of 2 cigarettes (e.g. with a scalpel)
  ☐   Cut off two cm of the tip of a 1ml pipettip.
  ☐   Insert the cigarette in the pipettip and connect the tip to the end of the tube. ☐
Insert the other end of the tube (connected to a rubber stop with 10ml plastic volume-pipets). ☐   Insert

 the stop in a 50 ml greiner-tube with 25 ml medium.

- ☐ Start the pump and set the speed at 70 rpm.
- ☐ The cigarette is smoked in approx. 5 minutes.
- ☐ Smoke 2 cigarettes in 25 ml medium
- ☐ 2 cigarettes in 25 ml medium is considered 100% Cigarette Smoke Extract.
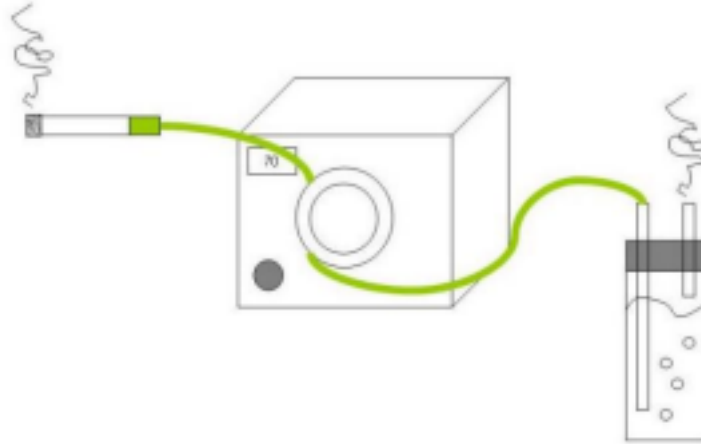
Update 2016   AF

Literature / extra info
Cigarette smoke-induced blockade of the mitochondrial respiratory chain switches lung epithelial cell apoptosis into necrosis. van der Toorn M, Slebos DJ, de Bruin HG, Leuvenink HG, Bakker SJ, Gans RO, Koëter GH, van Oosterhout AJ, Kauffman HF. Am J Physiol Lung Cell Mol Physiol. 2007 May;292(5):L1211-8. Epub 2007 Jan 5.

- Use the 100% CSE extract to create a CSE dilution series in separate tubes according to table below

CSE dilution series

CSE %	CSE extract added in mL	RIPM Growth medium in mL	Total mL
0	0,00	5,00	5
5	0,25	4,75	5
10	0,50	4,50	5
20	1,00	4,00	5

- The plates must have been serum deprived and incubated for 4 hours at 37°C before proceeding
- After incubation, remove leftover growth medium from the plate wells

- Pipette CSE dilution into plate wells according to the scheme below. Two of the wells on both the 10.000 cells plate and the 50.000 cell plate are treated with the same CSE concentration, as a duplicate of each other.
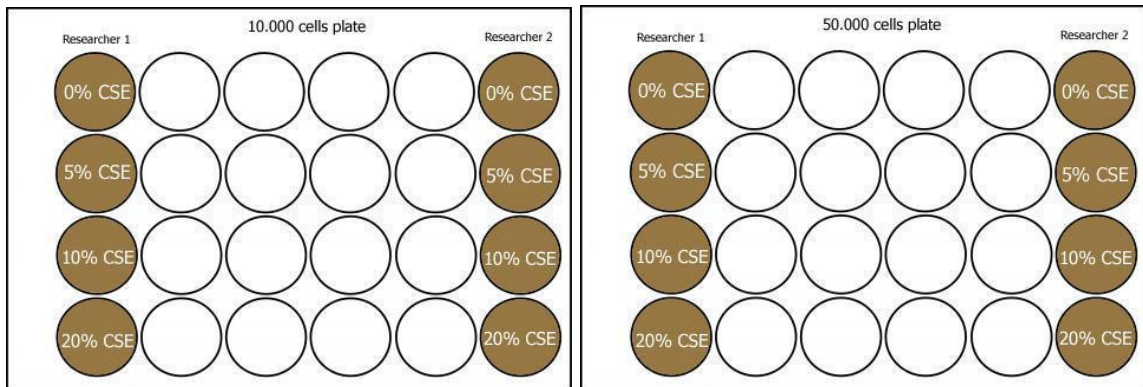
- Incubate plates for approx. 24 hours at 37°C.

Appendix C

SA-β-gal activity staining protocol

Materials:

- PBS+/+ (PBS + 1mM $CaCl_2$ + 0.5mM $MgCl_2$)
- Fixation solution (fresh): 2% formaldehyde + 0.2% glutaraldehyde in PBS+/+
- Staining solution:
    - Water
    - 0.2M citric acid/Na phosphate buffer (pH = 6.0 à very important)
        - 36.85ml of 0.1M citric acid solution (mw=210)
        - 63.15ml of 0.2M Na dibasic phosphate solution (mw=142)
    - 100mM potassium ferrocyanide (mw=422) (defrost from -20°C) (keep in the dark)
    - 100mM potassium ferricyanide (mw=329) (defrost from -20°C) (keep in the dark)
    - 5M NaCl (mw=58.4) (4°C)
    - 1M MgCl (mw=203) (4°C)
    - 20 mg/ml X-gal in DMF or DMSO (make fresh every time, light sensitive)
- 70% glycerol in PBS+/+

Preparation of plates before staining (approx. 48 hours before starting SA-β-gal activity staining protocol):

- Wash wells on the 10.000 plate to remove CSE
- Add 10% FCS growth medium to the wells
- Incubate 10.000 plate at 37°C for approx. 48 hrs to let the cells grow again after smoke exposure.

Preparation of fixation (fresh):

1. Make or defrost 2% paraformaldehyde (Stored -20C)
2. Add glutaraldehyde (25% à 0.2% = 125x dilution) (Use within 1 week)

Preparation of staining solution (20ml):

Volume/ Component	20ml	15ml	10ml	5ml
Water	12.4ml	9.3ml	6.2ml	3.1ml

Buffer	4ml	3ml	2ml	1ml
K ferrO	1ml	750ul	500ul	250ul add as last
K ferri	1ml	750ul	500ul	250ul add as last
NaCl	600ul	450ul	300ul	150ul
MgCl	40ul	30ul	20ul	10ul
X-gal (fresh)	1ml	750ul	500ul	250ul

Staining protocol:

1. Wash cells 2x with PBS+/+ (1ml) (Use PBS with Cal2 and MgCl2)
2. Add 500ul fixation solution to each well (12-wells plates) and incubate for 5 min (longer will affect staining) at RT
3. Aspirate fixation solution and wash 2x with PBS+/+ (1ml)
4. Add 500ul staining solution to each well (12-wells plates) and incubate overnight (± 18h) at 37°C in an incubator without CO_2
5. The next day, aspirate the staining solution and wash 2x with PBS+/+
6. For storage, add 500ul 70% glycerol and store at 4°C
7. Optional: counterstain nuclei with hematoxylin (1min + 1min Tap water) or DAPI (0.1-1ug/ml + 0.1% Triton X-100)
8. Capture min. 4 images per well (between the center and the edge) with U-lab mic or TissueFaxs
9. Count total and positive cells to calculate percentages of positive cells

Human Fibroblasts, Stained for SA-Bgal

Presenescent   Senescent
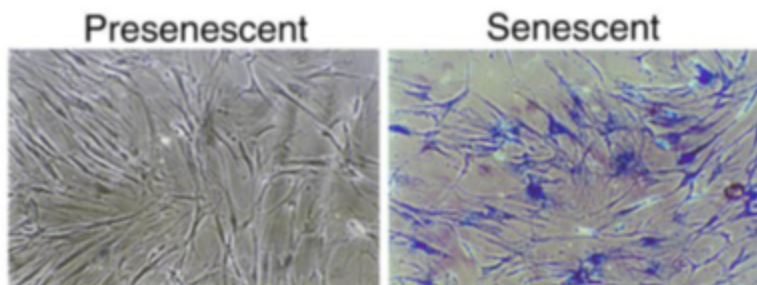
Citric acid/Na phosphate buffer, 0.2M (100 ml):

36.85 ml 0.1 M citric acid solution

63.15 ml 0.2 M sodium phosphate (dibasic) solution

Verify that pH is 6.0.

 Citric acid solution (0.1 M) = 2.1 g citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$)/100 ml water

Sodium phosphate solution (0.2M) = 2.84 g sodium dibasic phosphate (Na_2HPO_4) or 3.56g sodium

dibasic phosphate dihydrate ($Na_2HPO_4 \cdot H_2O$) (be sure to use dibasic)/100 ml water

Senescence Associated β-galactosidase Staining (Senescence assay)

Principle: The identification of senescent cells is based on an increased level of lysosomal β-galactosidase activity. Cells under normal growth conditions produce acid lysosomal β-galactosidase, which is localized in the lysosomes. The enzymatic activity can be detected at the optimal pH 4.0, using the chromogenic substrate 5- bromo-4-chloro-3-indolyl β D-galactopyranoside (X-gal). In comparison, upon senescence, the lysosomal mass is increased, leading to production of a higher level of β-galactosidase, termed senescence-associated β-galactosidase (SA-β-gal). The abundant senescence-associated enzyme is detectable over background despite the less favorable pH conditions (pH 6.0). The SA-β gal positive cells stain blue-green, which can be scored under bright-field microscopy (http://www.bio-protocol.org/e247).

Appendix D

RNA Isolation protocol

Materials

- Trizol samples
- Chloroform (Merck)
- Isopropanol (Merck)
- 75% Ethanol (Merck; Dilute 100% absolute ethanol with dH2O)
- RNAse-free water (Qiagen)

Methods

1. Defrost samples to Room temperature (RT)
2. Add 0.2 mL of chloroform per 1 mL of TRIzol™ Reagent used for lysis, then securely cap the tube
3. Invert tubes 10 times and incubate at RT for 5 minutes
4. Centrifuge the sample for 15 minutes at 12,000 × g at 4°C.
   NOTE! The mixture separates into a lower red phenol-chloroform, and interphase, and a colorless upper aqueous phase
5. Transfer the aqueous phase containing the RNA to a new tube by angling the tube at 45° and pipetting the solution out.
   I*MPORTANT! Avoid transferring any of the interphase or organic layer into the pipet when removing the aqueous phase, which will cause contamination of the RNA.*
6. Add 0.5 mL of isopropanol to the aqueous phase, per 1 mL of TRIzol™ Reagent used for lysis
7. Invert tubes 10 times and incubate at RT for 10 minutes
8. Centrifuge for 10 minutes at 12,000 × g at 4°C.
   NOTE! Total RNA precipitate forms a white gel-like pellet at the bottom of the tube, however this is not always visible by eye.
9. Discard supernatant
10. Resuspend the pellet in 1 mL of 75% ethanol per 1 mL of TRIzol™ Reagent used for lysis
11. Vortex the sample briefly then centrifuge for 5 minutes at 7500 × g at 4°C
12. Discard as much supernatant as possible with pipet and repeat step 10-12 (washing)
13. Let pellet air dry for 10 minutes to evaporate any remaining ethanol.
14. Resuspend pellet in 20 ul RNAse free water by pipetting up and down
15. Place tubes on ice and determine the RNA yield using a Nanodrop
16. Store samples at -20 or proceed with cDNA synthesis protocol

Appendix E

cDNA synthesis Protocol

Materials

- RNA samples
- RevertAid First Strand cDNA Synthesis Kit RevertAid First Strand cDNA Synthesis Kit # K1621 (Thermofisher)
  - RevertAid RT (200 U/µL)
  - RiboLock RNase Inhibitor (20 U/µL)
  - 5X Reaction Buffer
  - 10 mM dNTP Mix
  - Random Hexamer Primer, 100 µM
  - Water, nuclease-free

Methods

1. Defrost RNA samples and cDNA kit components on ice
2. Calculate the volume (ul) of RNA sample needed for 1 µg RNA/per sample
3. Add the following reagents into a sterile, nuclease- free tube on ice in the indicated order:

   - Template RNA   [1 ug]   (ul..)
   - Random Hexamer primer   1 ul
   - <u>nuclease-free to 12 µL</u>   <u>(ul…)</u>
     Total volume   12 ul/sample

4. For the Master mix add the following reagents into a sterile, nuclease- free tube on ice in the indicated order:

   - 5x Reaction buffer   4 ul
   - RiboLock RNase Inhibitor   1 ul
   - 10 mM dNTP Mix   2 ul
   - <u>RevertAid RT</u>   <u>1 ul</u>
     Total volume   8 ul/sample

     Total volume sample + mastermix = 20 ul
     Note! For Mastermix calculate how much is needed for all samples + 1

5. Pipet the samples (12 ul) and Mastermix (8ul) together in a PCR strip tube
6. Mix gently and centrifuge briefly to avoid/get rid of air bubbles.
7. Place samples in the Biorad Thermal Cycler and run samples on the following program:
   - 5 minutes at 25°C

- 60 minutes at 42°C
- 5 minutes at 70°C
- ∞ at 4°C
  8. Store samples at -20 or proceed with qPCR protocol

A calculation of the required concentration of our samples was done to eventually add up to the required 12 microliters. (amount of RNA has been normalized) see table below

Name	RNA ng/microL	Total ng	100 ng	H2O microL
cm00	48.92	978.4	2.04	8.96
cm05	60.12	1202.4	1.66	9.34
cm10	109.08	2181.6	0.92	10.08
cm20	9.64	192.8	10.37	0.63

Appendix F

qPCR Protocol

Materials

- cDNA
- iQ Sybr Green Mix (2x) (Biorad)
- RNAse free water
- Primers (Reverse and Forward)

Methods

1. Defrost cDNA samples, iQ Sybr Green mix and primers on ice
2. Prepare a cDNA mastermix:
   [A] Calculate the volume (ul) for 10 ng cDNA/Sample
   [B] Calculate the volume (ul) water needed to get 5 ul mastermix/sample (= 5-A)
   [C] Calculate the amount of samples needed (Different primers/duplicates)
   Calculate total volume cDNA needed = A*[C+1]
   Calculate total volume of water needed = B*[C+1]
   - and pipet together in a tube on ice
3. Prepare per primer a primer-Sybr green mastermix

   - Primer 1 (50uM)   0,2 ul
   - Primer 2 (50uM)   0,2 ul
   - <u>iQ Sybr Green</u>   <u>5 ul   </u>
     Total volume   5,4 ul/sample

   Calculate the volumes needed for each different primer set including all
   samples and duplicates +1 (for pipetting error)

4. Pipet 5 ul cDNA mix and 5 ul Primer mix together in a qPCR plate
   NOTE! Mix the iQ Sybr Green thoroughly to ensure homogeneity.

5. Seal the plate with an optical adhesive cover, avoid contact with your hands on the
   seal.
6. Centrifuge the plate for a few seconds to remove the air bubbles at the bottom.
7. Put the plate in the ViiA7

Short Protocol ViiA7:

- Start the ViiA7 software by clicking on the shortcut on the desktop.
- Click: new experiment
- Click the barcode, scan your plate, fill in your name at user name and give a unique experiment name furthermore use the standard run for the properties of the instrument and choose for reagent: SYBR Green Reagents
- In the assign tab, assign the reporter for the appropriate wells
- In the run method tab, adjust the volume to 10ul
- Save the template on the local (provided) USB stick and bring the plate and template to the Viia7 machine

ViiA7 Robotic loading

- Select the Instrument-Queue-tab for plate loading with the Sideloader robot. This option must be used if the run will not be completed before 16.00h.
- Start SDS Automation Controller by clicking on the shortcut on the desktop.
- Add your plate template to the plate queue
- Put your plate on the stack matching the A1 of your plate with the labeling of the stack
- Start Queue by clicking the Start Batch button.
- Click Run Status-tab to follow the run (cycles, temperature and signals).
- After your run is completed (next morning on working days); remove your plate from the Processed Plates-tab and from the robot-stack.
- Discard your plate in a blue bin in the lab.
- Copy your data from the computer using a USB-memory stick, in the Viia7 software you can choose which data to export (raw data, ct values etc)
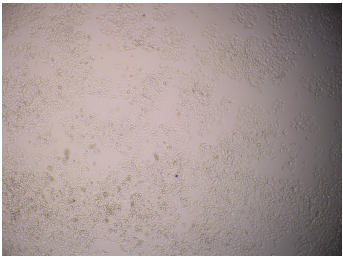
Appendix G

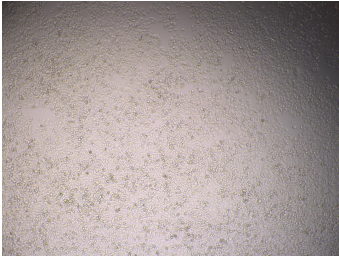**Pictures before and after CSE at 200x zoom**
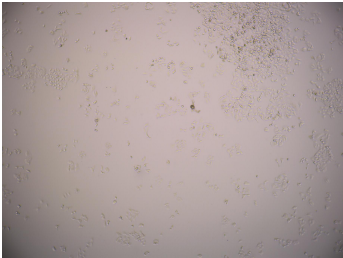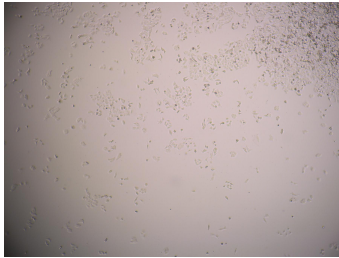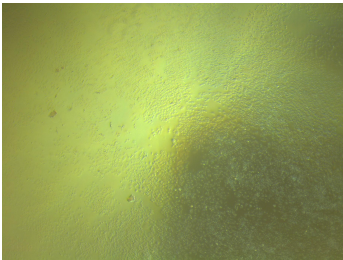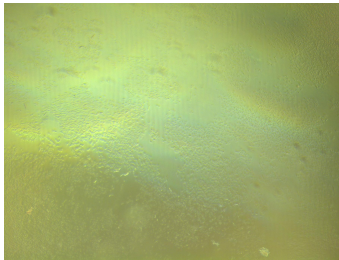At 0, 5, 10 and 20 percent smoke treatment.

Before CSE treatment

0

5

10

20

 After CSE treatment

0

5

10

20