High Content Screening (HCS) Approach to Characterize Phenotypic Changes Occurring During Long-term Treatment of Human Bronchial Epithelial Cells With Cigarette Smoke Total Particulate Matter

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Introduction

Chronic cigarette smoke exposure is responsible for airway epithelial changes which ultimately may cause lung cancer, although the current knowledge about the underlying molecular events are still not very detailed. Our aim was to establish an in vitro model which mimics chronic exposure conditions found in the airways of smokers and, utilizing BEAS-2B cells and an HCS approach, to more comprehensively characterize the chronological changes that occur in bronchial epithelial cells under those conditions. For this purpose, immortalized human bronchial epithelial BEAS-2B cells were continuously exposed to total particulate matter (TPM) from the reference cigarettes 3R4F for 4 weeks. Cell cycle, DNA damage, and oxidative stress were assessed weekly by high-content analysis. In addition, staining for epithelial-mesenchymal transition (EMT) markers was performed after 4 weeks of treatment.

Increased oxidative stress and DNA damage were noticeable within 1 week of treatment. At the same time a significant portion of cells arrested in S-phase, and by week 2 cells were dying or became senescent. However surviving treated BEAS-2B cells resumed normal cell cycle shortly thereafter. In addition, following a 4-week TPM treatment, BEAS-2B cells expressed lower levels of E-cadherin and higher levels of Vimentin and displayed fewer cell-to-cell contacts, although they were not yet invasive.

In summary, our experiments indicate that long-term exposure of bronchial epithelial cells to 3R4F TPM induces phenotypic changes that may eventually lead to carcinogenesis. The data here may lead to a better mechanistic understanding of the stepwise transformation of normal airway epithelial cells to full malignancy. The High Content Imaging platform provides sufficient throughput capacity and precision to track phenotypic signatures of individual cells/cell populations and enables longitudinal mechanistic in-vitro toxicology studies.

Material and Methods

Generation of TPM - 3R4F reference cigarettes were purchased from the University of Kentucky (http://www2.ca.uky.edu/refcig/) and stored in a cooling chamber at 5±3°C with uncontrolled humidity in the original packaging. The cigarettes were conditioned according to ISO standard 34021, i.e., for at least 48 h at 22±1°C and a relative humidity of 60±3% before total particulate matter (TPM) generation. TPM was collected on Cambridge filter pads using a 20-port Borgwaldt (Germany) RM20H smoking machine according to the ISO smoking regimen. Five mL DMSO were used to extract TPM from the filters, and aliquots of 25 mg/mL TPM were prepared and stored at -80°C until further use.

Cell Culture and Treatment - The human bronchial epithelial cell line BEAS-2B (LGC Standards GmbH, Wesel, Germany) was grown in complete BEGM™ (Lonza, Basel, Switzerland) on collagen A-coated plates. After two passages of the frozen stock, cells were seeded at an appropriate density and treated with TPM at a final concentration of 10 mg/mL. At a confluence of ca. 75%, cells were trypsinized and passaged in fresh treatment medium for a total treatment duration of 4 weeks. Cells cultured without TPM (medium only) and cells cultured in the presence of 0.2% DMSO (vehicle; Sigma Aldrich, Buchs, Switzerland) were included as controls. Additionally, treated and control cells were collected once a week for assessment of viability and high-content screening (HCS) as described in Figure 1. This experiment was repeated three times, giving a total sample number of 3 per time point/endpoint.

High-Content Screening (HCS) - Cellomics ArrayScan® VTI HCS reader (Thermo Fisher Scientific Inc., Berkshire, UK) was employed to assess oxidative stress (ROS and GSH), genotoxicity (DNA damage), cell cycle alterations and epithelial-mesenchymal transition (EMT). Briefly, BEAS-2B cells were seeded in black collagen I-coated, clear-bottom 96-well tissue culture plates (BD, Allschwil, Switzerland) at a density of 12,000 cells/well for all endpoints except the cell cycle assay, for which 6,000 cells/well were seeded. 24 h after the seeding, the cells were incubated for 24 h (or 72 h for the EMT assay) in the presence of positive control or vehicle control (DMSO or water). Reagent list: Nuclear staining: Hoechst 33342 (Fisher Scientific, Reinach, Switzerland) or DRAQ5 (for GHS only) (Fisher Scientific, Reinach, Switzerland). Bioapplication: Compartmental Analysis and Cell cycle.

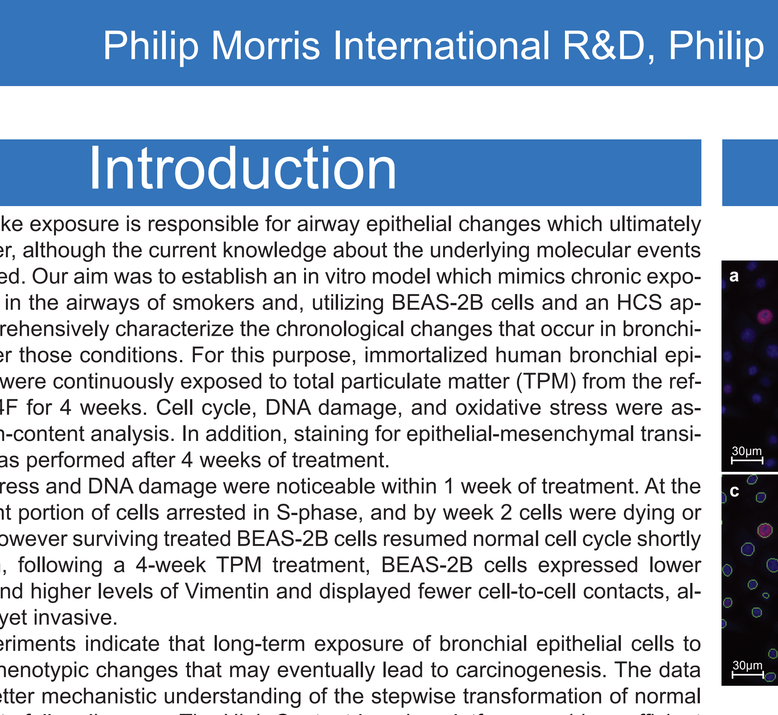


Figure 1. Experimental design (a) to study the effect of long-term exposure to 3R4F TPM. At the designated time points cells were collected for further analysis for a panel of different endpoints (b).

Assay Development

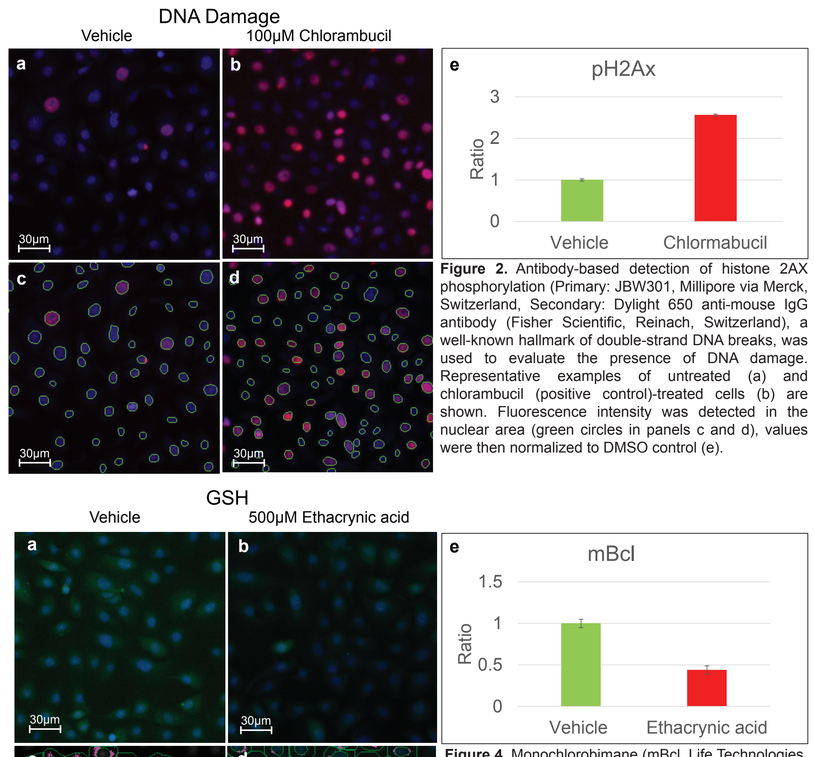


Figure 2. Antibody-based detection of histone 2AX phosphorylation (Primary: JBW301, Millipore via Merck, Switzerland, Secondary: Dylight 650 anti-mouse IgG antibody (Fisher Scientific, Reinach, Switzerland), a well-known hallmark of double-strand DNA breaks, was used to evaluate the presence of DNA damage. Representative examples of untreated (a) and chlorambucil (positive control)-treated cells (b) are shown. Fluorescence intensity was detected in the nuclear area (green circles in panels c and d), values were then normalized to DMSO control (e).

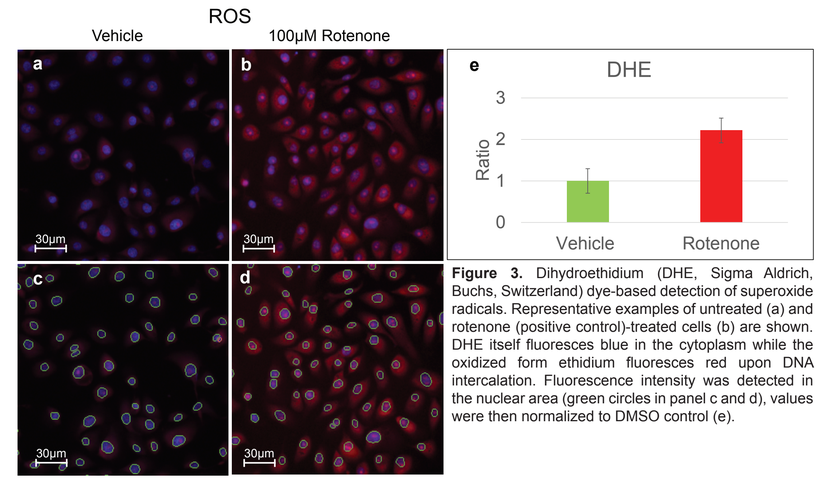


Figure 3. Dihydroethidium (DHE, Sigma Aldrich, Buchs, Switzerland) dye-based detection of superoxide radicals. Representative examples of untreated (a) and rotenone (positive control)-treated cells (b) are shown. DHE itself fluoresces blue in the cytoplasm while the oxidized form ethidium fluoresces red upon DNA intercalation. Fluorescence intensity was detected in the nuclear area (green circles in panel c and d), values were then normalized to DMSO control (e).

Figure 4. Monochlorobimane (mBcl, Life Technologies, Zug, Switzerland) dye-based detection of free GSH molecules. Representative examples of untreated (a) and ethacrynic acid (positive control)-treated cells (b) are shown. Fluorescence spot intensity (pink dots in panel c) was detected in the cytoplasmic area (ring area between the two green circles in panel c and d), values were then normalized to DMSO control (e).

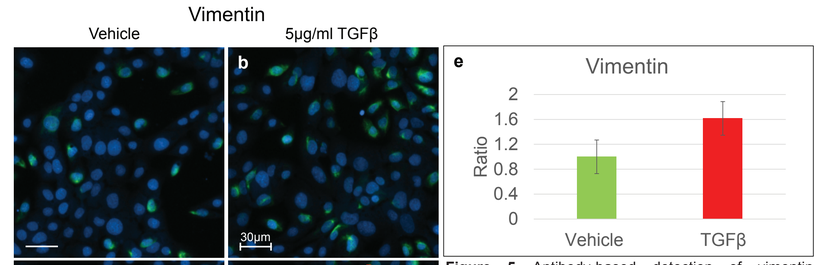


Figure 5. Antibody-based detection of vimentin (Primary: Ab45939, Abcam, Cambridge, UK, Secondary: Dylight 550 anti-rabbit antibody, Fisher Scientific, Reinach, Switzerland) a well-known hallmark of mesenchymal cells, was used to evaluate the EMT process. Representative examples of untreated (a) and TGFb (positive control)–treated cells (b) are shown. Fluorescence intensity was detected in the perinuclear area (red area in panels c and d), values were then normalized to DMSO control (e).

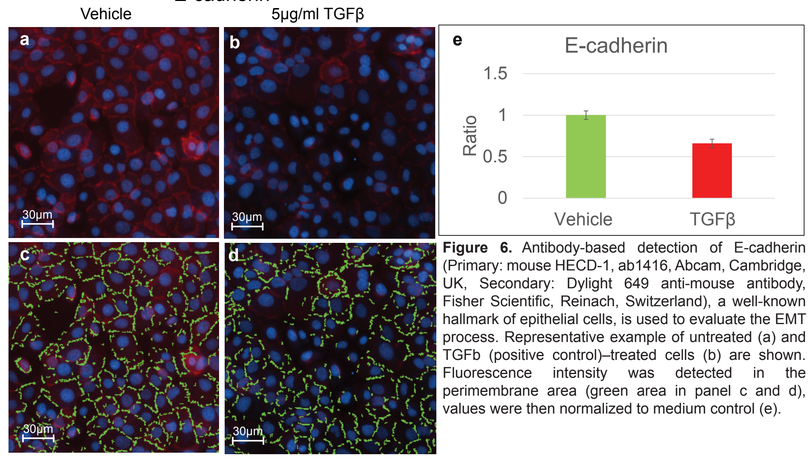


Figure 6. Antibody-based detection of E-cadherin (Primary: mouse HECD-1, ab1416, Abcam, Cambridge, UK, Secondary: Dylight 649 anti-mouse antibody, Fisher Scientific, Reinach, Switzerland), a well-known hallmark of epithelial cells, is used to evaluate the EMT process. Representative example of untreated (a) and TGFb (positive control)–treated cells (b) are shown. Fluorescence intensity was detected in the perimembrane area (green area in panel c and d), values were then normalized to medium control (e).

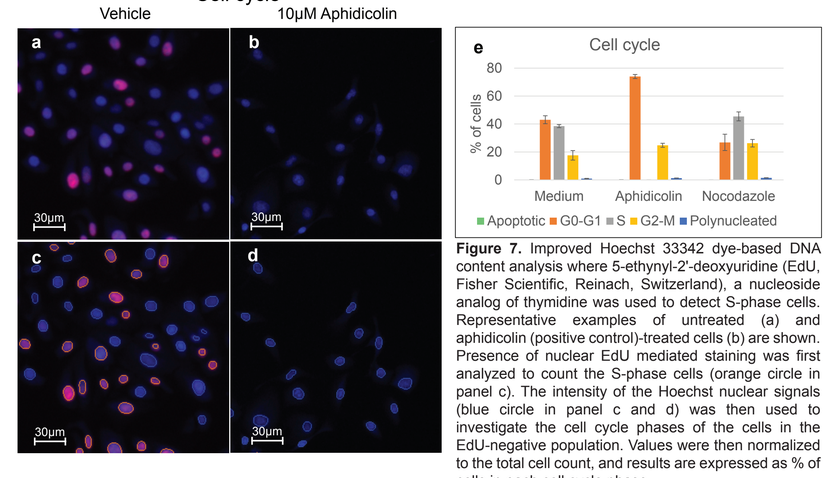
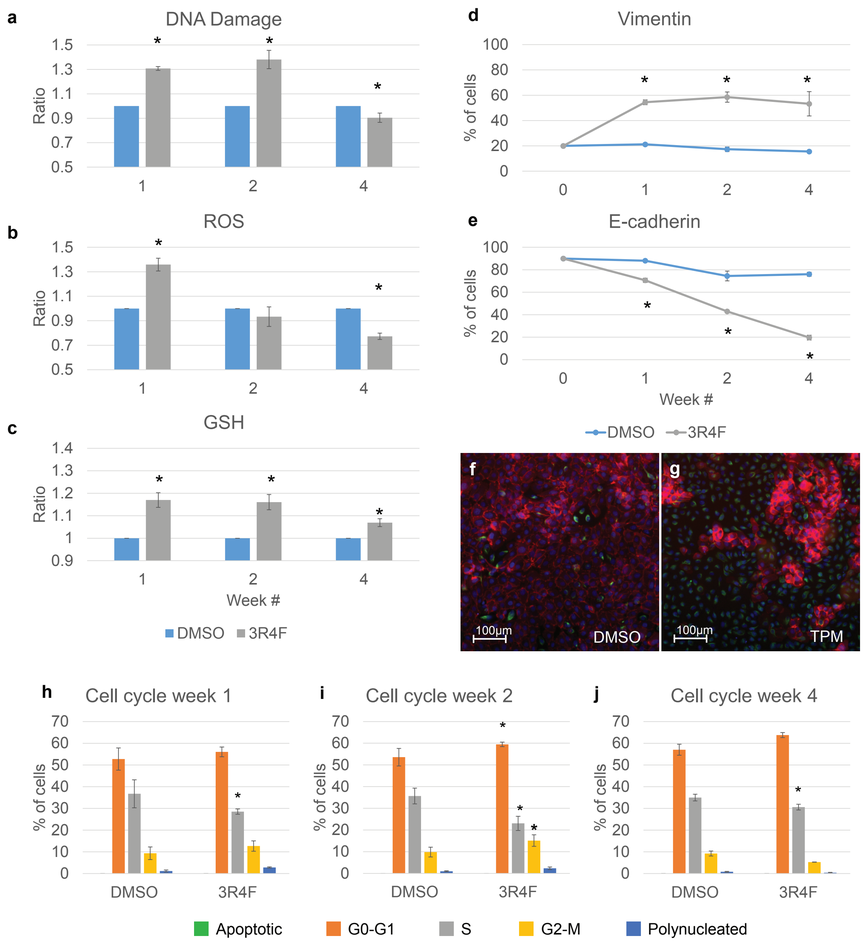


Figure 7. Improved Hoechst 33342 dye-based DNA content analysis where 5-ethynyl-2'-deoxyuridine (EdU, Fisher Scientific, Reinach, Switzerland), a nucleoside analog of thymidine was used to detect S-phase cells. Representative examples of untreated (a) and aphidicolin (positive control)-treated cells (b) are shown. Presence of nuclear EdU mediated staining was first analyzed to count the S-phase cells (orange circle in panel c). The intensity of the Hoechst nuclear signals (blue circle in panel c and d) was then used to investigate the cell cycle phases of the cells in the EdU-negative population. Values were then normalized to the total cell count, and results are expressed as % of cells in each cell cycle phase.

Results

- Chronic treatment of BEAS-2B cells with 3R4F TPM significantly increases phosphorylation of histone H2AX by 30% in the first 2 weeks of treatment while no difference were found after 4 weeks of treatment (Fig. 8a).
- Exposure to 3R4F TPM causes a significant increase of ROS only within the first week of treatment when compared to the vehicle (DMSO) control (Fig. 8b). Already after the second week, treated cells showed adaptation to the pro-oxidative insult as similar ROS level were detected when compared to vehicle control.
- Glutathione (GSH) is an important intracellular antioxidant which plays a key role in the cellular redox status of cells and tissues. In this study we revealed that long term treatment of BEAS-2B with 3R4F TPM increases the level of reduced GSH (Fig. 8c).
- Epithelial mesenchymal transition (EMT), is one of the first critical phenotypic alteration of cells that triggers invasion and metastasis. In this study, we show (figure 8d-e) that expression of E-cadherin (epithelial marker) is decreased while Vimentin (mesenchymal marker) starts to be expressed (both markers are expressed as % of positive cells). A clear altered phenotype is identified if 4-week TPM exposed cells (figure 8f) are compared to DMSO exposed cells (figure 8g).
- In figure 8, BEAS-2B cell cycle analysis (h-j) and cell growth numbers (k) are shown. Cell cycle of 3R4F TPM treated BEAS-2B shows a significant inhibition of DNA synthesis during the whole exposure time with a more pronounced effect after two weeks as both G1 and G2 phases were significantly increased. These findings were further supported by the reduced number of growing cells in the flask as a significant decrease was obtained at week one and two. However, after four weeks of treatment, BEAS-2B cells display a partial recovery as limited impact on the cell cycle and cell viability is detected.



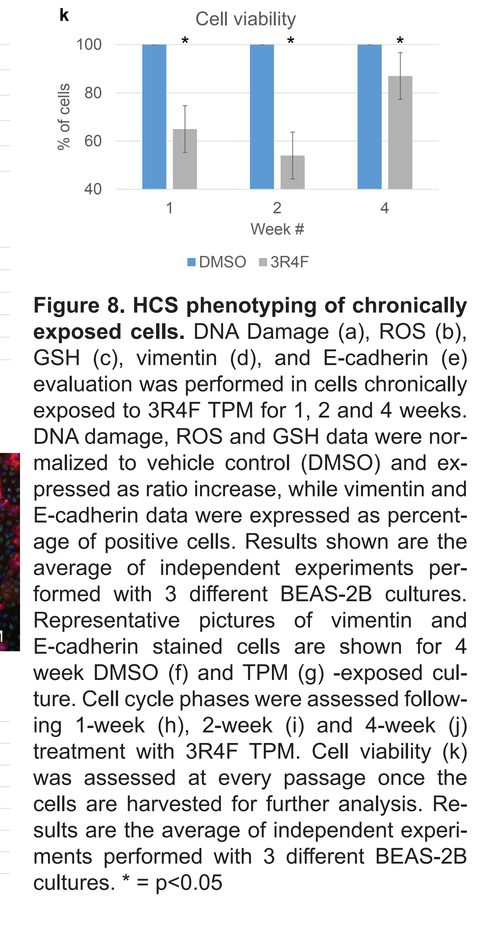


Figure 8. HCS phenotyping of chronically exposed cells. DNA Damage (a), ROS (b), GSH (c), vimentin (d), and E-cadherin (e) evaluation was performed in cells chronically exposed to 3R4F TPM for 1, 2 and 4 weeks. DNA damage, ROS and GSH data were normalized to vehicle control (DMSO) and expressed as ratio increase, while vimentin and E-cadherin data were expressed as percentage of positive cells. Results shown are the average of independent experiments performed with 3 different BEAS-2B cultures. Representative pictures of vimentin and E-cadherin stained cells are shown for 4 week DMSO (f) and TPM (g) -exposed culture. Cell cycle phases were assessed following 1-week (h), 2-week (i) and 4-week (j) treatment with 3R4F TPM. Cell viability (k) was assessed at every passage once the cells are harvested for further analysis. Results are the average of independent experiments performed with 3 different BEAS-2B cultures. * = $p<0.05$

Conclusion

- Repeated exposure of bronchial epithelial cells to 3R4F TPM induces ongoing phenotypic alterations related to tumorigenesis. In particular cellular oxidative stress and DNA damage are increased causing cell cycle alterations and evidence of epithelial-mesenchymal transition are detected.
- The data here may lead to a better mechanistic understanding of the stepwise transformation of normal airway epithelial cells to full malignancy.
- The High Content Screening imaging platform represents a powerful tool with sufficient throughput capacity and precision to allow detection of phenotypic changes in toxicological studies.