Volatile Compounds Are Involved in Cellular Crosstalk and Upregulation

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Cell–cell cross talk is of great importance in cancer research due to its major role in proliferation, differentiation, migration, and influence on the apoptotic pathway. Different cell–cell communication mechanisms have come mainly from proteomic and genomic approaches. In this paper, a new route is reported for cross talk between cancer cells that occurs, even when they are far away from each other. Single-cell and culture analysis shows that upregulation of cancer cells emits hundreds of volatile organic compounds (VOCs) into their headspace. Part of the VOCs remains without any change, disregarding the biological environment around it. The other part of the VOCs is exchanged between monocultures of the cells as well as between co-cultures of the cells with no physical contact between them, leading to different changes in growth than when left on their own. The chemical nature and composition of these VOCs have been determined and are discussed herein. Cell-to-cell cross talk has the advantage of being suitable for transfer/diffusion over relatively long distances. It would thus be expected to serve as a shuttling pad toward the development of advanced approaches that could enable very early detection of cancer and/or monitoring of metastasis and related cancer therapy.

1. Introduction

Studying cell-to-cell cross talk in a group of cells is indispensable because it provides a rich source of information. It is particularly important in cancer research since it has a major role in cancer development, tumorigenesis, metastasis, and apoptosis.1–7 Key factors in these communication networks are the cell-surface receptors, which are responsible for the transduction of signals through branched cascades of responses.

Cancer cells and the tumor microenvironment (TME) communicate with each other through numerous chemical mechanisms.8,9 This includes autocrine signaling where secreted chemical messenger binds to autocrine receptors on the same cell, which affects the way the cell functions; and paracrine signaling, which is a form of signaling in which one cell affects its neighbors by secreting chemicals into the intercellular space.10 Cells can also directly transfer ions or small molecules from one to another through pores in the cell membrane.11 Cell–cell cross talk and tumorigenesis are highly influenced by specific stromal factors and TME components.12

Different cell–cell communication mechanisms (e.g., cell proliferation, migration, cell recognition, and differentiation) have come from proteomic and genomic approaches. Though tremendous advances have been achieved, several limitations restrict the fulfillment of the approaches in diagnosis and therapeutic applications. These limitations include, but are not confined to 1) proteomics and genomics requiring prior and quite accurate knowledge of specific genes or proteins, and are exclusive to in vitro and in vivo trials—something that does not necessarily reflect real-life situations; and 2) proteomics and genomics continue to be expensive, of low specificity, and require complex analysis algorithms—that results in prolonged and cumbersome analysis.12–15 Cancer is a systematic disease (polygenetic) involving several mutations at different sites,16 (genetic, epigenetic, local to or at distance from the primary tumor, etc.), thus a more comprehensive observation and broad analysis is of interest. New biomarkers that can provide systematic knowledge of the disease without the need to isolate and explore specific genes or proteins would be highly beneficial.

There are different methods for evaluating cell-to-cell interaction, such as lab-on-chip or 3D microfluidics platforms. These methods have many advantages; however, they focus on analyzing liquid phase metabolites or using a range of optical evaluation.16,17 While these methods have great potential, they are currently unsuitable for gas phase interaction sampling of volatile metabolites. Here we report on a new route for cell-to-cell cross talk, which relies on the upregulation of volatile organic compounds (VOCs)—chemical compounds that have a low molecular weight and relatively high vapor pressure.
under ambient conditions. We demonstrate this finding by monitoring changes in nuclei and surface morphologies, as well as the apoptotic behavior, of lung cancer (LC) cells in co-cultured media that have no physical contact, and by characterizing the VOCs emitted at each stage of cancer growth. Single-cell analysis of the signaling VOCs showed that parts of the VOCs are emitted directly from the cancer cell whereas others result from intercellular communication.

2. Results and Discussion

In the course of preparation of cancer cells, they have been cultured in two adjacent Petri dishes that were not in physical contact and showed different growth changes compared with being on their own. To explore these phenomena, lung cancer (LC) cells in cocultured media that have no physical contact, and by characterizing the VOCs emitted at each stage of cancer growth. Single-cell analysis of the signaling VOCs showed that parts of the VOCs are emitted directly from the cancer cell whereas others result from intercellular communication.

2.1. Morphology of Physically Unconnected Cells in Co-cultures

Cancer and/or normal cell lines (1 × 10⁶ cell) were co-cultured under same headspace (i.e., the same gas environment above the cells) and incubated for up to 72 h. Morphological changes of all co-cultures of physically unconnected cells were examined by light microscopy (Figure 1A–D) at T₁₄, T₂₈, and T₇₂. The morphology of the monoculture and same-cell pair combination groups showed no significant changes (Figure 1E,F) at all time-points. In contrast, significant morphological changes (e.g., circular and floating cells) were seen in co-cultured combinations of different cell types in a time-dependent manner (Figure 1G). A wide variety of morphology changes occurred (Figure 1G). However, when normal cells were co-cultured with cancer cells
under the same headspace, normal cells became circular and rounded in shape compared with control (monoculture) after T_{48} and T_{72} (Figure 1G). The morphology of H1975M cells in co-cultured pairs of H1299 + H1975M and A549W+ H1975M combinations, H1975M cells were unchanged, but H1299 and A549W cells had altered morphology, for example, into rounded shapes and cell shrinkage compared with control (monoculture) at T_{72} (Figure 1G). Co-culture pair combinations of cancer cells showed strong morphological changes in H1299 and normal cell lines (Figure 1).

**2.2. Cell–cell Interactions in Connected and Unconnected Co-cultures Validated by Imaging Flow Cytometry**

Co-cultured cell combinations were also examined by digital imagery using flow cytometry (ImageStream).[18] For this analysis, Cell-A cultures were labeled with DiO and Cell-B with DiD before being incubated for 20 min. The cells were washed and co-cultured at a 1:1 ratio for T_{48}. Cells were analyzed by ImageStream (details in Experimental Section). Each cell was simultaneously imaged in Hoechst (435–505 nm), DiI (505–560 nm), DiO (435–505 nm), DiD (642–745 nm) and bright-field (660–720 nm). Quantitative measurement of apoptotic nuclear morphology (e.g., increased nuclear texture and fragmentation) used IDEAS software. The area of bright regions in the nuclear image and the intensity of small bright nuclear details within IDEAS were characteristic of apoptotic nuclei. During the analysis of these cell populations, we noticed that physically unconnected co-cultures of different cells as well as physically connected co-culture of different cells resulted in apoptotic and nuclear changes compared to monoculture of physically unconnected co-culture cells of the same type.

ImageStream analysis of different types of co-cultured cells at T_{48} had fewer nuclei in Hoechst stained cells (Figure 2A). Direct contact cell groups showed different sizes and shapes of nuclei compared to the control (Format 1) groups. Intensity analysis with software-based nucleus shape and nucleus staining indicated significant changes in morphology. The Hoechst dye-stained morphologically normal nuclei appear dimly blue, whereas co-culture physically unconnected cells (Formats 2 and 3) and co-culture physically connected cells (Format 4) with changes in nuclear morphology (nuclear shrinkage) were bright blue and had smaller nuclei after T_{48} (Figure 2A). These results show that two different cell types in co-culture induce morphological changes and apoptotic cell death due to soluble factors (chemical signals). Most interestingly, this also suggests that induction could be due to volatile signals (in the headspace of co-cultured unconnected cells). The level of apoptosis was verified by Hoechst staining of chromatin condensation, one of the hallmarks of apoptotic cell death.[19]

Image Stream analysis of apoptotic cell death assessed in different co-culture formats at T_{0} and T_{48} is given in Table 1. Low apoptotic levels were seen when cells were cultured alone (1.8%) (Figure 2B,C). This marginally increased (3.5%) in same-cell pair combinations at T_{48}. BEAS-2B cells co-cultured with other cell types increase apoptosis in unconnected cells by 3%, whereas in physically connected cell groups apoptosis was significantly (p < 0.05) increased (47.1%) at T_{48} (Table 1). The high level of apoptosis in the physically connected co-cultured cells may be due to cell type, but more likely is unrelated to the high cell density. This is probably because, in monoculture cells (also with high cell density), apoptosis is lower (1%). Co-culturing H1299 cells with H1975 at T_{48} resulted in 34.1% apoptosis, whereas co-culturing H1299 cells with A549 cells gave 21.2% (Table 1). When BEAS-2B and H1299 cells were co-cultured without physical contact between the co-cultures, apoptosis was drastically increased at almost 3–4-fold at T_{48} compared to T_{0}, as also to the same pair unconnected co-culture with H1299 cells at T_{48}. We confirmed that the H1975 and A549 cells mediated to H1299 and BEAS-2B apoptosis largely depended on contact between these two cell types. Apoptosis of H1299 and BEAS-2B cells was more induced by direct co-culture with H1975 and A549 cells than in the unconnected headspace method (Table 1). Analysis of the imaged flow cytometry results showed moderate changes in unconnected same-pair co-cultures. Direct contact co-culture of BEAS-2B and H1299 cells with H1975 cells had high levels of morphological changes and apoptosis (54.1 and 51.5%, respectively). These results suggest that the proliferative and apoptotic effects of H1975 and A549 cells is probably induced mainly by soluble factors which are dependent on direct cell contact cultured together, but most interestingly, is also affected by volatile signals to a lesser extent.

**2.3. Cell Growth in Co-cultures of Physically Unconnected Cells**

We examined cell growth after T_{72} regarding confluence in all combination as measured by trypan blue (Figure 3). Confluency was significantly increased (p < 0.05) in a time-dependent manner within each type of cell (A549+A549 [75.2%], H1299+H1299 [90.2%], and BEAS-2B+BEAS-2B [80.1%]), as well as in comparison with monoculture of the corresponding cell line when co-cultured in an unconnected manner (Figure 3A,B). In contrast, co-culture of unconnected different cells led to lower confluency levels than in same-cell type co-culture combination at all time-points (Figure 3C–E). Cell confluency (Figure 3E) was higher at T_{72} than in the monoculture groups (Figure 3A). It was also different from the confluency of co-cultures of the same cell type group in all conditions (Figure 3B).

H1975M cells had the highest growth rate in a co-cultured pair of H1299 and A549W cell combinations, but no significant morphological changes were seen. Interestingly, H1299 and A549W cells had the lowest growth rate. Their morphology was changed to more rounded shapes and was shrunken compared with the control (monoculture) at T_{72} (Figure 1G).

Since the kinetic and morphological characteristics of cells were significantly changed in the non-contact mode between the co-cultures, it was reasonable to assume that the main reason for changes and/or cross talk is due to the headspace of the gas phase above the cells. As such, we have explored the VOC composition of the headspace in the different samples. VOCs are chemical compounds that have a low molecular weight and relatively high vapor pressure at ordinary, room-temperature conditions. The VOCs can be either polar or non-polar, and can be found in body fluids, for example, blood, saliva, feaces, sweat, urine, and breath.[20–23] An intriguing feature of the VOCs is

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**Table 1.** Confluency analysis of co-cultures

| Format | Confluency (%) |
|--------|---------------|
| 1      | 75.2          |
| 2      | 90.2          |
| 3      | 80.1          |

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their widespread partition coefficient between fat and blood (i.e., a coefficient that is designed to estimate the equilibrium concentration of VOCs in fat tissue and (lipophilic) cell membranes with respect to fat/blood), indicating their (hypothetical) participation in the signaling pathways within and/or outside the cell.\textsuperscript{[26]}

2.4. Effect of Interspecific VOCs in Cell–Cell Interactions

To explore the potential role of VOCs in the cell–cell cross talk, we used the same set-ups described above (Figure 1A–D), taking samples for headspace analysis using the In-Tube Extraction (ITEX)-GC-MS methodology (see Experimental Section for details). A total of 104 VOCs was significantly identified together in all combinations. The results show that monocultured (Format 1) groups had different VOCs profile from that of the co-culture pair combination groups (Formats 2–4) (Figure 4; Tables S1–S3, Supporting Information). A higher number of VOCs were detected in the co-culture pair combinations of the cell lines (Figure 4). It is clear that same cell unconnected co-culture gave much more VOCs, as seen in Figure 4 (brown color of scale bar represents a greater abundance). However, the higher number of detected VOCs is most

Figure 2. Example of an ImageStream images from co-culture experiment, cells stained with Hoechst 33342. Nuclei morphology and apoptotic changes in lung cancer and normal cell lines. A) Nuclear morphology of cells treated with different formats. Hoechst 33342 dye stains morphologically normal nuclei pale blue, whereas apoptotic nuclei have every bright blue smaller nuclei. B) Images of apoptotic and non-apoptotic cells are in each format. The apoptotic index was measured for all treated formats at $T_{48}$. C) These panels show examples of apoptotic cells in each format. Bivariate plot measuring nuclear morphology based apoptotic index is given for co-cultured cells for $T_{48}$, using only the fluorescence intensity of Hoechst. These panels show the area threshold 50\% Area-Hoechst (Y-axis) versus fluorescence intensity signals (X-axis). Note: Format 1: Monoculture (alone), Format 2: Headspace co-culture physically unconnected same-cell pair, Format 3: Headspace co-culture physically unconnected different-cell pair, Format 4: physically connected co-culture different-cell.
probably due to the combination of the VOCs mixture of these four cell line isolates.

The effect of interspecific interactions on VOCs production and composition remains unknown.\(^{[21,22]}\) We therefore compared VOCs emitted from cancer and normal cell lines that are genetically different in p53 status, grown with no physical contact either in monocultures or in pair combinations. VOCs emitted during pair combinations differed from the VOCs of the respective monocultures; the VOCs mostly produced by monocultures were not detected in pair combinations. After T\(_{24}\) incubation period in monoculture, there was a difference in the peak area of 23 compounds in all cells compared with the control (Figure 1A), whereby 20 VOCs were significantly increased, with three compounds (2,5-dimethyl-1-heptene; 2,5-dimethyl-2,5-hexanediol; 4,6-dimethyl-2-heptanone) being significantly increased (\(p < 0.05\); Table S1, Supporting Information). After T\(_{48}\) differences in the peak area of 23 VOCs between all cell lines and the control medium were detected, whereby 22 VOCs were significantly increased and one VOC was significantly decreased (\(p < 0.05\)). At T\(_{72}\), 20 VOCs were identified, with 13 VOCs being significantly decreased and seven VOCs significantly increased (\(p < 0.05\)). Under our experimental conditions, the composition of the detected VOCs was different for each cell line, and also varied according to the incubation period. Ten of the VOCs in the T\(_{24}\), T\(_{48}\), and T\(_{72}\) incubations were common to all groups. Comparison of the results together show alterations during growth progression that may cause some VOCs to change between cells during cell–cell cross talk.

In same-cell pair combinations after T\(_{24}\) (Figure 1B), there was a difference in the peak area of 24 VOCs in all cells compared with the control, whereby 23 VOCs were significantly increased and one VOC (heptane, 4-methyl-) was significantly decreased (\(p < 0.05\); Table S2, Supporting Information). After T\(_{48}\), differences in the peak area of 25 VOCs between all cell lines and control medium were detected, whereby 19 VOCs were significantly increased, and six VOCs were significantly decreased (\(p < 0.05\)). At T\(_{72}\), 32 VOCs were identified, whereby five VOCs were significantly decreased, and 27 VOCs were significantly increased (\(p < 0.05\)). After T\(_{24}\), T\(_{48}\), and T\(_{72}\), 17 of the VOCs were common to all groups. Of these, seven VOCs were similar to the monoculture set-up, as expected due to the fact that the combination is of the same cell line. While not all VOCs found here could be related to endogenous sources, metabolism and signaling, others do. For example, both setups of monoculture and same cell unconnected co-culture show an increase in cell-metabolism related compounds, such as 3-methyl-3-buten-1-ol (The Human Metabolome Database, HMDB, registry HMDB003126), that can have both endogenous and exogenous source and relate to cell signaling. This compound may be part of the byproducts of dolichols in the mevalonate pathway,\(^{[27]}\) or byproducts of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) that can also be hydrolyzed by different Nudix hydrolase enzymes,\(^{[28]}\) like isopentenyl-diphosphate delta-isomerase 1 (HMDBP01541). Another interesting finding is the increase of 3-methyl-butanal (aka iso-valeraldehyde) in all same cells unconnected co-culture pairs (Table S11, Supporting Information). This increase can be one of several explanations of the increased proliferation rates in this co-culture set-up (Figure 3B). While 3-methyl-butanal is mainly associated with an external source, it is a leucine degradation product.\(^{[29,30]}\) It is known that branched-chain amino acids (BCAAs; e.g., leucine) are important regulators of many cell signaling pathways.\(^{[13]}\) Leucine deprivation inhibits cell proliferation in cancer.\(^{[12]}\) Increased levels of 3-methyl-butanal suggest increased participation of leucine in metabolic activity, leucine reduction, resulting (or indicating) increased proliferation. These results also correlate with another study showing a decrease in leucine levels in breast cancer cell headspace, which is usually regarded as being related to an increase in energy demand.\(^{[14]}\) Therefore, these results could be potentially attributed to a higher metabolic flux. This change could result from an increase in VOC signals that, in return, increases the number of by-product VOC metabolites arising due to the increased proliferation rate in cancer cells.

In co-culture pair combinations after T\(_{24}\) (Figure 1C), a difference in the peak area of 25 VOCs was seen in all groups compared with the control, whereby 20 VOCs were significantly increased and five compounds were significantly decreased (\(p < 0.05\); Table S3, Supporting Information). After T\(_{48}\), differences in the peak area of 31 VOCs between all groups and the control medium were detected, whereby 20 VOCs significantly increased and 11 VOCs significantly decreased (\(p < 0.05\)). At T\(_{72}\), 37 VOCs were identified, whereby five compounds were significantly decreased and 32 VOCs significantly increased (\(p < 0.05\)). These VOCs mostly consisted of ketones and hydrocarbons. Interestingly, five VOCs were commonly found at T\(_{24}\), T\(_{48}\), and T\(_{72}\) in all groups. Monocultured groups had a different volatile profile composition from that of the co-culture combination groups (Tables S1–S3, Supporting Information). The relatively higher number of VOCs detected is most probably due to the combination of the VOCs mixture of these four cell line isolates. The results show that some

### Table 1. Co-culture induced apoptosis (%) in lung cancer cells.

| Groups | Monoculture [Alone] | Co-culture unconnected Same-cell pair | Co-culture unconnected different-cell pair | Co-culture connected pair |
|--------|---------------------|--------------------------------------|-------------------------------------------|--------------------------|
| Time [h] | 0 | 48 | 0 | 0 | 48 | 0 | 48 |
| BEAS-28 | 1.07 | 1.7 | 2.1 | 4.1 | 4.4\(^{[4]}\) | 12.8\(^{[4]}\) | 4.5\(^{[4]}\) | 47.1\(^{[4]}\) |
| | | | | | 3.9\(^{[4]}\) | 17.9\(^{[4]}\) | 3.2\(^{[4]}\) | 45.2\(^{[4]}\) |
| | | | | | 4.3\(^{[4]}\) | 16.2\(^{[4]}\) | 4.6\(^{[4]}\) | 42.6\(^{[4]}\) |
| AS49 | 0.7 | 1.1 | 1.2 | 2.42 | 2.4\(^{[4]}\) | 3.4\(^{[4]}\) | 3.1\(^{[4]}\) | 12.7\(^{[4]}\) |
| | | | | | 2.7\(^{[4]}\) | 4.3\(^{[4]}\) | 4.1\(^{[4]}\) | 19.3\(^{[4]}\) |
| | | | | | 2.4\(^{[4]}\) | 3.4\(^{[4]}\) | 1.6\(^{[4]}\) | 12.5\(^{[4]}\) |
| H1299 | 0 | 1.04 | 1.74 | 3.01 | 1.0\(^{[4]}\) | 4.1\(^{[4]}\) | 1.8\(^{[4]}\) | 9.7\(^{[4]}\) |
| | | | | | 1.7\(^{[4]}\) | 4.5\(^{[4]}\) | 8.1\(^{[4]}\) | 21.2\(^{[4]}\) |
| | | | | | 2.4\(^{[4]}\) | 4.3\(^{[4]}\) | 6.7\(^{[4]}\) | 54.1\(^{[4]}\) |
| H1975 | 1.01 | 2.02 | 1.04 | 2.71 | 2.4\(^{[4]}\) | 4.6\(^{[4]}\) | 0.9\(^{[4]}\) | 2.2\(^{[4]}\) |
| | | | | | 1.1\(^{[4]}\) | 3.0\(^{[4]}\) | 4.3\(^{[4]}\) | 19.3\(^{[4]}\) |
| | | | | | 2.7\(^{[4]}\) | 4.3\(^{[4]}\) | 3.9\(^{[4]}\) | 19.7\(^{[4]}\) |

\(^{[4]}\)with H1299; \(^{[5]}\)with H1975; \(^{[6]}\)with AS49; \(^{[7]}\)with BEAS-28.
interesting VOCs could hypothetically be connected to the proliferation data (Figure 3C,D); for example, an increase in methyltartronic acid in all the co-culture unconnected cell samples that included the normal cell line (BEAS-2B) as one of the pair (Table S11, Supporting Information). This compound is a product of D-galacturonic acid in non-oxidative conditions.\textsuperscript{[14]} D-galacturonic acid, a sugar acid found endogenously, is part of specific polysaccharides shown to have anti-proliferative properties.\textsuperscript{[15,36]} This could potentially explain the marked reduction in proliferation of normal cells compared to co-cultures with

Figure 3. Volatile impact on growth kinetics in different co-culture headspace of unconnected cells: A) Monocultures for 24, 48, and 72 h; B) Same-cell pair combinations for 24, 48, and 72 h; C) Headspace co-culture for 24 h, D) Headspace co-culture for 48 h, E) Headspace co-culture for 72 h. Data are presented as mean values ± SD of three experiments (p < 0.05).
the cancerous cells, and might be related to a volatile signal. Interestingly, different levels of galacturonic acid are found in urine and feces of cancer patients.\[37\] Another interesting result is N-methyltaurine elevation in all bulk level H1299 samples (including monoculture and co-culture unconnected pairs). N-methyltaurine is a breakdown molecule of a number of N-acyl amides, specifically N-acyl taurine molecules. This family of molecules is involved in a range of physiological functions as lipid signaling molecules.\[38\] Specifically, N-acyl taurines can act anti-proliferatively in prostate cancer cells.\[39\] Therefore, increase in N-methyltaurine might be a direct result of raised levels of different N-acyl taurines that, in turn, could have inhibited proliferation of the cells in co-cultures. Yet another example is 2-methyl-2-hepten-6-one (aka Sulcatone) seen to be increased in all bulk level H1975 samples (including monoculture and co-culture unconnected pairs (Table S11, Supporting Information)). According to the HMDB registry, this molecule is found endogenously and can take part in cell signaling (HMDB0035915), and thus could explain the changes in proliferation rates seen in Figure 3 that relates to H1975 unconnected co-culture pairs.

Dimethyl succinate is another compound that is raised in all the different cell samples and is related to lipid metabolism and lipid peroxidation in the HMDB registry (HMDB0033837), which is associated with VOC production in cancer. These results indicate that different small chemicals take part in cell signaling and proliferation. Some of these molecules are also VOCs; as such, they have the potential of triggering changes when sharing the same headspace, implying that such molecules can move between liquid and gaseous phase, and thereby eventually inducing biochemical changes, such as the rate of proliferation (as in Figure 3).

A novel and unexpected finding is that, upon communication in co-culture pair combinations (Figure 1C), VOCs are released from the cells in the conditioned media, which can induce in vitro cross talk between cells. We detected a significantly augmented level of VOCs concentration in the samples collected from cells compared with control media. Together, the data indicate that cancer cells “talk to one another,” and this cross talk induces changes in different or same cell pair groups; we noted decreased hydrocarbons and increased ketone levels in different signaling pathway. These findings explain the significant contribution of VOCs in cell-to-cell cross talk with regard to cancer sign that could offer a possible therapeutic strategy to reduce tumor progress. Further investigations are necessary to follow the effect of VOC cross talk on these signaling pathways and strengthen the significance of these new observations.

For each cell type, we carried out an isolation process that enabled reaching two and four cells ahead of time. As the biochemical behavior of the cells changes with time, we also examined the VOCs profile at different time-points, $T_0$, $T_{24}$, and $T_{48}$. VOCs were analyzed by GC-MS as previously described.\[20\] The VOCs detected here are given in Table S4, Supporting Information. Cross-sectional comparison between one and two cell levels, out of the 18 different VOCs, 14 VOCs commonly appeared in different cell lines at all incubation times (Figure 4; Table S4, Supporting Information). Interestingly, four new VOCs (pentadecanal; nonane-4,5-dimethyl; tridecane;...
and 3-methyl-2-heptene) were identified only at the two and four cell levels, with significant changes in different cell lines (Figure 5A; Table S5, Supporting Information).

In more specific detail, these new compounds are decane, undecane, cyclohexanone, and hexadecanoic acid–butyl ester (Figure 5B; Table S6, Supporting Information). The experimental results are summarized in Tables S7A–D and S8A–D, Supporting Information; and Tables S9–S11, Supporting Information, summarizes the average concentration levels and calculated LOD of our system under experimental conditions in ppb. Interestingly, cyclohexanone (produced at the 2-cell level) was not detected in the co-culture and monoculture of bulk samples when cultured under the same headspace in physically unconnected and connected groups (Figure 1A–D). Cyclohexanone may be a potential breath biomarker for lung cancer patients. [40]

Similar results come from decane, undecane, and tridecane produced by the monocultures at the 4-cell level, but are not produced during interaction of these cell lines. Hence, the production of such potential signaling VOCs in nature seems to depend strongly on interspecific interactions. VOCs produced in cell culture experiments are very complex, consisting of a mixture of many compounds that are difficult to identify. [26,41]

Most of the VOCs that were tentatively identified within this study (~50%) are hydrocarbons (Table S11, Supporting Information). The composition of the VOCs depends significantly on media composition and growth conditions. [42,43] Interestingly VOCs emitted by the monocultures of all cell types at two and four cell levels were also found in the bulk cells and could be the cause of the induced changes in bulk cell morphology and growth rates, as explained above.

Throughout each cell life time, different metabolic processes occur to sustain life that have to adjust to both systemic and local stimuli. [43] A major part of these chemical changes is energy production, anabolism, catabolism, and the removal of waste. [20] These processes guarantee the health of the cells in sustaining critical functioning under normal conditions. It is mainly enzymes interacting with different biochemical compounds (proteins, lipids, carbohydrates) that drives these metabolic processes. [20] When stressed, cells typically response by triggering different intracellular cascades involving many enzymes and signaling pathways, [20] thereby adapting them to changing microenvironmental requirements. Such (patho) physiological processes arising during different diseases can alter metabolism from the single cell level right up to

Figure 5. Heat-map showing VOCs profile changes in three different single-cell levels: 1, 2, and 4 cell levels. Color coding shows the peak area of each compound measured in the 1, 2, and 4 cell levels, samples normalized with internal standard peak area calculated in all samples. A) BEAS-2B. B) A549. C) H1299. D) H1975. Note: VOC1–VOC26, Table S4, Supporting Information.
complete organ response, such as the liver, to sustain homeostasis. Some of these metabolites are at least VOCs and thus might have an important role in communication and disease metabolism.[44]

2.5. Limitations of Study

There have been some limitations in the present study. We decided to fix the background baseline of an experiment by using the same culture media for all cells, as in many previous studies. Thus, BEAS-2B cells were not cultured in the recommended media. This approach can ensure that the media per se is not the source of different compounds, something that cannot be done when using different media. Future studies need to evaluate the delta change of each cell line in its recommended media, which can provide additional information. Second, bulk data were calculated relatively to peak area, tentatively identified, and was not validated for absolute concentration, as it was not the main goal in this study. Third, we refer here to changes related to the p53 difference, as this is a major difference; however, other differences might be responsible for some of the changes in the results and as such should be interpreted cautiously in future studies. Fourth, in order to isolate and grow single cells, we have used serum-free media in the first growth step to slow proliferation. This might have affected VOC profiles; however, this step was done for all cell lines, thereby ensuring the same baseline in all. Fifth, VOC detection at the single cell level remains technically challenging due to many fundamental limitations, including the rapid changing of the VOCs, small sampling volumes, low number of VOCs and diverse range of volatiles present in the cell, along with at-present inadequacies in the sensitivity of analytical instruments. Thus, the metabolic mechanism of these different compounds needs further investigation. Finally, p < 0.05 was considered as significant for the different potential VOCs. This value is the accepted standard; however, the authors are aware that more restrictive values and correction would be preferable. Because this is a first-of-its kind experiment within a new and unknown field, we considered that a 5% restriction is better to avoid losing important data at this stage. Subsequent experiments will require additional statistical restrictions and correction to strengthen results, and remove additional potential false discoveries. Many of the VOCs presented here had a p-value < 0.01. However, the technology used to analyze the volatiles from single cells can provide valuable insights into biological interactions, which the fields of genomics and transcriptomics themselves might be unable to deliver. This allows a better understanding of the unique properties of cells, cell-to-cell communication and cell–environment interactions. Significant single cell volatolomic data will provide insights to permit the development and testing of hypotheses that might identify the fundamental biological mechanisms and address clinical issues in diagnostics and diseases. Nevertheless, current developments indicate a forthcoming model-shift from the analysis of volatolomics to the study of single cell volatolomics, which will enhance other “omics” approaches on the path toward better combined systems biology of single cells.

3. Conclusions

This study shows the potential role of VOCs as signaling/communication agents that exchange with neighboring cells. Based on the results, it is evident that cancer cells can release VOCs that are taken up by neighbor cells. An unexpected finding has been the novel demonstration, that upon cross talk in culture, VOCs are released from cells in conditioned media that can induce in vitro cross talk between cells (Figures 4 and 5). This indicates that cancer cells communicate with one another, and that this cross talk induces changes in different co-culture arrangements of cells. These findings can potentially provide alternative and/or complementary perspectives to proteomic and genomic approaches in monitoring cancer development. Evidence for VOC-like endocrine secretion signaling would open a new avenue for developing smart sensors/devices that can detect cell communication mechanisms and genetic alterations through simple, painless, quick, and highly accurate breath or skin test, thus enabling inexpensive monitoring of metastatic or therapy response to cancer. It could also potentially serve as a target for novel therapies. The VOC-based cell-to-cell cross talk has the advantage of being suitable for transfer/diffusion over relatively long distances between the cells, and/or from one cell to another destination inside/outside the human body.

4. Experimental Section

Methods are included in Supporting Information.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

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