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Research paper

Multi-omics analysis of ten carbon nanomaterials effects highlights cell type specific patterns of molecular regulation and adaptation

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ABSTRACT

New strategies to characterize the effects of engineered nanomaterials (ENMs) based on omics technologies are emerging. However, given the intricate interplay of multiple regulatory layers, the study of a single molecular species in exposed biological systems might not allow the needed granularity to successfully identify the pathways of toxicity (PoT) and, hence, portraying adverse outcome pathways (AOPs). Moreover, the intrinsic diversity of different cell types composing the exposed organs and tissues in living organisms poses a problem when transferring *in vivo* experimentation into cell-based *in vitro* systems.

To overcome these limitations, we have profiled genome-wide DNA methylation, mRNA and microRNA expression in three human cell lines representative of relevant cell types of the respiratory system, A549, BEAS-2B and THP-1, exposed to a low dose of ten carbon nanomaterials (CNMs) for 48 h. We applied advanced data integration and modelling techniques in order to build comprehensive regulatory and functional maps of the CNM effects in each cell type.

We observed that different cell types respond differently to the same CNM exposure even at concentrations exerting similar phenotypic effects. Furthermore, we linked patterns of genomic and epigenomic regulation to intrinsic properties of CNM. Interestingly, DNA methylation and microRNA expression only partially explain the mechanism of action (MOA) of CNMs. Taken together, our results strongly support the implementation of approaches based on multi-omics screenings on multiple tissues/cell types, along with systems biology-based multi-variate data modelling, in order to build more accurate AOPs.

1. Introduction

Since the rapid expansion of the nanotechnology, nanotoxicology has emerged as an important discipline to ensure safe innovation. Nanomaterial toxicity is currently investigated by extensive animal studies, but more and more emphasis has been put into *in vitro*-based approaches as well as in understanding the relevant molecular mechanisms involved in engineered nanomaterials (ENMs) exposures. Several studies have already addressed the importance of profiling the transcriptome of exposed biological systems for understanding the molecular alterations caused in response to ENMs exposure (Nel, 2013; Nel et al., 2013; Marx-Stoelting et al., 2015; Jennen et al., 2011; Robinson et al., 2012; Tralau et al., 2015). However, the separate investigation of certain molecular domains does not allow to build a thorough landscape of the ENMs mechanism of action (MOA). Hence, to better model and predict the long-term adaptation of an exposed biological system, more comprehensive and integrative analyses, interrogating multiple molecular districts, need to be carried out. Still, to the best of our knowledge, there have not yet been to date comprehensive attempts to study ENMs MOA using a multi-omics approach.

In humans, exposure to ENMs mainly happens in production environments *via* the airways. We and others already showed that ENMs are able to exert toxic effects on the respiratory system, by using animal exposure models (Kinaret et al., 2017a; Rydman et al., 2015; Rydman et al., 2014). Apart from the pathological responses, ENMs MOA has been investigated in different tissues (Kinaret et al., 2017b; Fröhlich, 2017; Costa and Fadeel, 2016), by analyzing the molecular perturbations that these materials are able to induce on the normal

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transcriptional program of exposed cells and tissues.

However, responding to the constant pressure in developing reliable and efficient alternative screening methods, many studies tried to predict the potential ENMs effects *in vivo* by analyzing their effects *in vitro* (Drasler et al., 2017; Braakhuis, 2015; Törnqvist et al., 2014; Bachler et al., 2015). We recently demonstrated that carbon based nanomaterials have distinct transcriptional MOA between *in vivo* and *in vitro* experimental settings and that commonalities are to be found by using comprehensive gene network models (Kinaret et al., 2017b).

Changes in the levels of gene transcription can be usually appreciated at short term and acute responses, but they are less effective in explaining longer effects of ENMs exposure, which are of greater interest to model the real-life exposure scenarios. More recently, a number of studies have focused on the alterations caused by ENMs exposure at the level of DNA methylation (Chen et al., 2017; Sierra et al., 2017). Investigating epigenomic mechanisms, such as alteration of DNA methylation and microRNA expression, can indeed capture more persistent molecular changes underlying long-term transcriptional programs.

In this study, we exposed three human lung-derived cell lines, representative of major cell types of the respiratory system, to sublethal doses of ten different carbon nanomaterials for two days, assessed their genome-wide effects on three distinct molecular layers simultaneously, the DNA methylation, the microRNA and mRNA expression, and modelled their functional interactions.

By performing a comprehensive integrative analysis, we provide a broad picture of the cross-talk between regulatory factors (DNA methylation and miRNAs) and mRNA deregulation following the exposure to carbon nanomaterials. Further, we show how the MOA of the same carbon nanomaterials vary according to the cell type/tissue of origin, thus highlighting the importance of considering a heterogeneous and representative set of cell types for a target organ when testing the effects of engineered nanomaterials *in vitro*.

2. Results and discussion

2.1. Experimental setup

In this study, we investigated the effects of ten well characterized carbon nanomaterials (CNMs, Table 1) on three cell lines of human alveolar epithelium (A549), bronchial epithelium (BEAS-2B) and macrophages (PMA-differentiated THP-1). We focused on low dose exposure for 48 h and thoroughly characterized their effects by interrogating three different molecular districts genome-wide, the mRNA, microRNA, and DNA methylation.

2.2. Cell viability and cytotoxicity

Most of the *in vitro* exposure studies concentrate on acute effects (usually within 24 h), with relatively high ENM concentrations that do not correlate well with *in vivo* conditions or with the human long-term exposure scenarios (Landsiedel et al., 2014). Gangwal et al. estimated that a life-time (45 years) exposure duration of the alveolar mass retention for TiO_2, Ag and CNT ranges from 10 to 50 μg/cm^2. Based on the proposed model, the mass retention corresponds to *in vitro* studies with relatively high ENM concentrations ranging from 30 to 400 μg/ml (Gangwal et al., 2011).

Here, nanomaterial exposures to THP-1, BEAS-2B and A549 cell lines decreased the cell viability in dose-dependent manner, especially with the three highest tested doses of 50, 100 and 500 μg/ml (Figs. S1–S3). Lactate Dehydrogenase (LDH) release was concordant with the cell viability measures in BEAS-2B and THP-1 showing a clear LDH increase after 48-h CNM exposure at the highest doses (Figs. S2, S3), while A549 seemed to be the least responsive cell line (Fig. S1). In particular, THP-1 was the most sensitive (Fig. S3), showing slightly more elevated LDH levels, when compared to other cell lines. This was especially observed with GNF and MIT_MW, that have a known toxic potential. The lower doses, ranging from 0.1 to 5 μg/ml, did not to cause major responses, with highly similar LDH levels and consistent cell viability. Given these results, the 10 μg/ml concentration was chosen for showing no significant cell death, but still indicating some responses as compared to the baseline, thus corresponding more to a subchronic rather than acute response.

2.3. Molecular effects

In addition to a variety of *in vitro* end point assessments, OMICS approaches allow for comparison of different *in vitro* systems as well as better correspondence to *in vivo* exposure scenarios (Kinaret et al., 2017b; Drasler et al., 2017). Combination of different OMICS methods can inform more thoroughly about ENMs MOA, which could be further used in ENM grouping approaches, as suggested by Riebeling et al. (2017). Gene expression profiling of co-cultures and 3D cultures, as well as systems taking into account exposure route such as Air Liquid Interface cultures (Latvala et al., 2016; Kletting, 2017), better resemble the *in vivo* exposure scenarios, but before clear conclusions about the ENM MOA can be drawn, cell type-specific responses need to be addressed by omics approaches (Drasler et al., 2017; Snyder-Talkington et al., 2015). The importance of multi-omics approaches in the study of MOA of toxicants has been demonstrated in several toxicogenomics studies (Jayapal, 2012; Gavin, 2016). On the other hand, focusing on one molecular layer alone might not be sufficient to fully describe the MOA of an external stimulus (Huang et al., 2017). The analysis of a single molecular layer can reveal information that is mainly related to

Table 1
Tested nanomaterials and their characteristics.

Material name	Producer	Acronym	Type	Length (nm)	Diameter (nm)	Surface area (m2/g)	Aspect ratio	References
Carbon black (Evonik)	Evonik industries/ Degussa	CBL	Particle	14	14	265	1	(Vippola et al., 2009)
Fullerene C60 (MTR)	MTR Ltd.	FUL	Sphere	100	100	20	1	(Lehto et al., 2014)
Graphite nanofibers (Sigma)	Sigma-Aldrich	GNF	Fiber	10,000	140	32	71.4	(Vippola et al., 2009)
Singlewalled carbon nanotube (Sigma)	Sigma-Aldrich	SIG_SW	Tube	50,000	1.1	567	45,454	(Vippola et al., 2009)
Singlewalled carbon nanotube (SES)	SES research	SES_SW	Tube	1500	2	436	750	(Vippola et al., 2009)
Multiwalled carbon nanotube (Bayer)	Bayer material science	BAY_MW	Tube	1000	14.5	204	68.9	(Vippola et al., 2009)
Multiwalled carbon nanotube (Mitsui)	Mitsui & Co.	MIT_MW	Tube	13,000	50	22	260	(Kinaret et al., 2017a)
Multiwalled carbon nanotube (SES)	SES research	SES_MW	Tube	1500	20	60	75	(Vippola et al., 2009)
Multiwalled carbon nanotube (cheaptubes)	Cheaptubes Inc.	CHT_MW	Tube	30,000	11.5	180	2608	(Rydman et al., 2015)
Multiwalled carbon nanotube (Sigma)	Sigma-Aldrich	SIG_MW	Tube	100,000	15	119	6666	(Vippola et al., 2009)