

1 **No genome-wide DNA methylation changes found**
2 **associated with medium-term reduced graphene oxide**
3 **exposure in human lung epithelial cells**

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30 **Keywords**

31 Epigenetics; DNA methylation; nanomaterials; graphene; exposure

32

33 **Abstract**

34 The presence of nanomaterials in our everyday life is ever increasing, and so too are
35 concerns about the possible health consequences of exposure to them. While evidence
36 of their biological activity is growing, there is still scant knowledge of the epigenetic
37 mechanisms that could be at play in these processes. Moreover, the great variability in
38 the chemical and physical structures of these compounds handicaps the study of their
39 possible health risks. Here we have synthesized reduced graphene oxide (rGO) through
40 the thermal exfoliation/reduction of graphite oxide, and characterized the resulting
41 material. We have then made use of Illumina's MethylationEPIC arrays and bisulfite
42 pyrosequencing to analyze the genome-wide and global DNA methylation dynamics
43 associated with the medium-term exposure of human lung epithelial cells to rGO at
44 concentrations of 1 and 10 $\mu\text{g}/\text{mL}$. The results show no genome-wide or global DNA
45 methylation changes associated with either condition. Our observations thus suggest

46 that medium-term rGO exposure does not have significant effects on the DNA
47 methylation patterns of human lung epithelial cells.

48

49 **Introduction**

50 Nanomaterials, those with dimensions below the 100 nm limit, are a growing
51 component of our everyday life. Emanating from sources such as food, cosmetics or
52 the chemical and biomedical industries, human exposure to nanomaterials has
53 substantially increased during recent decades. Some of the most fundamental players
54 in the nanomaterial “revolution” of recent times have been carbon-based materials,
55 such as fullerenes, carbon nanotubes (CNTs) and graphene. Their unique electrical,
56 mechanical and thermal properties have led to their use in the development of a wide
57 spectrum of technologies, like electrochemical storage (e.g. solar cells, batteries),
58 electronics (e.g. transistors, field emission) and even biological technologies such as
59 photothermal therapy and drug delivery ¹. Thus, directly or indirectly, these
60 components are, or will soon be, present in our everyday lives, and in tandem with this
61 increasing exposure, concern about their potential threat to human health has risen, in
62 the case of both naturally occurring and engineered nanomaterials (ENMs) ².

63 The potential toxicity of nanomaterials can be influenced not only by their composition,
64 but also their size, the dose involved, time of exposure and target tissue ³. Although
65 the interest in the potential adverse effects caused by nanomaterials is recent, there is
66 already considerable evidence of their biological activity in both *in vitro* and *in vivo*

67 models ³⁻⁵, while, however, more limited evidence exists regarding direct associations
68 with human exposure ⁶.

69 Epigenetic mechanisms constitute molecular pathways which can integrate
70 environmental inputs to produce genomic responses ⁷ and thus are interesting targets
71 for the study of the biological effects of nanomaterial exposure. To date, research has
72 been carried out on the main epigenetic marks: DNA methylation (the covalent
73 addition of methyl groups to DNA cytosines), histone modification (various post-
74 translational modifications such as acetylation or methylation that occur at histone
75 tails) and non-coding RNAs. The observed effects are varied and depend on a multitude
76 of variables, although general trends have been observed in some works, especially
77 regarding DNA methylation, such as a tendency towards DNMT (DNA
78 methyltransferase) downregulation and DNA hypomethylation ^{8,9}. However, most
79 studies have focused on changes either from a local perspective or have looked at total
80 cellular levels of epigenetic marks. While providing necessary and informative results,
81 genome-wide studies using up-to-date technologies are essential in order to uncover
82 the genomic dynamics of epigenetic processes such as DNA methylation, which is
83 context-specific ^{10,11}, and also to detect more subtle changes which could in fact be the
84 consequences of nanomaterial exposure in real-life settings.

85 Graphene derivatives such as reduced graphene oxide (rGO) and graphene oxide (GO)
86 are currently being investigated for their biomedical applications, which cover a wide
87 range of technologies such as their biofunctionalization with proteins, their use as drug
88 delivery carriers, and the development of graphene-based biosensors ¹². It is therefore

89 important to characterize the possible biological effects that these materials could have
90 on living beings, and particularly to study the impact of these nanomaterial in human
91 contact applications. In the present study we have focused on rGO, which is a
92 graphene-like material produced by the reduction of oxidized graphene/graphite ¹³.
93 The main advantages of this method are its low-cost and the high-yield obtained,
94 meaning that, ideally, rGO could constitute an advantageous methodology for
95 generating graphene-like materials. In general, rGO effects in various *in vitro* and *in*
96 *vivo* settings are usually smaller as compared to GO, or indeed non-existent ¹⁴,
97 indicating that rGO could be a more biocompatible material, and that its biological
98 characterization is therefore important. All the same, although various biochemical
99 parameters have been studied, epigenetic analyses, especially on a genome-wide scale,
100 remain to be carried out.

101 In this study we have synthesized rGO by the thermal exfoliation/reduction of graphite
102 oxide and characterized the resulting material. Subsequently we analyzed the genome-
103 wide DNA methylation dynamics associated with mediun-term exposure to different
104 concentrations of rGO in human lung epithelial cells using the Illumina MethylationEPIC
105 platform, which covers more than 800000 CpG sites and serves to potentially identify
106 DNA methylation changes across the genome. Furthermore, we have also analyzed
107 global levels of DNA methylation by bisulfite sequencing of representative repetitive
108 DNA *loci*.

109

110

111 **Materials and methods**

112

113 *Synthesis and characterization of reduced graphene oxide*

114 The material was prepared from a commercial synthetic graphite (Sigma-Aldrich) by an
115 initial oxidation using a previously described modified Hummers' method ¹⁵ to obtain
116 graphite oxide, followed by a thermal exfoliation/reduction process similar to that
117 described by Álvarez P. and colleagues ¹⁶ (see Supplementary Methods for additional
118 information).

119 The elemental composition of the sample was determined in a LECO-CHNS-932 micro-
120 analyzer and the oxygen content was determined directly in a LECO-TF-900 furnace
121 coupled to the previous equipment ¹⁵. The analyses were performed using 1 mg of
122 ground sample. The results are quoted from an average of the values of four
123 determinations. In all cases, experimental error was < 0.5 % of the absolute value.

124 X-ray diffraction (XRD) analysis of the powdered sample was performed using a Bruker
125 D8 Advance diffractometer. The radiation frequency employed was the K α 1 line from
126 Cu (1.5406 Å), with a power supply of 40 kV and 40 mA ¹⁵. The interlaminar distances
127 (d_{002}) of sheets and the average crystallite size along the c-axis (L_c) were obtained from
128 the (002) reflection of the XRD patterns, which were recorded at steps of 0.01° and
129 intervals of 6 s per step, using the Bragg's law and the Scherrer equation respectively.

130 X-ray photoelectron spectroscopy (XPS) analyses were carried out in a VG-Microtech
131 Mutilab 3000 device. The XPS C1s peak was analysed using a peak synthesis procedure
132 that employs a combination of Gaussian and Lorentzian functions ¹⁷ in order to identify

133 the functional groups and their respective percentages. The binding energy profiles
134 were deconvoluted as follows: undamaged structures of Csp²-hybridized carbon (284.5
135 eV), damaged structures or sp³-hybridized carbons (285 eV), C–OH groups (285.7 eV),
136 C–O–C functional groups (287 eV), C=O functional groups (287.5 eV) and C(O)OH
137 groups at 288.7 eV).

138 The specific surface area was calculated from the N₂ adsorption isotherm at 77 K using
139 the Brunauer–Emmett–Teller (BET) equation ¹⁸. The isotherm was obtained using an
140 ASAP 2020 Micromeritics equipment. The sample was outgassed at 300 °C for 3 h under
141 vacuum conditions prior to the test.

142 SEM (scanning electron microscopy) images were obtained using a field emission gun
143 scanning electron microscope (QUAN-TAN FEG 650, FEI) operating at 5 kV, and TEM
144 (transmission electron microscopy) observations were performed with a JEOL 2000 EX-
145 II instrument operating at 160 keV ¹⁵.

146

147 ***Cell culture, nanomaterial preparation and exposure***

148 Human airway epithelial BEAS-2B cells were provided by Dr. G.M. Albaiceta's group
149 (Instituto de Investigación Sanitaria del Principado de Asturias). The cells were cultured
150 as a monolayer with serum-free BEGM medium (Bronchial epithelial cell growth medium,
151 Lonza) in culture dishes. This medium is complemented with the following factors:
152 retinoic acid, insulin, hydrocortisone, transferrin, epinephrine, triiodothyronine, Bovine
153 Pituitary Extract, hEGF (human epidermal growth factor) and GA-1000 (Gentamicin and
154 Amphotericin). Cultures were maintained at 37°C in a humidified atmosphere of 5 % CO₂.

155 The rGO stock solution was prepared by pre-wetting with ethanol followed by
156 dispersion in BSA-water (Nanogenotox protocol 2011, see Supplementary Methods for
157 additional information). For rGO exposure, the working dilutions of 1 and 10 $\mu\text{g}/\text{mL}$
158 were prepared directly in cell culture medium. 150 cm^2 dishes were seeded with 10^6
159 cells, which were left to attach for 4-8 hours prior to the addition of the nanomaterials.
160 Exposure medium was renewed every 4-5 days.

161

162 ***Global and repetitive DNA methylation analyses***

163 Bisulfite pyrosequencing was used for the evaluation of the DNA methylation status of
164 5 different families of repetitive DNA: LINE1¹⁹, D4Z4 and NBL2²⁰, SAT α and AluYb8²¹
165 (see primer sequences in Table S2). First, genomic DNA was isolated by standard
166 phenol-chloroform extraction. Secondly, bisulfite conversion was performed with the
167 EZ DNA methylation-gold kit (Zymo Research) following the manufacturer's
168 instructions. Thirdly, modified DNA was amplified by PCR and, finally, pyrosequenced
169 using PyroMark 24 reagents and a vacuum prep workstation, equipment and software
170 (Qiagen).

171

172 ***Genome-wide DNA methylation analyses***

173 The Illumina Infinium MethylationEPIC BeadChip was used for the genome-wide
174 interrogation of the DNA methylation status of more than 800000 CpG sites in the
175 human BEAS-2B cell line genome. A total of 12 samples were analyzed, which consisted
176 of 3 exposure groups (control, 1 and 10 $\mu\text{g}/\text{mL}$ rGO) at 2 time intervals (15 and 30

177 days), each condition having 2 technical replicates. DNA samples were hybridized to
178 the BeadChip following the Illumina Infinium HD methylation protocol, and this service
179 was provided by the Centro Nacional de Genotipado (CEGEN-ISCI, Spain,
180 <http://www.cegen.org>).

181

182 ***Infinium MethylationEPIC BeadChip data preprocessing***

183 All the data were preprocessed by means of the statistical software R (version 3.4.2).
184 The IDAT files from the Illumina MethylationEPIC array were processed following a
185 pipeline built on the R/Bioconductor package minfi (version 1.22.1)²². Probes which
186 have been shown to be potentially crossreactive or multimapping²³, those overlapping
187 genetic variants²⁴ and those with a detection p-value > 0.01 in at least one sample
188 were filtered out. The methylation data was normalized in a two-step procedure: first
189 using the Noob method²⁵ as implemented in minfi for background correction and
190 subsequently using the BMIQ method²⁶ to correct for probe design bias, implemented
191 in the R/Bioconductor package ChAMP (version 2.8.9)²⁷. β -values were extracted with
192 the *getBeta* minfi function and M-values were obtained by the logit transformation of
193 the normalized β -values with the R/Bioconductor package lumi (version 2.28.0)²⁸.

194

195 ***Genomic annotation of array probes***

196 MethylationEPIC BeadChip probes were mapped to different CpG island-associated or
197 gene-associated regions using the annotation of the R/Bioconductor package
198 IlluminaHumanMethylationEPICanno.ilm10b2.hg19 (version 0.6.0). To annotate array

199 probes to genomic repeats, because most of the probes do not cover repetitive
200 elements, their locations were expanded to 100 bp windows and checked for overlaps
201 with repetitive DNA families extracted from RepeatMasker libraries (hg19 - Feb 2009 -
202 RepeatMasker open-4.0.5 - Repeat Library 20140131) ²⁹. This strategy, adapted from
203 Lai et al. ³⁰, uses close-proximity probes as surrogates for the methylation status of
204 repeat elements.

205

206 *Characterization of BEAS-2B's DNA methylation patterns and definition of differentially*
207 *methyated probes in the exposure experiments*

208 To characterize the genome-wide DNA methylation patterns of the BEAS-2B cell line
209 we collapsed the measurements for the 4 control samples used in the rGO exposure
210 experiments, which allowed us to have highly reliable DNA methylation measurements
211 for the CpG sites. To achieve this, we first filtered out CpG sites which varied more than
212 10 % between the different samples (3 % of all probes) and then averaged the DNA
213 methylation values of the remaining sites (a total of 774728) between the 4 controls.

214 To perform gene ontology analyses, the R/Bioconductor package missMethyl (version
215 1.10.0) ³¹ was used (see Supplementary Methods for additional information). To better
216 visualize the results obtained, the significant ontologies detected were semantically
217 summarized using the online tool ReViGO (<http://revigo.irb.hr/>) ³².

218 To study differentially methylated CpG sites between the conditions we collapsed the
219 two technical replicates for each condition into a single measurement. In this case we
220 first filtered out probes with differences in β -values between the pairs of technical

221 replicates larger than 5 % (on average, 5 % of the probes) and then averaged the β -
222 value of each remaining CpG site between the two replicates (a stricter threshold was
223 used because only 2 samples were collapsed). Finally, we defined as differentially
224 methylated those probes with absolute differences in β -value between different
225 conditions equal to or over 30%. We further looked for lesser differences, such as 20 %
226 or 10 %.

227

228 **Results**

229

230 *Characterization of reduced graphene oxide*

231 The oxidation of the synthesized graphene material (see Methods) was confirmed by
232 XRD (Figure 1A); the interlayer distance (d_{002}) was 0.35 nm, the average crystallite size
233 along the c-axis (L_c) was 0.89 nm, and the average number of stacked layers, estimated
234 by the equation $(L_c/d) + 1$, ranged from 3 to 4. Representative SEM and TEM images
235 showing the morphology of the rGO are shown in Figure 1B. The rGO showed an
236 oxygen content of up to 24 wt. %, and low contents of sulfur (1.1 wt. %), nitrogen (0.3
237 wt. %), and hydrogen (0.9 wt. %), determined by elemental analysis. This oxygen was
238 mostly in the form of epoxy groups (11.3 at. %), followed by alcohols (8.6 at. %),
239 carboxylic groups (6.4 at. %), and ketonic groups (4.8 at. %), according to XPS spectra
240 (Figure 1C). The calculated BET surface area of the rGO was 397 m²/g, and the isotherm
241 (Figure 1D) showed a steady increase in nitrogen adsorption and hysteresis in the

242 desorption branch, which is indicative of the presence of mesopores (mainly several
243 tens nm), as a result of the mis-stacking of single layers (or small packs of a few layers).

244 **[FIGURE 1 HERE]**

245

246 *Genome-wide DNA methylation characterization of human lung epithelial cells*

247 We used the Illumina MethylationEPIC platform to characterize the genome-wide DNA
248 methylation patterns of BEAS-2B human lung epithelial cells, and observed the
249 expected bimodal distribution of DNA methylation (Figure 2A, upper panel) whereby
250 most of the probes were either unmethylated or highly methylated. To better
251 characterize the epigenomic patterns observed, we calculated gene ontology
252 enrichments for the genes associated with highly methylated (β -values > 0.8) or
253 unmethylated (β -values < 0.2) CpG sites.

254

255 **[FIGURE 2 HERE]**

256

257 DNA methylation can have different values within one particular gene and, specifically,
258 we found that as much as 75 % of the genes interrogated by the array contained both
259 highly- and lowly-methylated probes in the BEAS-2B cells, and therefore we focused
260 on genes which exclusively contained high- or low-methylation CpG sites (a total of
261 3357 and 2491 genes, respectively, see Supplementary Methods). Interestingly, while
262 lowly-methylated genes were associated with several distinct pathways, some relating
263 to the negative regulation of biological processes (Figure 2A, lower left panel), highly-

264 methylated genes were associated with many fewer pathways (Figure 2A, lower right
265 panel; see Table S1 for full information), suggesting that genes with low levels of DNA
266 methylation are associated with specific functional pathways in the BEAS-2B cell line.

267

268 Next, we studied the DNA methylation state of the lung epithelial cells at different
269 genomic sites. Regarding CpG island membership (Figure 2B, upper panel), the lowest
270 DNA methylation values were found at CpG islands, followed by immediate border
271 elements (island shores), while high methylation values were found at farther elements
272 (island shelves) and open sea sites. Furthermore, when looking at gene-region
273 classifications (Figure 2B, lower panel), low DNA methylation was found at transcription
274 start sites and first exons – but not at 5'UTR regions – while high DNA methylation
275 values were mainly observed at gene bodies and 3'UTR regions, and intermediate-high
276 values at intergenic regions (the median methylation of all of the interrogated probes
277 was 0.59). Thus, DNA methylation levels are correlated with CpG-density and specific
278 genic regions at a genome-wide level in the BEAS-2B cell line.

279

280 *Genome-wide DNA methylation changes upon reduced graphene oxide exposure*

281 In order to characterize DNA methylation changes associated with rGO exposure in
282 human lung epithelial cells, we designed an experimental setup with 2 different
283 nanomaterial concentrations and 2 different exposure times (Figure 3A, left panel): 1
284 or 10 $\mu\text{g}/\text{mL}$ for either 15 or 30 days (as well as control groups with no exposure). To
285 capture DNA methylation states at a genome-wide level, we used the Illumina

286 MethylationEPIC platform on 2 technical replicates per condition, for a total of 12
287 arrays. We collapsed the replicates in each condition to define site specific DNA
288 methylation values by retaining sites with differences between measurements of less
289 than 5 % (Figure 3A, right panel; see methods), resulting in a final tally of 696365
290 evaluated CpG sites across all conditions.

291

292 [FIGURE 3 HERE]

293

294 Next, we compared the different experimental conditions in order to look for
295 differentially methylated sites. Interestingly, we found no DNA methylation changes
296 associated with rGO exposure at concentrations of either 1 or 10 µg/mL at either 15 or
297 30 days of exposure. Correlation analyses (Figure 3B) showed a very strong epigenomic
298 similarity between all of the conditions, especially within the same time groups. We
299 lowered the threshold to 10 % difference in methylation and still found no differentially
300 methylated CpG sites. Our results therefore indicate that lung epithelial cells do not
301 suffer significant DNA methylation changes when confronted with rGO during
302 medium-term exposure.

303

304 ***Global and repetitive DNA methylation changes upon reduced graphene oxide exposure***

305 Finally, we looked for global DNA methylation differences by performing bisulfite
306 pyrosequencing on 5 different families of repetitive DNA: LINE1¹⁹, D4Z4 and NBL2²⁰,
307 SATα and AluYb8²¹ (see primer sequences in Table S2 and pyrosequencing

308 measurements in Table S3). These multi-represented sequences cover large portions
309 of the whole genome, and have been regularly used as proxies for the evaluation of
310 the global levels of DNA methylation across the whole genome (e.g. LINE1, AluYb8), or
311 at specific regions, e.g. subtelomeric D4Z4 repeat or the centromeric SAT α repeats.

312

313 **[FIGURE 4 HERE]**

314

315 In consonance with the earlier genome-wide specific approach, we found no DNA
316 methylation changes associated with rGO exposure at either concentration or exposure
317 time for any repeat family (Figure 4). Furthermore, we made use of our previously
318 generated genome-wide data to validate the bisulfite pyrosequencing observations by
319 mapping the array probes to different repetitive DNA locations and looking at the DNA
320 methylation β -values measured by the array (see methods). We again found no
321 differences between conditions for a variety of genomic repeat families (see Figure S1).
322 Thus our results suggest that lung epithelial cells do not experience extensive global
323 and repetitive-associated DNA methylation changes when exposed to reduced
324 graphene oxide.

325

326

327 **Discussion**

328 Interest in the potential consequences of the biological activities of nanomaterials has
329 risen dramatically in the last few years. However, while biochemical and physiological

330 studies are starting beginning to be more common, genomic studies, especially on a
331 genome-wide scale are more scarce, not to mention those looking at epigenetic marks
332 ⁸. Thus there is a wide knowledge gap to fill in terms of the epigenetic mechanisms that
333 might mediate the biological effects of nanomaterials. Of these, carbon-based
334 materials are among the most important compounds application-wise, and we have
335 previously studied the impact of MWCNTs on the epigenome of human lung epithelial
336 cells ³³ because inhalation is among the most common and most studied routes of
337 exposure to nanomaterials. We also focused on medium-term exposures at low
338 concentrations, because these conditions might better mimic real human subchronic
339 exposure.

340 In the present study we have focused on rGO because it is a low-cost and promising
341 graphene-like derivative. However, the structure of rGO is far from that of perfect
342 graphene sheets, and, importantly, oxygen is still present at relevant quantities in the
343 structure of rGO, such that the hydrophilic groups present could alter its behavior in
344 biological environments and even provide functionalities different to those of GO.

345 We have investigated the genome-wide and global DNA methylation dynamics
346 associated with rGO exposure in human lung epithelial cells, utilizing 2 different
347 concentrations (1 and 10 $\mu\text{g}/\text{mL}$) with medium-term exposure times of 15 and 30 days.

348 We have used concentrations in the range of those previously reported for *in vitro*
349 studies using this and other graphene-based nanomaterials ¹⁴. These studies, which
350 evaluate physiological changes, usually choose shorter exposure windows (on a scale
351 of hours). However, we sought to characterize altered epigenetic states which are

352 maintained through larger time-scales because of the role they may play in the
353 regulation of gene expression ³⁴. Nonetheless, because in general the influence of
354 graphene-based nanomaterials (and in particular of rGO) on an epigenome-wide scale
355 has not been studied, the exposure conditions (time and concentration) which can
356 damage cells, at least on an epigenetic level, are still to be established.

357 We found no notable changes in DNA methylation associated with rGO exposure under
358 any of the conditions evaluated, and regardless of the analysis method used (i.e. global
359 or locus-specific approaches). Moreover, we used repetitive DNA-associated array
360 probes to validate the lack of differences found in the analysis of repetitive DNA
361 sequences in LINE1, D4Z4, NBL2, SAT α and AluYb8, all of which have been widely used
362 for the estimation of global levels of DNA methylation ^{19–21}. Our analyses thus indicate
363 that medium-term *in vitro* exposure to rGO at concentrations up to 10 $\mu\text{g}/\text{mL}$ has no
364 apparent effects on the epigenome of human lung epithelial cells.

365 Interestingly, a recent *in vitro* study also found no adverse short-term cytotoxic or
366 genotoxic effects induced by GO or rGO in murine lung cells at 5–200 $\mu\text{g}/\text{mL}$ doses ³⁵
367 and rGO at the same concentrations as used in our study did not cause any noticeable
368 effects in murine spermatogonial stem cells ³⁶. On the other hand, rGO nanosheets
369 were shown to have short-term cytotoxic effects on human lung cancer cells ³⁷,
370 pointing towards the choice of transformed versus normal *in vitro* models being an
371 important variable. However, another recent study has found no effects of rGO
372 nanosheet of up to 100 $\mu\text{g}/\text{mL}$ concentrations on the cell viability of human lung cancer
373 cells, although effects were found for a murine macrophage cell line ³⁸. These mixed

374 findings point to the importance of standardizing the experimental assays used to
375 evaluate cellular parameters. Our work provides insight into how doses similar to those
376 used in other *in vitro* studies produce no effects even after up to 30 days of exposure.
377 Moreover, while *in vivo* studies do show adverse effects related to the exposure of
378 graphene-based nanomaterials (GNMs), in general, rGO effects observed in various *in*
379 *vivo* settings are usually smaller compared to GO or other types of GNMs^{14,39}. This
380 effect has also been observed *in vitro*: it has been hypothesized that the higher
381 oxygenated functional group content of GO increases its cellular toxicity, as compared
382 to rGO toxicity, through ROS pathways⁴⁰. Nevertheless, a recent study using BEAS-2B
383 cells has shown that GO compounds in general generate less genotoxicity than other
384 graphene-derived materials, albeit they were found to cause slight DNA methylation
385 changes at the global level⁴¹.

386 Thus, our findings of no DNA methylation changes caused by rGO at low doses with
387 medium-term exposures are not entirely surprising. Our observations, when
388 accompanied by the aforementioned results on non-existent or generally lower cellular
389 effects of rGO versus GO suggest that rGO could have considerable biocompatibility.
390 It is important to stress that at different doses, or time exposures, to those used in this
391 work it is possible that effects might be observed, and that our results would be
392 strengthened if extended to other lung epithelial cell lines. Moreover, we have used an
393 *in vitro* model of airway epithelium as an initial approximation, but our observations
394 should be expanded upon with the use of *in vivo* models, which can more accurately
395 model the process of exposure to nanomaterials through inhalation. We have also not

396 looked at other epigenetic marks such as histone modifications, which are interrelated
397 ⁴². Our study is a starting point towards future analyses which should try to combine
398 and integrate different technologies and, we think, focus on genome-wide approaches
399 in order to uncover more subtle changes which could be associated with exposure to
400 nanomaterials.

401

402 **Availability of data and materials**

403 The raw IDAT and preprocessed data generated in this study by Illumina Infinium
404 MethylationEPIC BeadChip technology are available in the ArrayExpress public
405 repository under accession E-MTAB-7719.

406 **Disclosure of interest**

407 The authors report no conflict of interest.

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427

428 Figure and table legends

429

430 **Figure 1.** *A) XRD pattern for the rGO; intensity is expressed in arbitrary units (a.u.). B)*
431 *Representative SEM images (upper panels) and TEM images (lower panels) of the rGO. C)*
432 *High-resolution XPS spectra; intensity is expressed in arbitrary units (a.u.), binding energy*
433 *(B.E.) is measured in electronvolts (eV). D) Nitrogen adsorption/desorption isotherm of rGO;*
434 *P/P0 is the relative pressure.*

435

436 **Figure 2.** *A) Density plot showing DNA methylation β -values from all of the CpG sites*
437 *analyzed by the Illumina MethylationEPIC platform in the BEAS-2B control cells. Highlighted*
438 *from the density plot are two treemap plots indicating the semantic summaries of gene*

439 *ontologies found for those genes containing either low- or high-methylation CpG sites. B)*
440 *Violin plots indicating the DNA methylation β -value distribution of CpGs mapped to CpG*
441 *Island-related regions (upper panel) or gene-related regions (lower panel). The median values*
442 *of the distributions are highlighted by a black dot. (N_Shelf: north shelf, N_Shore: north shore,*
443 *Island: CpG Island, S_Shelf: south shelf, S_Shore: south shore; TSS1500, TSS200: 1500 or*
444 *200 bp from transcription start site, nogene: intergenic).*

445

446 **Figure 3.** *A) Experimental set-up of the rGO exposure experiments and collapsing of the*
447 *replicates. B) Left panel: heatmap depicting Spearman correlation values for the correlation*
448 *between the profiled β -values of the CpG sites of the different collapsed experimental*
449 *conditions. Right panel: density scatter plot showing an example of the correlation between the*
450 *β -values of the CpG sites of the rGO 15-day 1 $\mu\text{g}/\text{mL}$ condition and the 15-day control*
451 *condition. The color indicates the density of the points, from low (blue) to high (yellow).*

452

453 **Figure 4.** *Line/point plots describing the DNA methylation values of different repeat elements*
454 *measured by bisulfite pyrosequencing. For each experimental condition, the 2 technical*
455 *replicates (“a” and “b”) are plotted as independent lines. For each DNA repeat region, a*
456 *different number of CpGs were analyzed. Values for the 15-day treatments at 0, 1 and 10 $\mu\text{g}/\text{mL}$*
457 *concentrations and 30-day treatments at 0, 1 and 10 $\mu\text{g}/\text{mL}$ concentrations are separated in 2*
458 *different plots.*

459

460 SUPPLEMENTARY INFORMATION

461 **Supplementary methods.** *Expanded descriptions of the materials and methodology used in*
462 *this study.*

463 **Figure S1.** *Violin plots indicating the DNA methylation β -value distribution of CpGs within*
464 *a distance of 100 bp to different repeat elements, for the different conditions (technical*
465 *replicates have been collapsed).*

466

467 **Table S1.** *Gene ontology enrichment analysis of genes exclusively containing high-*
468 *methylation or low-methylation CpG sites in the BEAS-2B cell line. All of the analyses include*
469 *“molecular function”, “cellular component” and “biological process” terms.*

470

471 **Table S2.** *Primer sequences for bisulfite pyrosequencing of the different repetitive DNA*
472 *families.*

473

474 **Table S3.** *DNA methylation measurements obtained by bisulfite pyrosequencing of the*
475 *different repetitive DNA families.*

476

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