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Comparison of the DNA damage response in BEAS-2B and A549 cells exposed to titanium dioxide nanoparticles

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Abstract

For some decades production of TiO₂ nanoparticle (TiO₂-NP) has been increasing at a considerable rate; concerns as to the toxicity of these particles upon inhalation have been raised. Indeed, TiO₂-NPs have been shown to induce significant genotoxicity and to adversely affect both major DNA repair mechanisms: base excision repair (BER) and nucleotide excision repair (NER).

The aims of the present study were to i) compare the genotoxicity of TiO₂-NPs and their impact on DNA repair processes on A549 alveolar carcinoma and BEAS-2B normal bronchial lung cell lines and ii) delve deeper into the mechanisms leading to these effects. To achieve these goals, TiO₂-NPs effects on cytotoxicity, genotoxicity, DNA repair activity and DNA repair gene expression were investigated in both cell lines upon exposure to 1-100 µg/mL of anatase/rutile, 21 nm TiO₂-NPs. Our results show that TiO₂-NPs induce comparable cyto- and genotoxic responses in BEAS-2B and A549 cells. Functional response to DNA damage is observed in both cell lines and consists in a general downregulation of DNA repair processes. When evaluating the relative importance of the two DNA repair pathways, we observed a lower impact on BER compared to NER activities, suggesting that repair of oxidatively-generated DNA damage is still triggered in these cells. This response becomes measurable at 4 h of exposure in BEAS-2B but only after 48 h of exposure in A549 cells. The delayed response in A549 cells is due to an initial overall and intense downregulation of the genes encoding DNA repair proteins. This overall downregulation correlates with increased methylation of DNA repair gene promoters and downregulation of NRF2 and BRCA1, which may thus be considered as upstream regulators.

These results strengthen the evidence that TiO₂-NP induces indirect genotoxicity in lung cells, via modulation of DNA repair processes, and shed some light on the mechanisms behind this effect.

Introduction

TiO₂ nanoparticles (NPs) are among the top five NPs used in consumer products (1). Their annual production is estimated at 3800-7800 tons in the US alone (2) and is continuously increasing (1). It therefore appears necessary to characterise the impact of these NPs on the health of those who handle them and are likely to inhale them; inhalation was shown to be the main human exposure route in occupational settings. *In vitro* and *in vivo* experiments indicated that the main toxicity mechanisms induced by TiO₂-NPs include induction of inflammation and oxidative stress, as well as genotoxicity (1,3-5). In rodents, exposure of the lungs to high doses of TiO₂-NPs led to tumours development (1). Based on this evidence, TiO₂ was classified by the IARC (International Agency for Research on Cancer) in group 2B, possibly carcinogenic to humans (6).

Genotoxicity is often considered as a hallmark of NP-induced carcinogenesis. The effect can be direct, when the target of the genotoxic agent is DNA itself, or indirect, when the target something other than DNA (7). *In vitro*, TiO₂-NPs have been shown to accumulate in cells mainly by endocytosis and to distribute in intracytoplasmic compartments (8,9). Some reports also indicated their presence in the nucleus of cells (10-14). However, the proportion was minor compared to the amount in cytoplasmic compartments, making direct interaction between DNA and TiO₂-NPs unlikely. The main mechanism reported in relation to TiO₂-NP genotoxicity is the generation of reactive oxygen species (ROS) (15). Production of ROS is considered as direct genotoxicity (7), since these species may migrate to the nucleus where they can cause single or double-strand breaks in DNA or cause the formation of oxidised bases such as 8-oxo-dGuo. These phenomena were reported to occur in cells exposed to TiO₂-NPs following both acute (12,16) and chronic exposure (17,18). TiO₂-NPs have also been reported to induce indirect genotoxicity via mechanical interference with microtubules (7) which, among other outcomes, hinders mitotic progression (19). In addition, TiO₂-NPs have been reported to induce epigenetic modification (7), to modulate the expression of DNA repair genes and proteins (15,20,21), and to reduce DNA repair ability through the nucleotide excision repair (NER) and base excision repair (BER) pathways (12).

In the human respiratory tract, based on their size, NPs are predicted to mainly deposit in the alveoli (22). Alveolar effects are frequently studied using the A549 cell line, derived from a human adenocarcinoma of the alveolar basal epithelium. However, the use of A549 cells in studies of DNA damage response has been criticised because these cells are hypotriploid and they have an increased oxidative stress response (23) due to a mutation in the gene encoding Kelch-like ECH-associated protein 1 (KEAP1). *KEAP1* encodes a cytoplasmic protein that sequesters Nuclear factor (erythroid-derived 2)-like 2 (NRF2) in the cytoplasmic compartment. When not tethered by KEAP1, NRF2 locates to the cell nucleus where it promotes the expression of genes encoding redox regulation proteins. Since the accepted paradigm for particle-induced genotoxicity is oxidative stress, the A549 response to NPs may therefore differ from that of other cell types.

The aims of the present study were i) to compare the response of non-cancerous bronchial epithelial BEAS-2B cells to that of A549 epithelial alveolar cancer cells, particularly their DNA damage response when exposed to TiO₂-NPs, and ii) to delve deeper into the mechanisms of DNA damage response induced by TiO₂-NPs. These two cell lines were exposed to 1-100 µg/mL NM105 TiO₂-NPs with an average primary diameter of 21 nm (from the Joint Research Centre (JRC) library) for 4 h, 24 h or 48 h. The exposure medium consisted in cell culture medium without foetal bovine serum (FBS), as in our previous studies (9,12). Cell viability, DNA integrity, DNA repair ability and expression of DNA repair genes were evaluated in both cell lines. The mechanisms through which DNA repair genes were repressed was then further investigated in A549 cells by analysing the methylation state of DNA repair gene promoters and expression levels for three upstream regulators of DNA repair gene expression.

Material and methods

Chemicals and nanoparticles

Unless indicated otherwise, chemicals were purchased from Sigma-Aldrich and were > 99% pure.

NM105 TiO₂-NPs were obtained from the nanomaterial library at the JRC (Ispra, Italy). Their physico-chemical characteristics were determined to be as follows: spherical shape, primary particle size 21±9 nm,

anatase:rutile ratio 86:14%, specific surface area 46 m²/g (<http://bookshop.europa.eu/fr/titanium-dioxide-nm-100-nm-101-nm-102-nm-103-nm-104-nm-105-pbLBNA26637/>) and point of zero charge 7.0 (9).

Nanoparticle suspension

Nanoparticles were dispersed in ultrapure water as previously described, by ultrasonication for 30 min at 4°C on a Vibra Cell 75043 sonicator (Bioblock Scientific) operated in pulse mode (1s on/1s off) at 28% amplitude (9), i.e. 16.7 W (24). After dispersion in water, the number-based hydrodynamic diameter was 70 ± 20 nm, with a polydispersity index (PDI) of 0.18 ± 0.04. Just before cell exposure, these water suspensions were diluted in FBS-free cell culture medium at a final concentration of 20 µg/mL, a concentration compatible with the Malvern zetasizer. In these conditions, the hydrodynamic diameter shifted to 720 ± 20 nm and the PDI to 0.50 ± 0.02, suggesting agglomeration. 48 h after dilution in exposure medium, the hydrodynamic diameter was >1, indicating that NPs were totally agglomerated.

Cell culture and exposure conditions

BEAS-2B cells were purchased from ATCC (CRL-9609) and cultured in DMEM (Gibco, 11960-044) containing 10% FBS, antibiotics and glutamine on collagen-coated Petri dishes and plates. Cells were exposed to 0-100 µg/ml of TiO₂-NPs in FBS-free cell culture medium for 4, 24 or 48 h.

Cytotoxicity assay

TiO₂-NPs effect on cell viability was investigated on A549 and BEAS-2B cells using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were grown in 96-well plates, exposed to TiO₂-NPs for 4 h, 24 h or 48 h; exposure media were then replaced with 100 µL of MTT and cells were incubated for a further 1 h at 37 °C. The formazan crystals that formed inside cells were then dissolved in 100 µL of DMSO. To limit possible NP interference with the assay, plates were centrifuged for 5 min at 3200 × g and 50 µL of the supernatant was transferred to a new plate for

absorbance reading, as previously described (24). Assays were independently reproduced three times, with six independent replicates for each experiment (i.e., n=6).

Genotoxicity assays

Comet assay

DNA strand breaks and alkali-labile sites induced by TiO₂-NPs were visualised using the alkaline version of the comet assay (25). About 20000 cells from each sample were deposited on each slide, and three slides were analysed per sample. Lysis was performed overnight at 4 °C and electrophoresis was carried out at 25 V (300 mA) for 30 min. Comets were stained using ethidium bromide and measured using comet assay IV (Perceptive Instrument, Bury St Edmunds, UK). This assay was independently reproduced three times, with three independent biological replicates in each case, each of which was loaded on two different comet slides.

8-oxo-dGuo and 5-Me-dC quantification by HPLC-MS/MS

The levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dGuo) and 5-methyl deoxycytosine (5-Me-dC) were measured by HPLC-MS/MS. DNA from frozen cell pellets (-80 °C) was extracted using the DNeasy® Blood and Tissue kit (Qiagen) according to the manufacturer's procedure. An RNase treatment step was added after cell lysis, samples were incubated for 2 min with 400 µg of RNase A. DNA was eluted in 100 µL of 0.1 mM deferoxamine to prevent spurious oxidation (26). DNA from 50 µL of sample was digested in two incubation steps. First, the pH was adjusted to pH 5.5 by addition of 5 µl of buffer (100 mM succinic acid, 50 mM CaCl₂, 150 mM, 5 µM ZnSO₄, pH 5.5). Samples were then incubated for 2h at 37°C in the presence of 2.5 U of nuclease P1, DNase II and 0.05 U of phosphodiesterase II. Then, Tris buffer (6 µl, 500 mM Tris, 1 mM EDTA, pH 8) was added together with 2 U of alkaline phosphatase and 0.05 U of phosphodiesterase I. The sample was incubated for a further 2 h at 37 °C. Hydrochloric acid (3 µl, 0.1 N) was added and the tubes were centrifuged at 7000xg for 5 min. The aqueous phase was collected and transferred into HPLC injection vials for HPLC-MS/MS analysis. Chromatographic

separation was performed on a C18 reverse-phase Uptisphere ODB column (Interchim, Montluçon, France). Analytes were eluted using a gradient of methanol in 2 mM ammonium formate at a flow rate of 0.2 ml.min⁻¹. Upstream of the mass spectrometer, the HPLC eluate was analysed in a UV detector set at 280 nm to quantify the amount of unmodified nucleosides. The retention time was around 29 min. MS/MS was carried out on an API 3000 mass spectrometer (SCIEX) in multiple reaction monitoring mode with positive electrospray ionisation, as previously described (12,26). The monitored fragmentation were m/z 284 [M + H]⁺ → m/z 168 [M + H -116]⁺ for 8-oxo-dGuo and m/z 242 → m/z 126 for 5-Me-dC. Levels of 8-oxo-dGuo were expressed as a number of lesions per million normal bases. Levels of 5-Me-dC were expressed as % of lesions per dC. This assay was reproduced three times independently, with n=3 in each replicate.

Analysis of DNA repair gene expression

DNA repair gene expression was assessed by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Briefly, RNA was extracted and reverse-transcribed using the GenEluteTM mammalian total RNA miniprep kit with the optional DNase treatment step and the SuperScript II Reverse Transcriptase kit (Life Technologies), according to the manufacturers' protocols. qPCR was performed on a MX3005P Multiplex Quantitative PCR thermocycler (Stratagene). Primer sequences are reported in Table S1. Three reference genes, GAPDH, S18 and CycloB were chosen for normalisation using BestKeeper (27). Relative quantification of gene expression and statistical analyses were performed with the REST 2009 software (28), based on the $\Delta\Delta C_q$ method. A theoretical value of 2 was assumed for all PCR efficiencies. RT-qPCR was reproduced three times independently, with three independent biological replicates loaded twice on the qPCR plate.

Multiplex array for DNA repair activity

The BER and NER abilities of BEAS-2B cells exposed to TiO₂-NPs were assessed using a multiplexed excision/synthesis repair assay (LXRepair, Grenoble, France). Nuclear extracts were prepared as

described previously (29) and excision/repair reactions were run for 3 h at 30 °C at a final protein concentration of 0.3 mg.ml⁻¹ on damaged plasmid microarrays (29). The reaction medium contained 1 mM adenosine triphosphate and 0.25 μM dCTP-Cy3 (GE Healthcare). The lesions assessed were photoproducts (cyclobutane pyrimidine dimers and (6-4) photoproducts (CPD-64)), 8-oxodGuo (8oxoG), alkylated bases (AlkB), abasic sites (AbaS), and pyrimidine glycols (Glycol). For each lesion, the total fluorescence incorporated into a lesion-containing plasmid was quantified using the Innoscan 710AL scanner (Innopsys, Toulouse, France) and normalised relative to the fluorescence incorporated into an undamaged plasmid. Each extract was tested twice and the experiment was reproduced three times. The Glyco-SPOT assay (LXRepair, Grenoble, France) was used to quantify the excision activities of excision-resynthesis repair. In this assay, a multiplexed array of oligonucleotides (ODNs) containing specific lesions is used to monitor several glycosylases/AP endonuclease activities in cell extracts. The lesions comprised 8-oxoguanine paired with C (8oxoG-C), A paired with 8oxoguanine (A-8oxoG), ethenoadenine (EthA-T), thymine glycol (Tg-A), uracil (paired either with G or A (U-G and U-A respectively), hypoxanthine (Hx-T), abasic sites (THF-A). Cleavage of the lesions by the enzymes contained in the extracts released the fluorescence attached to the lesion-containing ODNs (30). Repair reactions were allowed to proceed for 1 h at 37 °C with 20 μg/mL of protein in 80 μL of excision buffer (10 mM HEPES/KOH pH 7.8, 80 mM KCl, 1 mM EGTA, 0.1 mM ZnCl₂, 1 mM DTT, 0.5 mg/mL BSA). After washing three times for 5 min at room temperature in PBS containing 0.2 M NaCl and 0.1% Tween 20, the fluorescence emitted by spots was quantified using the Innoscan 710AL scanner. Each extract was run in duplicate. Data were normalized using the NormalizeIt software as described previously (29).

Percentage of excision of each lesion was calculated using the following formula: $(100 \times (1 - \text{percentage of fluorescence of Lesion_containing_ODN} / \text{percentage of fluorescence of Lesion_Free ODN}))$. This calculation determines a percentage of residual fluorescence in the wells incubated with the extracts with respect to wells incubated with the buffer alone. Possible non-specific cleavage of the control ODN

(Lesion_Free ODN) was also taken into account by the formula. These assays were performed once, with three independent biological replicates.

Methylation in the promoters of DNA repair genes

DNA from frozen cell pellets was extracted using the DNeasy® Blood and Tissue kit (Qiagen), following the manufacturer's instructions with an extra RNase treatment step after cell lysis (2 min of incubation with 400 µg of RNase A, Sigma-Aldrich). DNA was eluted in nuclease-free water before digestion using the EpiTect® Methyl II DNA Restriction Kit (Qiagen) according to the manufacturer's recommendations. Samples were stored at -20 °C. The methylation profile was analysed using the EpiTect® Methyl II Signature PCR Array Human DNA Repair kit (Qiagen) according to the manufacturer's protocol. qPCR was performed with MESA Blue qPCR Mastermix Plus for SYBR® Assay Low ROX (Eurogentec) on a MX3005P thermocycler (Stratagene). Data were analysed using the EpiTect Methyl II PCR Array Excel-based data analysis template, version 2.0 (Qiagen). This assay was independently reproduced three times, with n=3 on each replicate.

Statistical analyses

For all experiments except qPCR, statistical significance was assessed with both non-parametric one-way analysis of variance on ranks (Kruskal-Wallis) and pair-wise comparison by applying a Mann-Whitney U-test using Statistica 8.0 software (Statsoft). Results were considered statistically significant when the p-value was < 0.05. The Relative Expression Software Tool (REST 2009) software was used to analyse qPCR data (28).

Results

Cytotoxicity

In both A549 and BEAS-2B cell lines, cytotoxicity induced by TiO₂-NPs was determined using the MTT assay, which is based on the measurement of cellular NAD(P)H-dependent oxidoreductase enzymatic activity in viable cells. TiO₂-NPs did not interfere with the MTT assay as previously shown (24).

Cytotoxicity was observed only after 24 h or 48 h exposure times, never at 4 h (Fig. 1). However, more than 70% of the cells remained viable after 48 h of exposure, suggesting that TiO₂-NP is moderately cytotoxic.

Genotoxicity

Induction of strand breaks and alkali-labile sites by TiO₂-NPs was assessed by performing the alkaline comet assay. Then 8-oxo-dGuo, which is the most frequent oxidized DNA base, was measured by HPLC-MS/MS. Cells were exposed to 10 or 100 µg/mL TiO₂-NPs. At these concentrations cell viability was >70%, as required for genotoxicity assays. These concentrations also limited possible interference of NPs with the assays. No DNA damage was induced at 10 µg/mL (Fig. 2), in contrast at 100 µg/mL DNA damage was observed at all time-points, in both cell lines and using both assays (Fig. 2). Thus, comet tail intensity increased from 2-10% in control cells to 18-28% in cells exposed to 100 µg/mL TiO₂-NPs (Fig. 2A-B). Significantly higher levels of DNA damage were observed in A549 cells than in BEAS-2B cells at 4 h of exposure. However, at 48 h, damage was more extensive in BEAS-2B cells than in A549 cells, although the difference was modest. The number of 8-oxo-dGuo lesions ranged from 15 to 26 8-oxo-dGuo per million bases in exposed cells, while only three to eight 8-oxo-dGuo per million bases were detected in control cells (Fig. 2C-D). This level of oxidation is within the acceptable range defined by the European standards committee on oxidative DNA damage (ESCODD) (31). The number of 8-oxo-dGuo lesions was moderately, but statistically significantly, higher in BEAS-2B than in A549 at 24 h of exposure (Fig. 2C-D).

DNA repair activity in cells exposed to TiO₂-NPs

In A549 cells, we previously reported that a 24 h of exposure to 100 µg/mL TiO₂-NPs induces a slight increase in DNA repair activity through the BER and NER pathways, followed by a drastic reduction in DNA repair activity at 48 h post-exposure (12). This result was obtained using a microarray (ExSy-SPOT microarray) that measures repair efficiency for five DNA lesions, repaired either via the BER (8oxoG, AbaS, AlkB) or the NER (CPD-64, glycol) pathways (29). Here, the same experiment was carried out on BEAS-2B cells, exposed to 100 µg/mL TiO₂-NPs for 4 h, 24 h or 48 h. At all three time-points, exposure to TiO₂-NPs induced a drastic reduction in cell's ability to repair the five lesions (not shown).

Normalisation for the total fluorescence detected on the array was used to reveal whether a specific repair pathway played a more extensive role when compared to the overall DNA repair activity of the cell (Fig. S1). As indicated above, AlkB, 8oxoG and AbaS are typically repaired by BER, while CPD-64 (cyclobutane pyrimidine dimers and 6-4 photoproducts) are repaired by NER. However, photoproducts and glycols (thymine and cytosine glycols) are transcription-blocking lesions and tend to cluster together in this assay. After normalisation of BEAS-2B data (Fig. 3B), TiO₂-NP exposure was found to induce a shift in the relative importance of two groups of repair activities. Thus, in control (unexposed) cells, CPD-64 and Glycols were more frequently repaired than AlkB, 8oxoG and AbaS, whereas the opposite effect was noted in TiO₂-NP-exposed cells. A similar shift in repair activity was observed in A549 cells, but only after 48h of treatment. These results suggest that although the DNA repair activity is decreased overall in BEAS-2B cells, the relative ability of the cell to repair oxidative lesions via the BER pathway is somehow preserved, while the NER pathway is more severely affected.

To further investigate the mechanisms by which TiO₂-NP exposure modulates BER activity, we assessed their impact on the first step of this pathway, excision by glycosylases/AP endonucleases. To do this, we focused on A549 cells exposed for 24 h or 48 h to TiO₂-NPs. The rationale for conducting this assay is that glycosylases - particularly OGG1, which is responsible for the excision of 8-oxo-dGuo - are susceptible to oxidative stress (33) and may thus be inactivated in TiO₂-NP-exposed cells. Another multiplexed assay was used (GlycoSPOT), based on oligonucleotide cleavage (30). No significant impact

was observed at 24 h of exposure to TiO₂-NPs. However, after 48 h of exposure, the ability of A549 cells to excise adenine paired with 8-oxo-dGuo and ethenoadenine (EthA) was decreased (Fig. 4). Conversely, TiO₂-NP-exposure did not affect the cell's ability to excise uracil (U), 8-oxo-dGuo, thymine glycol (Tg), hypoxanthine (Hx) or abasic sites (THF-A) (Fig. 4).

Modulation of the expression of genes encoding DNA repair proteins in TiO₂-NP-exposed cells

To examine the mechanisms behind this deregulation of DNA repair activities, we measured the expression levels of 15 genes encoding DNA repair proteins from the BER or NER pathways by RT-qPCR. For these experiments, both cell lines were exposed to 1, 10 or 100 µg/mL TiO₂-NPs. No modulation of gene expression was observed in cells exposed to 1 µg/mL TiO₂-NPs and only one gene, *CSB*, was affected at 10 µg/ml (data not shown).

The most intense modulation of gene expression was observed in A549 cells exposed to 100 µg/mL TiO₂-NPs for 4 h or 24 h. In these conditions, 12 genes out of the 15 monitored were downregulated, of which seven at 4 h (*XRCC1*, *PCNA*, *LIG3*, *XPC*, *DDB1*, *DDB2* and *CSB*) and 11 at 24 h (*APE1*, *PARP1*, *POLB*, *PCNA*, *LIG3*, *hHR23B*, *XPC*, *DDB1*, *DDB2*, *CSA* and *CSB*) (Table 1). The transcriptional response was much less intense at 48 h of exposure in this cell line - with downregulation of only one gene, *PARP1* - and in BEAS-2B cells at all time-points. Indeed, in BEAS-2B cells exposed to 100 µg/mL TiO₂-NPs, only 3 out of 15 genes were downregulated, two of them at 4 h and 24 h of exposure (*POLB* and *PCNA*) and one at 48 h (*hHR23B*) (Table 1).

Deregulated expression of three genes was common to both cell lines. These genes were, *POLB* at 4 h, *PCNA* at 4 h and 24 h, and *hHR23B* which was downregulated at 24 h in A549 and at 48 h in BEAS-2B (Table 1, bold). The fold-change in gene expression for these genes was consistently lower in A549 cells than in BEAS-2B cells, indicating a more intense transcriptional regulation in A549.

Because DNA repair gene expression was more intensively modulated in A549 cells, we chose this cell line to further study the effects of TiO₂-NP exposure on genes encoding other repair proteins involved in

the BER and NER pathways, as well as genes linked to non-homologous end-joining (NHEJ), homologous recombination (HR) and mismatch repair (MMR). Modulation of expression levels was primarily observed in cells exposed to 100 µg/mL TiO₂-NPs for 4 h or 24 h. A schematic representation of these DNA repair pathways is presented in Fig. 5, together with the proteins for which gene expression was found to be deregulated (downregulated genes are depicted in blue and upregulated genes in red). As shown in this figure and in Table S2, the general trend was for downregulation, with 19 out of 29 genes tested (65%) downregulated, with fold-changes ranging between 0.36 and 0.90 (Table S2). The causes of this trend were investigated by measuring the expression levels of three genes encoding upstream regulators of DNA repair protein expression, BRCA1, E2F1 and NRF2. Expression of *BRCA1* and *NRF2* was decreased at 24 h of exposure (Table S2).

Overall DNA methylation and specific methylation of DNA repair gene promoters

Since epigenetic modification, in particular DNA methylation, is well known to regulate gene expression (34), the overall methylation level of DNA was monitored, by 5-Me-dC quantification in A549 cells exposed for 4 h, 24 h or 48 h to TiO₂-NPs (Table 2). No change in the overall DNA methylation level was detected by this assay. We therefore used RT-qPCR (EpiTect assay) to examine the methylation profile of 20 DNA repair gene promoters in cells exposed to TiO₂-NPs. The promoters tested were those of *APE1*, *ATM*, *BRCA1*, *LIG3*, *MLH1*, *MRE11*, *MSH2*, *PMS2*, *POLB*, *RAD50*, *UNG*, *XPC* and *XRCC1*. Exposure of A549 cells to 100 µg/ml of TiO₂-NPs for 4 or 24 h led to a moderate, but statistically significant, increase in methylation levels of *APE1* (at 4 and 24 h), *POLD3* (at 4 h), *MRE11A* (at 24 h) and *PMS2* (at 24 h) (Fig. 6).

Discussion

This article compares the impact of TiO₂-NPs on human bronchial BEAS-2B cells and alveolar A549 cells in terms of cell mortality, genotoxicity, DNA repair activity and expression of DNA repair genes. It is

important to note that the same procedure was used to expose both cell lines - the same exposure medium, duration and TiO₂-NP concentration. Thus, both cell lines received the same dose of NPs, initially coated with the same corona. TiO₂-NPs were observed to be moderately toxic to cells, with equivalent mortality rates in A549 cells and BEAS-2B cells. Oxidative damage to DNA was also observed in both cell lines, with DNA strand breaks and/or alkali-labile sites as well as oxidative damage, producing 8-oxo-dGuo. Similar levels of these types of lesion were observed in A549 and BEAS-2B, with lesions appearing as soon as 4 h after exposure in both cell lines. However, strand breaks and/or alkali-labile sites tended to appear earlier in A549 than in BEAS-2B cells. With increased exposure durations, the frequency of this damage decreased slightly in A549 cells, in line with the increase in DNA repair activity previously observed (12). In BEAS-2B, extended exposure duration increased the number of strand breaks and/or alkali-labile sites slightly, a result that is compatible with the decreased DNA repair ability observed in these cells. Our results are thus in agreement with the vast majority of studies published to date. Indeed, most previous studies also reported only moderate cell mortality (4) which often reaches a plateau not exceeding 30% (9,12,35-38). In addition, most genotoxicity studies performed on BEAS-2B or A549 cells exposed to comparable TiO₂-NP concentrations also reported a TiO₂-NP-induced increase in numbers of DNA breaks and/or DNA oxidation sites (12,20,39-47). Moreover, except in A549 at the earlier time-point (24 h), the results presented here indicate that DNA repair activity is reduced in both cell lines upon exposure to TiO₂-NPs. Delving deeper into the cellular response to TiO₂-NP exposure, we observed that the relative importance of NER decreased considerably, while the relative decrease of the BER pathway was less extensive. This suggests that BEAS-2B and A549 cells preserve BER, which is necessary to repair oxidative damage caused by TiO₂-NPs. This phenomenon is not linked to transcriptional regulation since at these time-points very few DNA repair genes are modulated. Based on these endpoints for assessing NP toxicity, we conclude that the response of the two cell lines is equivalent. A similar response of A549 and BEAS-2B cells following NP exposure was previously reported by Kain et al. (48), in their case the NP tested were MnO₂, Ag, CeO₂, Co₃O₄, Fe₃O₄, NiO and SiO₂(48). Thus, either cell line is

appropriate for use as a biological model for nanotoxicity studies investigating cytotoxicity and genotoxicity.

The most striking difference between the two cell lines is the early response observed in A549 cells but not in BEAS-2B. At 4 h and 24 h of exposure to TiO₂-NPs, a slight increase in DNA repair activities was observed in A549, this decrease correlated with generalised, intense downregulation of DNA repair genes. A549 cells thus respond to TiO₂-NPs in a biphasic way, with i) a first phase of intense deregulation of gene expression and ii) a second phase, that can be interpreted as an adaptive response, during which cells amplify their response towards oxidatively-generated DNA lesions. In the first phase, 31 DNA repair genes or upstream regulators out of 44 are downregulated in at least one TiO₂-NP-exposure condition (exposure duration or NP concentration). Strikingly, these genes are all downregulated. This unexpected profile could be an indication that TiO₂-NPs impact transcription overall, in a non-specific way. However, unpublished transcriptomic analyses conducted in our laboratory refute this hypothesis; in these experiments, exposure of A549 cells to the same TiO₂-NPs as in the present study induced an overall upregulation of gene expression. A similar generalised downregulation of DNA repair gene expression was reported in lung cells exposed to silver NPs (49), and in liver and lymphoma cells exposed to Ag-NPs (50,51). In these other cell types, expression of *OGG1*, *NRF2*, *XPA* and *ERCC2* was decreased (50,51). This inhibition may be related to the fact that Ag-NPs are thought to dissolve in the intracellular compartment, and to the affinity of Ag ions for thiol groups. Some DNA repair proteins and transcription factors are Zn-finger proteins, in which Zn is bonded to the protein via –SH groups, the hypothesis is that Ag ions may replace Zn in the active site of these proteins, causing their inactivation (48). In contrast, TiO₂-NPs are considered to be very chemically inert (e.g. they do not dissolve) they would not therefore engage in the same type of interactions as Ag-NPs, and consequently must act via a different mechanism. Recently, DNA repair gene expression profiles in various species exposed to a range of nanomaterials showed that TiO₂-NPs induce downregulation of five out of six DNA repair genes at 24 h of exposure (20). This effect may be produced by sequestration of transcription factors in the protein corona that forms on the surface of NPs. However, the fact that downregulation of gene expression is restricted to DNA

repair genes suggests that one or more upstream regulator(s) of DNA repair processes may be directly affected by TiO₂-NP exposure. Attempts to identify this (these) upstream regulator(s) led us to analyse the expression of *BRCA1*, *E2F1* and *NRF2* in TiO₂-NP-exposed cells, as well as the overall methylation levels, and more specifically the methylation profile of DNA repair gene promoters. Contrary to what was observed by Li et al. (52) in A549 cells exposed to carbon-based nanomaterials, we did not detect any overall change in DNA methylation levels. Moreover, when examining the promoter methylation profile, although some promoters were found to be more methylated in TiO₂-NP-exposed cells than in control cells, the methylation levels observed in this study remained low compared to the levels reported in cancer cells (53). Only 4 gene promoters out of the 20 tested here showed increased methylation levels. This suggests that methylation is unlikely to be the only factor behind the overall downregulation of DNA repair genes observed. Nevertheless, the *APE1* promoter shows increased methylation in NP-exposed cells, which is consistent with its decreased expression. While epigenetic alterations are known to be a major causative factor in diseases or adverse effects of environmental toxicants, especially those found in ambient air and due to ultrafine particles (54), based on our results we believe that it is not a key mode of action of TiO₂-NPs.

Bai et al. (55) reported altered methylation of the *PARP1* gene promoter in TiO₂-NP-exposed cells, which correlated with oxidative stress (55). Although we did not assess methylation of the *PARP1* promoter in the present study, we did observe downregulation of this gene. Hypermethylation of *PARP1* promoter was also reported in HaCaT cells exposed to SiO₂-NPs, where it was correlated with *PARP1* gene repression (56). Our data thus confirm the implication of epigenetic regulation, in particular methylation of gene promoters, in regulating gene expression associated with TiO₂-NP exposure, although it may not be the only causative factor.

Decreased expression of *NRF2* and *BRCA1* could also partly explain the reduced DNA repair activity observed here. Indeed, inactivation of *NRF2*, in addition to its antioxidant activities, has been shown to significantly slow down DNA repair processes (57). It especially affects HR as it binds to the antioxidant-response-element sequences of some DNA repair proteins involved in this pathway (58). Similarly, breast

cancer type 1 susceptibility protein (BRCA1) is known to be involved in the HR, BER and NER pathways (59), as it regulates transcription and has E3 ubiquitin ligase activity. Mutation of *BRCA1* compromises the repair of oxidatively damaged DNA and makes cells more sensitive to the alkylating agent methane methylsulfonate (59). We also reported similar effects in A549 cells following chronic exposure to TiO₂-NPs (18).

The genes downregulated encode proteins from all the DNA repair pathways and from every stage of each pathway from damage recognition to DNA resynthesis and ligation. However, the excision stage appears to be less affected than others, as only one glycosylase (UNG) was downregulated out of the three tested. This reduced sensitivity was confirmed by a multiplexed array of excision activities of A549 cells, which showed that only excision of A paired with 8-oxo-dGuo and EthA were affected in TiO₂-NP-exposed cells. Contrary to expectations, OGG1 activity is not impaired in TiO₂-NP-exposed cells, even though oxidative stress has been described as a potent and reversible inactivator of this protein (33). However, elimination of A paired with 8-oxo-dGuo, which occurs when 8-oxo-dGuo is bypassed by DNA polymerases during replication - causing G-to-T transversion mutations - is impaired in TiO₂-NP-exposed cells. These lesions are primarily excised by MUTYH glycosylase which is considered as a backup system for the removal of 8-oxo-dGuo (60). Inactivation of this repair pathway raises concerns as mutation of the *MUTYH* gene has been described in a common form of familial colorectal cancer (60).

Downregulation of the expression of this DNA repair gene, together with impaired DNA repair activity, prove that high concentrations of TiO₂-NPs cause intense indirect genotoxicity, while lower concentrations do not. The TiO₂-NP exposure doses used here were 0, 1, 10, or 100 µg/ml, which correspond to 0.17-17 µg/cm² or 1.1 pg- 0.11 ng/cell. The lower end of this dose range roughly corresponds to the alveolar deposition theoretically obtained after 24 h of exposure to a 1 mg/m³ TiO₂-NP aerosol (61). This equivalent aerosol concentration is between the maximum exposure concentrations recommended by the NIOSH (National Institute for Occupational Safety and Health) and the maximum recommended by NEDO (New Energy and industrial technology Development Organization) for TiO₂-NPs, which are 0.3 and 1.2 mg/m³, respectively. The highest end of the dose range tested here could

represent a worst-case-scenario, but it is nevertheless a very high exposure concentration which could be considered unrealistic. However, occupational exposure is often chronic in nature, and we previously reported that chronicity increases the impact of TiO₂-NPs as it is correlated with increased intracellular accumulation (17,18). Consequently, even if the high concentration is unrealistic as an acute dose, we consider that the acute exposure model used in the present study could nevertheless be informative from a risk assessment perspective.

To summarise, in this article we show that A549 and BEAS-2B cell lines display similar responses to TiO₂-NPs, characterised by moderate mortality, significant oxidatively-generated DNA damage, and impaired DNA repair activities. Although several DNA repair activities are dramatically affected, BER activities are somehow preserved, suggesting attempts by the cells to repair oxidatively-generated DNA damage. Before the onset of this adaptive response, a first phase is observed in A549 cells, during which DNA repair genes are intensely deregulated. This transcriptional modulation mainly consists in downregulation affecting almost every stage of the different DNA repair pathways. Attempts to identify upstream causative factor(s) led to the conclusion that gene promoter methylation as well as NRF2 and BRCA1 might be involved in the effects of TiO₂-NP exposure on DNA repair. Thus, our results describe indirect genotoxicity of TiO₂-NPs in two lung cell lines due to impaired DNA repair.

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Table 1. Comparison of the modulation of expression of proteins involved in NER and BER in A549 and BEAS-2B cells exposed to TiO₂-NPs^a

	4h		24h		48h	
	A549	BEAS-2B	A549	BEAS-2B	A549	BEAS-2B
OGG1						
APE1			0.48±0.16			
PARP1			0.72±0.09		0.55±0.11	
POLB		0.62±0.10	0.56±0.06	0.67±0.15		
XRCC1	0.81±0.07					
PCNA	0.64±0.08	0.76±0.14	0.54±0.08	0.58±0.06		
LIG3	0.75±0.11		0.62±0.12			
POLE						
LIG1						
hHR23B			0.70±0.07			0.57±0.16
XPC	0.54±0.05		0.48±0.06			
DDB1	0.66±0.18		0.59±0.15			
DDB2	0.66±0.09		0.46±0.13			
CSA			0.59±0.08			
CSB	0.49±0.14		0.47±0.13			

^aExpression levels for genes encoding DNA repair proteins were analysed by RT-qPCR in A549 and BEAS-2B cells exposed to 100 µg/mL TiO₂-NPs, and compared to levels in unexposed (control) cells. Reference genes: GAPDH, S18 and CycloB. Fold-change is indicated only when expression was significantly different in exposed cells vs. unexposed cells. Bold typeface indicates that expression changed in both A549 and BEAS-2B cells. Statistical significance was assessed using REST 2009 software (28).

Table 2. Overall DNA methylation level in A549 cells exposed to TiO₂-NPs^a

	4 h	24 h	48 h
Ctl	0.95±0.70	1.00±0.55	0.80±0.30
10 µg/mL TiO ₂ -NPs	1.02±0.57	0.98±0.55	0.91±0.05
100 µg/mL TiO ₂ -NPs	1.04±0.68	0.88±0.34	0.92±0.18

^aOverall DNA methylation was assessed by measuring 5-methyl-deoxycytosine (5-mdC) in A549 cells exposed for 4, 24 or 48 h to 0, 10 or 100 µg/mL TiO₂-NPs. Results show DNA methylation rate, average ± standard deviation, n=3.

Figure 1. Cytotoxicity of TiO₂-NPs towards A549 and BEAS-2B cells. A549 (A) and BEAS-2B (B) cell mortality was assessed using the MTT assay in cells exposed to 0-100 µg/mL TiO₂-NPs for 24 or 48 h. The assays were independently reproduced three times, with n=6 in each independent experiment.

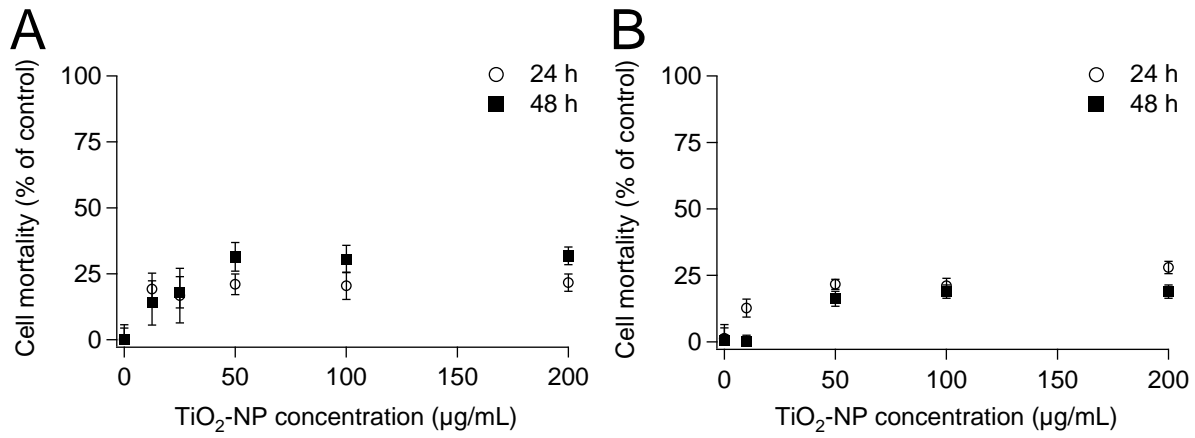


Figure 2. TiO₂-NP-induced genotoxicity in BEAS-2B and A549 cells. Genotoxicity was assessed using the alkaline comet assay (A, B) and by assaying 8-oxo-dGuo (C, D). A549 cells (A, C) and BEAS-2B cells (B, D) were exposed to 100 µg/mL of TiO₂-NPs for 4, 24 or 48 h. Experiments were independently repeated three times with three biological replicates each time. Statistically significant (*p* < 0.05) results are indicated: (*) exposed vs. control; (#) A549 vs. BEAS-2B in the same exposure conditions.

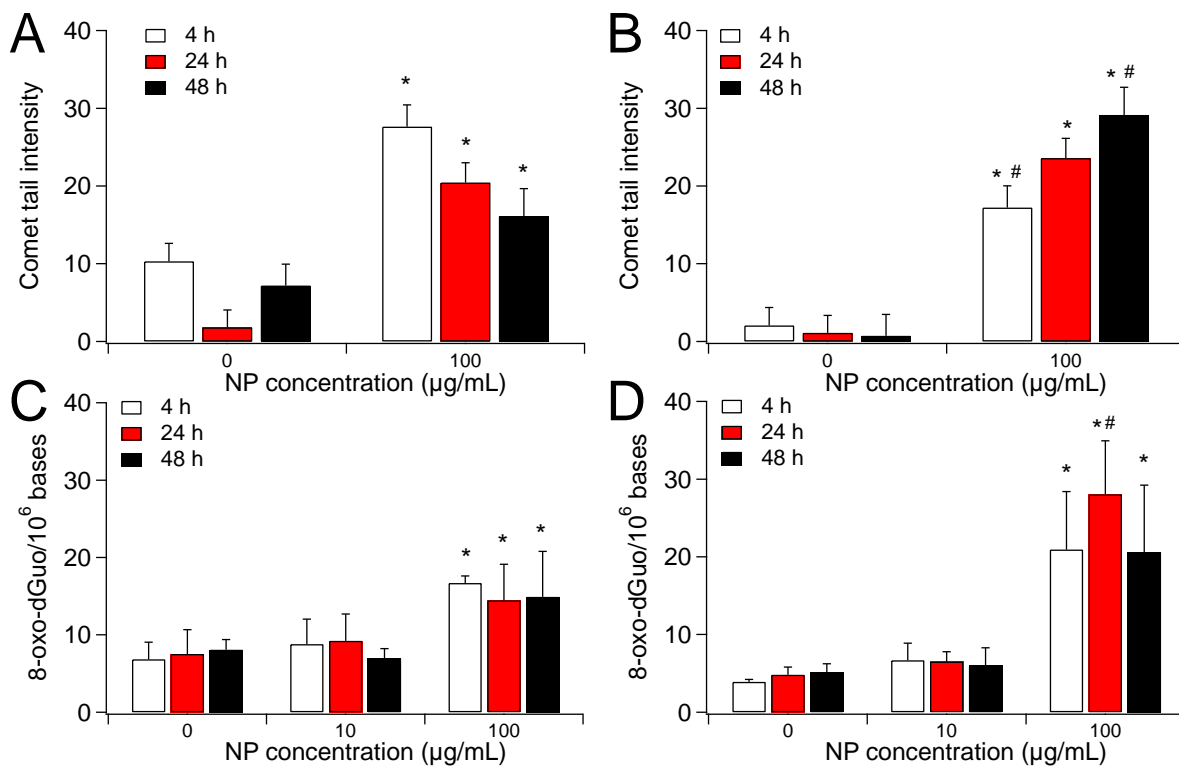


Figure 3. Heat-map representation of DNA excision/synthesis repair activities in A549 and BEAS-2B cells exposed to TiO₂-NPs. Cells were exposed to 100 µg/mL TiO₂-NPs for 4, 24 or 48 h (BEAS-2B); 24 or 48 h (A549). NT: non-treated cells (i.e., control cells); T: treated cells; 24 and 48 for 24 h and 48 h, respectively. Colour scale ranges between blue (lower value) and red (higher value). Each value represents the relative contribution of a specific repair pathway to total DNA repair activity in the cell after normalisation of data. For instance when considering DNA repair activity in a chosen condition (NT or T, 24 or 48), the pathway with the reddest colour is the most active, while the bluest pathway is the least active. The experiment was performed once with three independent replicates.

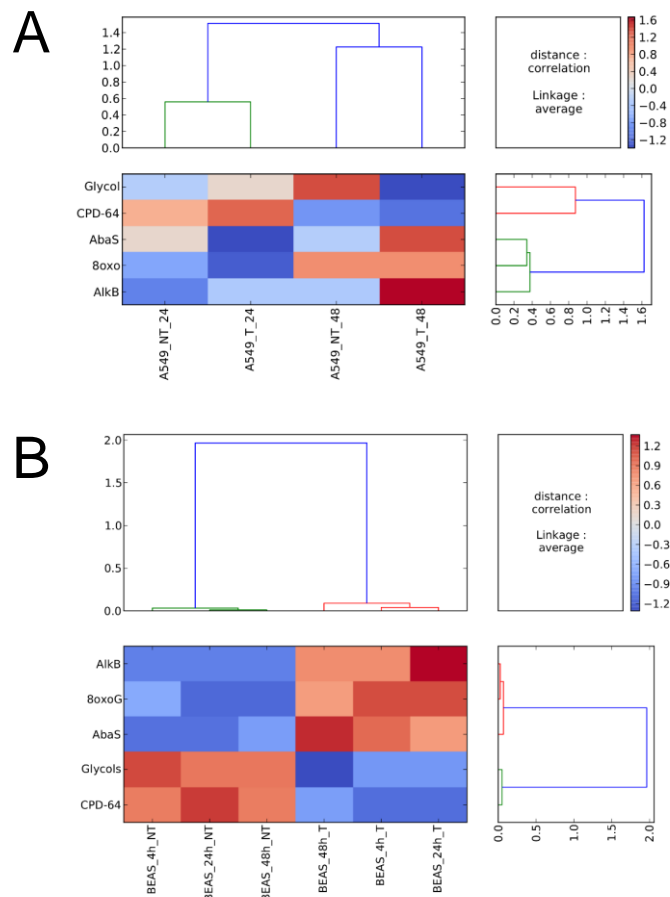


Figure 4. DNA lesion excision activity in A549 cells exposed to TiO₂-NPs. A549 cells were exposed to 100 µg/mL TiO₂-NPs for 24 or 48 h. *In vitro* excision activity was assessed using the GlycoSPOT assay. The fluorescence of each spot was normalised for the total fluorescence measured on the slide, to determine the relative contribution of each repair pathway. This experiment was performed once with three independent replicates (n=3). Statistical significance, p < 0.05, is indicated for exposed vs. control (*).

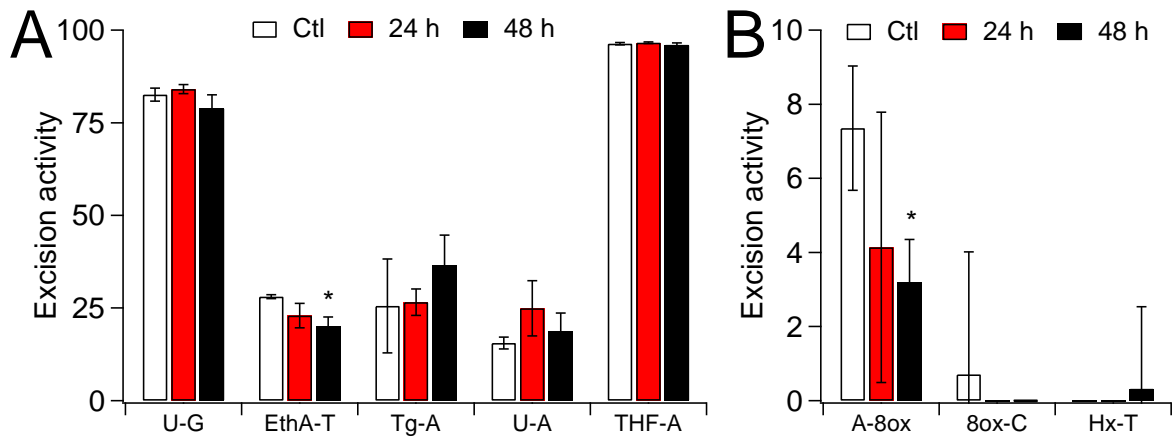


Figure 5. Schematic representation of DNA repair pathways and modulation of the gene expression profile for the proteins involved in each pathway. Protein name is indicated in blue when expression of its gene is downregulated, in black when expression of its gene is unchanged.

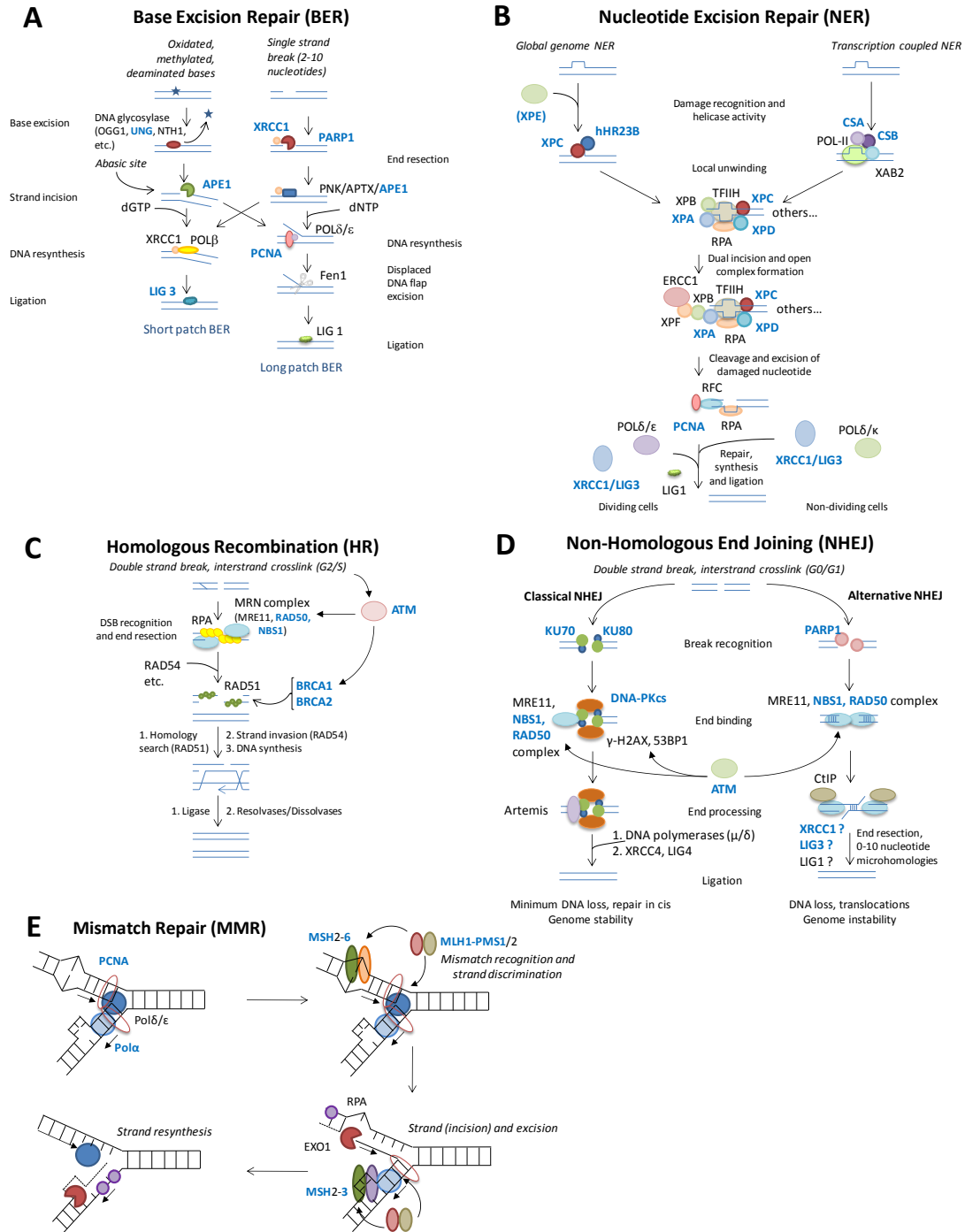


Figure 6. DNA methylation profile of DNA repair gene promoters. The methylation level of each promoter was measured using the EpiTect® assay (Qiagen), on A549 cells exposed for 4 h (A, B) or 24 h (C, D) to 0 (Ctrl) or 100 µg/ml of TiO₂-NPs. Statistical significance, $p < 0.05$, is indicated for exposed vs. Ctrl (*).

