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Identification through microarray gene expression analysis of cellular responses to benzo(a)pyrene and its diol-epoxide that are dependent or independent of p53

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ABSTRACT

Human colon carcinoma cells (HCT116) differing in p53 status were exposed to benzo(a)pyrene (BaP) or *anti*-benzo(a)pyrene-*trans*-7,8-dihydrodiol-9,10-epoxide (BPDE), and their gene expression responses compared by cDNA microarray technology. Exposure of cells to BPDE for up to 24 h resulted in gene expression profiles more distinguishable by duration of exposure than by p53 status, although a sub-set of genes were identified that had significantly different expression in p53-wild type (WT) cells relative to p53-null cells. Apoptotic signalling genes were up-regulated in p53-WT cells but not in p53-null cells and, consistent with this, reduced viability and caspase activity were also p53-dependent. BPDE modulated cell cycle and histone genes in both cell lines and, in agreement with this, both cell lines accumulated in S-phase. In p53-WT cells, G2 arrest was also evident, which was associated with accumulation of CDKN1A. Regardless of p53 status, exposure to BaP for up to 48 h had subtle effects on gene transcription and had no influence on cell viability or cell cycle. Interestingly, DNA adduct formation after BaP, but not BPDE, exposure was p53-dependent with 10-fold lower levels detected in p53-null cells. Other cell lines were investigated for BaP-DNA adduct formation and in these the effect of p53 knock-down was also to reduce adduct formation. Taken together these results give further insight into the role of p53 in the response of human cells to BaP and BPDE and suggest that loss of this tumour suppressor can influence the metabolic activation of BaP.

INTRODUCTION

The p53 tumour suppressor functions as a key player in determining cell fate after occurrence of various types of DNA damage and its gene is mutated in more than 50% of human tumours [1]. Upon genotoxic stress, cellular levels of p53 protein increase via post-transcriptional mechanisms [2] and the ability of p53 to bind specific DNA sequences is activated. Induction of p53 in response to DNA damage has been suggested as a means of genotoxicity testing [3,4]. Transcriptional modulation of p53-target genes leads to the regulation of downstream cellular processes, primarily cell-cycle arrest, apoptosis and DNA repair, that protect the cell from DNA damage and transformation [2,5].

Benzo(a)pyrene (BaP), a carcinogenic polycyclic aromatic hydrocarbon (PAH), exerts its genotoxic effects through metabolic bioactivation to *anti*-benzo(a)pyrene-*trans*-7,8-dihydrodiol-9,10-epoxide (BPDE), which binds covalently to DNA [6]. There is strong evidence linking this DNA damage directly with carcinogenesis [7]. For example, the distribution of BPDE-DNA adducts along the p53 gene in cultured cells strongly correlated with p53 mutation sites of human lung cancers [7-9]. In addition, the expression of p53 and one of its major transcriptional targets, CDKN1A (p21), after exposure to BaP, has been found to correlate with DNA adduct formation in human lung diploid fibroblasts [10]. The importance of p53 in cellular protection against tumourigenesis is highlighted by studies in mice with a mutated p53 genotype in which carcinogens induce more tumours than in mice expressing wild-type (WT) p53 [11,12].

The accumulation of p53 after carcinogen exposure has been observed in many studies and in a recent one, in which we analysed gene expression changes induced in cultured human cells exposed to BaP, we found that p53 plays an important role in this transcriptional response [13]. In another study [14] gene expression profiles of cells differing in p53 status were compared after exposure to genotoxic and non-genotoxic agents. p53-dependent gene

expression was identified for the genotoxic, but not for the non-genotoxic, agents, although PAHs were not included in this study. To investigate further the role of p53 in the cellular response to BaP we have analysed a pair of human colorectal cell lines (HCT116) that differ in p53 status (i.e. WT and knock-out) after exposure to BaP or BPDE. In addition, other biological parameters were investigated including DNA adducts, cell cycle, and protein expression. Expression changes together with biological outcomes were identified in cells exposed to BPDE that were dependent or independent of p53 status. DNA adduct formation by BaP was p53-dependent, with 10-fold lower adduct levels detected in p53-null cells. The influence of p53 expression on BaP-DNA adduct formation in other cell lines with partial knock-down of p53 function, confirmed that loss of p53 *in vitro* has effects on the metabolic activation of BaP.

MATERIALS AND METHODS

Cell culture and chemical treatment

The HCT116 human colorectal carcinoma cell line pair [15], one expressing wild-type p53 (p53-WT) and one with complete knock-out of p53 (p53-null), were kindly provided by Professor Bert Vogelstein, Johns Hopkins University School of Medicine, Baltimore MD. Stable shRNA p53-knock-down A549 human lung carcinoma cells and empty vector control cells were kindly provided by Mr Gunnar Jahnke and Professor Andrea Hartwig (Technical University of Berlin, Germany). Details concerning the construction and characterisation of the p53 knock-down A549 cell line will be published elsewhere (G. Jahnke and A. Hartwig, in preparation). Briefly, A549 cells were transfected with pRNA-H1.1/Neo siRNA expression vector (GenScript Corp., USA) with or without a small DNA insert encoding a short hairpin RNA targeting p53 [16] and using Lipofectamine in combination with Plus Reagent (Invitrogen, Germany) according to the manufacturer's recommendations. E6-expressing

A2780 human ovarian carcinoma cells and empty vector control cells were kindly provided by Dr. Michael Walton, Cancer Research UK Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, Surrey, UK. These cells were constructed as described previously [17] but using a pEFIRES-P vector system [18].

HCT116 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with Glutamax™ I, 1000mg/L D-glucose and sodium pyruvate (Invitrogen) and supplemented with 10% foetal bovine serum and 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma Aldrich). A549 cells were grown similarly, but with geneticin (Invitrogen) (0.3mg/ml) instead of penicillin/streptomycin for selection of plasmid containing cells. A2780 cells were maintained under similar conditions to those for HCT116 cells with the addition of purimycin (Invitrogen) (0.6 µg/ml) for selection of plasmid containing cells. Cells were grown as adherent monolayers and sub-culturing performed every 72 h when cells were 80% confluent and incubated in a humidified 5% CO_2 atmosphere at 37°C.

BaP was obtained from Sigma Aldrich and BPDE was synthesised by the method of R.G. Harvey and P.P. Fu [19]. 3-Nitrobenzanthrone (3-NBA) was synthesised as previously reported by Arlt *et al.* [20]. For chemical exposure, cells were grown for 48 h until 70% confluent and then BaP, BPDE or 3-NBA dissolved in DMSO (Sigma Aldrich) were added. DMSO alone was added to control cultures and its volume kept at 0.2% of the total culture medium. Cells were harvested by trypsinisation and washed with PBS.

Cell viability and DNA adduct measurement

Cells were exposed to BaP (0.25-5 µM), BPDE (0.1-1 µM), 3-NBA (5 µM), or DMSO alone (controls) in duplicate and cell viability was measured as described previously [13]. Cells were spun down, and DNA was isolated by a standard phenol chloroform extraction method. DNA adducts were measured for DNA extracted from BaP and BPDE treated cells using the

nuclease P1 version of the ^{32}P-postlabelling method as described previously [13]. For DNA prepared from cells treated with 3-NBA the butanol enrichment method was used to measure DNA adducts as described previously [20].

RNA extraction and cDNA synthesis for microarray analysis

Cells were exposed to BaP (2.5 and 5 μM) for 6, 24 and 48 h, or BPDE (0.5 and 1 μM) for 2, 6 and 24 h and control cells were treated with DMSO. All incubations were performed in triplicate. Cell pellets were collected and total RNA extracted using the Qiagen RNeasy Mini Kit protocol (RNeasy Mini Handbook, Qiagen, UK) and quantity and quality assessed as described [13]. Total RNA (4 μg) was reversed transcribed into cDNA and labelled with Cy3 or Cy5 mono-reactive dyes (Amersham Biosciences, UK) using the Invitrogen Indirect cDNA Labelling Kit protocol (Invitrogen, UK) as described [13].

cDNA microarray hybridisations

Gene expression analysis was performed using the Cancer Research UK DNA Microarray Facility (CRUKDMF) Human 22K Genome-Wide Array v1.0.0. The full probe list for this array can be found on the CRUKDMF website (**http://www.icr.ac.uk/array/array.html**). The majority of the clones have been sequence verified and are 800-2,000bp in length. The arrays were gridded onto Type 7* silanised slides (GE Healthcare, UK) and hybridisation, washing and scanning of the slides were performed as described previously [13]. RNA from exposed cells and time-matched vehicle-treated control cells were hybridised against each other for each dose and time-point from each triplicate biological experiment.

Microarray analysis

Image analysis using GenePix Pro v-5.1 software (Axon Instruments, USA) and data normalisation and analysis were performed within GeneSpring v-7.2 as described previously [13]. Briefly, after Lowess normalisations and filtering out of unreliable data, triplicate biological replicates were averaged to identify genes with significantly ($p < 0.05$) altered expression by at least 1.4-fold. Log_2 transformed data were used for any correlation (hierarchical clustering, principal components analysis [PCA]) or statistical algorithms (1-Way ANOVA) performed within GeneSpring.

The gene expression data discussed in this publication have been deposited in NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) (awaiting GEO accession number).

Transient transfection of p53-targeted siRNA

Approximately 3 x 10^5 HCT116 cells per well in a six-well plate in 2 ml medium containing 10% fetal calf serum (FCS), but without antibiotics, were cultured for 24 h to give 40-60% confluence. Cells were transfected with an siRNA duplex (siGENOME Duplex D-003329-05, Dharmacon, Thermo Fisher Scientific, UK) that targets the mRNA sequence encoding p53. The siRNA was introduced into the cells by complex formation with Dharma*FECT* 1 lipid transfection reagent (Dharmacon) according to the recommendations of the manufacturer. Briefly, 200 pmol of siRNA oligonucleotides were mixed with 5 μl of Dharma*FECT* 1 in a final volume of 400 ml of serum-free medium. Complexes were allowed to form for 20 min at room temperature after which the samples were diluted in 1.6 ml of serum-containing medium, to give a final siRNA concentration of 100 nM, and then added directly to the cells after removal of old medium. Control cells were exposed to transfection reagent alone. Cells were harvested 24 -72 h after transfection for Western blot analysis. For chemical exposure, 24 h after transfection, BaP or BPDE dissolved in DMSO was added to the cells. Cells were

harvested after 24 and 48 h exposure for Western blot and DNA adduct analysis. As an added control, cells were transfected with functional, non-targeting siRNA (si*CONTROL™,* Dharmacon) to confirm that knock-down of *p53* transcript was specific to the p53-targeting siRNA molecule.

Real-time quantitative PCR (RTqPCR)

Reverse transcription-PCR was used to generate cDNA from total RNA for relative quantitation analysis using real-time fluorescent PCR on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, UK) performed as previously described [13]. To detect the modulated expression of *CYP1A1* and *CYP1B1* 20x Assays-On-Demand™ gene expression primers and probes (Applied Biosystems) were used (CYP1A1-Hs00153120_m1, CYP1B1-Hs00164383_m1). Relative gene expression was calculated using the comparative threshold cycle (C_T) method as performed previously [13]. Transcript levels were compared between BaP-exposed cells and time-matched vehicle-treated control cells (calibrator sample) in order to measure gene induction.

Western blot analysis

Cells were exposed to BaP (2.5 µM) or BPDE (0.5 µM) for up to 48 h, and control cells were treated with DMSO. Cell pellets were collected and lysis and immunoblotting were performed as described previously [13]. Monoclonal antibody against p53 (Ab-6) was purchased from Calbiochem (Darmstadt, Germany) and diluted 1:5000. Detection of CDKN1A (p21) was by monoclonal antibody sc817 purchased from Santa Cruz Biotechnology (CA, USA), diluted 1:500. For detection of CYP1A1 we used a polyclonal antibody (PAI-340) in a 1:500 dilution (Affinity BioReagents, Golden, CO, USA). Monoclonal antibody to detect GAPDH (6C5)

was purchased from Chemicon (Chemicon, Millipore, USA), diluted 1:2000 and used as a loading control.

Flow cytometry

Cells were exposed to 2.5 µM BaP, 0.5 µM BPDE or DMSO alone for up to 48 h. Harvested cells were fixed and stained with propidium iodide as described previously [13] in preparation for cell cycle analysis performed using a Beckman Coulter EPICS Elite ESP (Beckman Coulter, Buckinghamshire, UK) at 488 nm. The relative number of cells in each phase of the cell cycle was determined using Cylchred v1.0.2 and WinMidi v2.8 software (**http://www.cardiff.ac.uk/medicine/haematology/cytonetuk/documents/software.htm**).

Caspase Glow® 3/7 assay

The activities of caspase-3 and -7 were measured in HCT116 p53-WT and p53-null cells after exposure to 0.5 µM BPDE for up to 24 h in 96-well plates using a Caspase-Glo assay kit (Promega, Southampton, UK) and following the manufacturer's instructions. Luminescent signal was detected using a Microtiter Plate Luminometer (DYNEX Technologies, Worthing, UK). Luminescence is proportional to the amount of caspase activity present and expressed as relative light units (RLU).

RESULTS

HCT116 p53-WT and p53-null gene expression analysis

BaP-induced transcription: In total, the expression of 24 genes was modulated significantly by at least 1.4-fold in p53-WT cells and 21 in p53-null cells after BaP exposure (Supplementary Table 1). Seven genes were modulated in the same direction in both cell lines (Supplementary Figure 1A and Supplementary Table 1), suggesting that these genes are

altered by p53-independent mechanisms with a larger proportion of the expression changes being p53-dependent. When hierarchical clustering is performed on the data (Figure 1A) this difference is not so apparent and suggests that the gene expression profiles are relatively similar regardless of time, concentration, or p53 status. BaP had very subtle effects on gene expression in both cell lines with few genes exceeding a 2-fold change in expression. Induction of the xenobiotic metabolism gene *NQO1* was independent of p53 status. A probe for *CYP1A1* was not present on the microarrays but its induction by BaP in these cells was demonstrated by RTqPCR (Supplementary Table 4). Induction of *CYP1B1* was not identified from the array data. The inability of the microarrays to detect changes in this transcript seems to be a common phenomenon and may be related to low basal expression levels in cells, as described in our previous study [13]. Up-regulation of *CYP1B1* in HCT116 cells was however, detected by RTqPCR (Supplementary Table 4).

Consistent with the subtle effects on gene expression, HCT116 cell viability was not significantly reduced by up to 5 µM BaP for up to 48 h exposure (Supplementary Figure 2A and B). Although an approximate 2-fold accumulation of p53 in the p53-WT cells was observed in response to BaP exposure (Figure 2A) a significant p53 gene expression response was not identified from the microarrays. Closer inspection of the expression data revealed that a key p53-regulated gene, *CDKN1A,* did have altered expression (1.4-fold) in p53-WT but not p53-null cells after 24 h exposure to 5 µM BaP, although it was not identified as significant. Whilst this observation was confirmed by RTqPCR (data not shown) it was not seen consistently at the protein level (Figure 2A).

BPDE-induced transcription. The lack of a robust p53-gene expression response in the HCT116 cells after BaP exposure prompted us to investigate gene expression induced by the potent DNA binding metabolite of BaP, BPDE, in order to gain more information on the role of p53 in the BaP-gene expression response. Whilst BPDE exposure (up to 1 µM for up to 48

h) did not affect the viability of p53-null cells, a concentration-dependent reduction in viability of p53-WT cells of up to 40% was observed (Supplementary Figure 2C and D). BPDE exposure modulated significantly a total of 67 genes by at least 1.4-fold in p53-WT cells and 66 in p53-null cells (Supplementary Table 2) with an overlap of 25 genes altered in both cell lines (Supplementary Table 2 and Supplementary Figure 1B). As with the BaP-induced expression profiles, hierarchical clustering revealed that the BPDE-induced gene expression profiles were not affected greatly by p53 status, but rather differences between the profiles were dependent on time of exposure to BPDE (Figure 1B). PCA confirmed these effects (Supplementary Figure 1C) and also showed that p53 status had a greater influence on the gene expression profiles after 24 h BPDE exposure. 1-Way ANOVA was performed on the list of all genes modulated by BPDE in the two cell lines (108 genes), which identified 22 genes that were most significantly ($p < 0.05$) differentially expressed between the p53-WT and p53-null cells (Supplementary Table 3). This list includes p53-regulated genes, such as *CDKN1A, DDB2,* which were induced in p53-WT cells but not p53-null cells. In addition, tumour necrosis factor receptor (*TNFR*) genes, including p53 regulated *FAS* and *TNFR10B*, linked to apoptosis were up-regulated only in p53-WT cells.

Affected biological processes

To determine if any biological themes exist within the expression data of the HCT116 cells exposed to BPDE or BaP, Expression Analysis Systematic Explorer (EASE) analysis was performed to identify biological processes that are significantly over-represented (EASE score <0.05) in the gene lists when compared to their total representation on the microarrays. In concordance with the subtle effect that BaP had on gene expression in these cells, few biological processes were significantly over-represented in the BaP-gene lists and no overlap was observed between p53-WT and p53-null cells (not shown). Analysis of the BPDE-

induced gene expression changes, however, revealed that apoptotic processes were significantly up-regulated in p53-WT cells but not in p53-null cells (Table 1), confirming that apoptotic signalling in response to BPDE exposure is dependent on p53. This is consistent with the cell viability data for these cells (Supplementary Figure 2C and D). Cell-cycle processes were up-regulated regardless of p53 status, although the genes linked to these processes differed between the p53-WT cells and p53-null cells with a number of genes linked to progression through mitosis up-regulated in p53-null cells (Table 1). Interestingly, processes linked to chromosome organisation were significantly affected through gene repression in both cell lines with down-regulation of *HIST1H1C*, *HIST1H2AC*, *HIST1H2BJ*, *HIST1H3D* and *TAF6L*. DNA repair processes were significantly up-regulated in p53-null cells, but not in p53-WT cells.

DNA adduct analysis of HCT116 p53-WT and p53-null cells

A time- and concentration-dependent increase in DNA adduct formation was observed in p53-WT, but not in p53-null cells, exposed to up to 5 μM BaP for up to 48 h (Figure 3A). A striking observation was that BaP-induced DNA adduct levels were around 10-fold lower in p53-null cells relative to p53-WT cells. In contrast, similar or lower adduct levels in p53-WT cells relative to p53-null cells, were observed after exposure to BPDE (Figure 3B). This suggests that the difference in adduct levels in the two cell lines when exposed to BaP may be through a p53-dependent effect on the metabolic bioactivation of this compound.

Cell cycle analysis of HCT116 p53-WT and p53-null cells

To determine if p53 plays a role in the cell cycle effects induced by BaP and BPDE exposure, p53-WT and p53-null HCT116 cells were exposed to BaP (2.5 μM) or BPDE (0.5 μM) for up to 48 h and DNA content was measured by FACS analysis (Figure 4). In agreement with the

lack of cell cycle-related gene expression alterations in HCT116 cells exposed to BaP, this compound had no effect on their cell cycle parameters. Exposure to BPDE resulted in an accumulation of cells in S phase and a reduction of cells in G1 that was independent of p53 status. After 48 h the number of exposed cells in S phase had almost returned to control levels. In p53-WT, but not p53-null cells, an arrest in G2 phase was evident at 48 h.

Apoptotic activity of HCT116 p53-WT and p53-null cells

BPDE had a greater effect on cell viability in p53-WT cells than in p53-null cells and, in agreement with this, apoptotic signalling was evident from the gene expression analysis of p53-WT cells, but not p53-null cells, exposed to BPDE. To confirm this differential response of the two cell lines to BPDE exposure, the activities of two key effector enzymes caspase-3 and caspase-7 that are involved in apoptosis in mammalian cells, were measured by luminescence detection. This particular caspase assay was chosen as caspase-3 activation has been linked to BaP-induced apoptosis [21,22]. Figure 5 illustrates the level of luminescence detected in cells exposed to 0.5 and 1 μM BPDE for 6 and 24 h, which is proportional to the amount of caspase-3 and caspase-7 activity in the cells. While there is only a small difference in caspase activity after 6 h exposure in both cell lines there is a large increase in activity in p53-WT cells after 24 h exposure to 1 μM BPDE that is not seen in p53-null cells. This is consistent with the cell viability data where greatest loss of viability in p53-WT cells occurred after exposure to 1 μM BPDE (Supplementary Figure 2C). In these cells, the expression level of *TNFR* apoptosis signalling genes was greatest after 1 μM BPDE exposure, but predominantly after 6 h, not 24 h (Supplementary Table 3), indicating that apoptotic mRNA levels increase early followed by biological outcome at 24 h.

DNA adduct analysis in other cell line pairs differing in p53 status

To determine if the apparent effect of loss of p53 on the activation of BaP and its DNA adduct forming capability was a consequence of the p53 knock-out procedure, two other cell line pairs differing in p53 status were investigated. A549 lung carcinoma cells stably expressing p53-targeting siRNA (with approximately 50% reduction in p53 protein expression, Figure 2C) and A2780 ovarian carcinoma cells stably expressing either of two different E6 clones (with approximately 70% reduction in p53 protein expression, Figure 2D) were compared against empty vector control cells. E6 is a viral oncoprotein that can complex with p53 and target it for degradation, the result of which can be equivalent to inactivating p53 by mutation [23]. In addition, we silenced the expression of p53 transiently with siRNA in the HCT116 p53-WT cells and compared them with mock-transfected cells. A reduction of approximately 80% in p53 protein expression was achieved in these cells (Figure 2B) specific to the p53 siRNA, as confirmed by parallel transfection with a functional, non-targeting siRNA control (not shown). Reduction of CDKN1A was observed in all cells with reduced p53 expression (Figure 2A-D), confirming the p53 status of the cells.

DNA adduct levels were measured in all cell lines after exposure to BaP or BPDE for 24 and 48 h (Figure 6A-D). Significantly lower adduct levels were detected in all cell lines with reduced p53 expression, relative to their controls, after 48 h exposure to BaP. This was also observed at 24 h for the HCT116 with complete p53 knock-out and for the A2780 cells with knocked-down p53 expression. This provides evidence that the observed effects on BaP-induced DNA adduct formation are not artefacts of the cells' manipulation, but are genuinely p53-dependent. In contrast, BPDE exposure of the cells, in the majority of cases, resulted in similar DNA adduct levels regardless of p53 status (Figure 6) suggesting that the effect seen with BaP is not related to DNA repair, but to the metabolic activation of BaP. Interestingly, exposure to 3-NBA, a nitro-PAH that also requires metabolic activation to form DNA adducts

[24-26], resulted in similar adduct levels in p53-WT and p53-null cells (Figure 6). Bioactivation of 3-NBA is catalysed primarily by cytosolic nitroreductases such as NQO1 rather than cytochrome P450 (CYP) enzymes [25,26] indicating that basal levels of p53 might be linked to the expression of CYP enzymes, such as CYP1A1 and/or CYP1B1. To determine if loss of p53 affects the levels of key enzymes involved in the bioactivation of BaP, the induction of *CYP1A1* and *CYP1B1* expression was assessed by RTqPCR (Supplementary Table 4). Whilst an approximate 6-fold lower induction of these transcripts was observed in HCT116 p53-null cells relative to p53-WT cells, equal induction of these genes was seen for the other cell line pairs. In addition, no difference in the basal or induction levels of CYP1A1 protein, as assessed by Western blot, was observed between the HCT116 p53-WT and p53-null cells (data not shown).

DISCUSSION

The tumour suppressor protein p53 protects cells from genotoxic stress by activating cellular processes such as cell cycle inhibition, DNA repair and apoptosis [2,5]. The importance of this protein is highlighted by the fact that it is mutated in approximately 50% of human cancers [1] and this figure increases to 60% for lung cancers [27]. There are examples of correlation between sites of carcinogen-DNA damage in the *p53* gene in cells in culture and the sites of p53 mutations in human tumours [7-9], strongly indicating that this DNA damage is directly linked to carcinogenesis. Cellular accumulation of p53 in response to PAH exposure has been observed in many studies and recently we identified p53 as an important component of BaP-induced transcriptional responses in human cell lines [13]. To further understand the role of p53 in the cellular response to BaP exposure we have compared the transcriptional responses to BaP or BPDE in human cells that differ in p53 status.

Gene expression analysis of HCT116 cells

BaP had very subtle effects on HCT116 cells, with little evidence of a p53 response. This may be due to the levels of DNA damage not being high enough to elicit such a response. Exposure to BPDE resulted in greater transcriptional signatures that were most distinguishable by duration of exposure. A small sub-set of p53-dependent gene expression changes were identified and p53 status of the cells was most apparent after the longer exposure time of 24 h, which may be a result of downstream gene expression alterations, dependent on p53 activation, coming into effect over time. Included in the p53-dependent gene expression signature were known p53-regulated genes, including *CDKN1A* and *DDB2* that were also identified by Amundson et al. [14] as discriminating p53 status in TK6 cells.

Amundson's study [14] investigated the participation of p53 in gene expression signatures induced by toxic stress in TK6 cells differing in p53 status. Although they identified a small p53-dependent signature for genotoxic agents, all profiles were largely independent of p53 status, but were, however, distinguishable by mechanistic differences between the stresses. Similarly Park et al., who analysed the expression of a relatively small set of genes in cells exposed to γ-irradiation, UV, doxorubicin and cisplatin did not identify any p53-dependent expression. The current study extends these studies to consider the important class of PAHs and also shows that expression profiles induced by BaP or BPDE exposure are largely p53-independent, although a sub-set of p53-dependent expression was identified. Several PAH compounds, including BaP, have been shown to induce apoptosis *in vitro* [28-30], a process in which p53 plays an important role [28]. The expression of three genes, *FAS*, *TNFRSF10B* and *TNFRSF10D,* which encode members of the tumour necrosis factor receptor family of death receptors, was identified as dependent on p53 in cells exposed to BPDE in this study. Upon ligand binding these death receptors are capable of signalling for

apoptosis through a 'death domain' contained within their intracellular portion and transactivation of these receptors by p53 has been shown to mediate apoptosis *in vitro* [31,32]. The effect of BPDE on cell viability in HCT116 cells may be through signalling of these receptors that are up-regulated in a p53-dependent manner. Apoptotic signalling was also seen as p53-dependent at the level of affected biological processes. Functional analysis of the cells also confirmed this response with a greater increase in apoptotic signalling, as measured by caspase activity, observed in the cells expressing p53. We have also seen the induction of members of these death receptors in two other cell lines, MCF-7 and HepG2, after BPDE exposure [33], indicating that this is an important p53-dependent cellular response to this compound *in vitro*.

Previously we detected the repression of histone genes in response to BaP exposure in MCF-7 and HepG2 cells and histone expression levels showed a strong inverse correlation with DNA adduct formation in MCF-7 cells [13]. BPDE exposure of HCT116 cells also led to the repression of a number of histone genes and, interestingly, this effect was independent of p53 status. It is therefore likely that regulation of histone gene expression is important in the cellular response to PAH exposure. The expression of histone genes is tightly coupled to DNA synthesis [34], the delay of which is evident in cells exposed to BaP that undergo an accumulation in S phase of the cell cycle [13,35,36]. The repression of histone expression has also been observed in response to ionising radiation-induced DNA damage [37,38] and in yeast cells exposed to aflatoxin B_1 that were also seen to arrest in S phase [39]. The conservation of this response across species suggests that this cellular response is important in maintaining DNA integrity after genotoxic insult. In this study we have shown that, consistent with histone gene repression, the accumulation of cells in S phase after BPDE exposure is also independent of p53 status, further validating the link between these two events. This stealth property of cells to evade a protective G1 arrest initiated by p53 activation, after BaP

or BPDE exposure, has been seen in response to other PAHs [40,41] and has also been observed in p53-deficient human cells exposed to aflatoxin B_1 [42], consistent with our finding that this effect is independent of p53. It has been suggested that the ability of cells to overcome G1 arrest and accumulate in S phase leaves them more susceptible to DNA damage and transformation [36,43] and that lack of G1 arrest is through inability to induce the expression of the potent G1/G2 cell cycle inhibitor, CDKN1A [43]. In this study, however, we saw accumulation of CDKN1A protein in p53-WT cells at time-points at which S phase accumulation was also observed, but not G1 arrest. G2 arrest was also evident in p53-WT cells, which may be a result of p53-dependent expression of CDKN1A [15] coming into play in these cells as an added protective mechanism against DNA damage. In addition, a large number of genes linked to the mitotic phase of the cell cycle were induced in p53-null cells but not p53-WT cells, suggesting that p53 is involved in the repression of these genes thus preventing progression into mitosis after S phase arrest.

The effect of p53 on DNA adduct formation

DNA adduct analysis of HCT116 cells exposed to BaP revealed that adduct levels were significantly lower in cells not expressing p53. This was not seen after BPDE exposure suggesting that basal levels of p53 are linked to the metabolic activation of BaP. The gene expression data suggest that off-target effects of the p53 knock-out procedure by homologous recombination are minimal at the gene expression level. The observed effect of loss of p53 on BaP metabolism and DNA adduct formation in HCT116 cells was confirmed through the investigation of other cell lines with partial knock-down of p53 by different methods, which also saw lower DNA adduct levels in cells with lower p53 levels. The difference in adduct levels in cells with partial loss of p53 was smaller than that observed in cells with no p53 expression, suggesting that the effect is related to the level of p53 expression. The

observations made in this *in-vitro* study are in contrast to *in-vivo* studies in which loss of p53 results in increased DNA adduct levels and tumour formation [11,12,44]. Higher induction of *CYP1A1* and *CYP1B1* expression could only explain the difference in BaP metabolism in the HCT116 cells with complete loss of p53, although this was not observed at the protein level. Further investigation of levels of other key xenobiotic metabolism enzymes and their activities, together with global gene expression analyses of the additional cell lines, need to be carried out.

Conclusion

We have characterised cellular responses to BaP and its metabolite BPDE and gained greater insight into the role of p53 in these responses. Although a p53-dependent signature was identified, many genes were altered independently of p53. Whilst apoptotic responses have been identified as dependent on p53, early cell-cycle effects were seen to be independent of this tumour suppressor. The effect of loss of p53 on the metabolic activation of BaP *in vitro* was unexpected and requires further investigation.

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Table 1. Biological processes significantly (EASE Score < 0.05) over-represented within the gene expression profiles induced after BPDE exposure in HCT116 p53-WT and p53-null cells.

Biological Process	p53-WT	p53-null
Cell cycle/cell proliferation	BTG3, CDC2, CDKN1A, PCNA, RPS27L, RRM2, TOB1 [1]↑	CCNA2, CDC2, CDC6, MAD2L1, MCM3, MCM4, PCNA, PPP2CA, RBBP4, RBBP6, RRM2, SMC1L1, TOB1, UBE2C ↑
M phase of mitotic cell cycle		CCNA2, CDC2, MAD2L1, PPP2CA, SMC1L1, UBE2C ↑
DNA replication/S phase of mitotic cell cycle		CDC6, MCM3, MCM4, PCNA, PPP2CA, RBBP4, RRM2, SMC1L1, UBE2C, UNG ↑
Apoptosis	CDKN1A, TNFRSF10B, TNFRSF6 ↑	
Response to DNA damage stimulus		PCNA, RBBP4, SMC1L1, TNKS2, UBE2C, UNG ↑
DNA repair		PCNA, RBBP4, SMC1L1, UBE2C, UNG ↑
Cytokinesis		CCNA2, CDC2, CDC6, SMC1L1, UBE2C ↑
Cell growth and/or maintenance		CCNA2, CDC2, CDC6, K-ALPHA-1, MAD2L1, MCM3, MCM4, PCNA, PPP2CA, RBBP4, RBBP6, RRM2, SMC1L1, STMN1, TNKS2, TOB1, UBE2C ↑ AREG, EREG, HIST1H1C, HIST1H2AC, HIST1H2BJ, HIST1H3D, IGFBP3, PLAU, SLC38A2, TAF6L, XPO1 ↓
Microtubule-based process		K-ALPHA-1, PPP2CA, SMC1L1 ↑
Assembly/maintenance of chromatin	HIST1H1C, HIST1H2AC, HIST1H2BJ, HIST1H3D, TAF6L ↓	HIST1H1C, HIST1H2AC, HIST1H2BJ, HIST1H3D, TAF6L ↓
Cell organisation and biogenesis	HIST1H1C, HIST1H2AC, HIST1H2BJ, HIST1H3D, TAF6L, TTK ↓	HIST1H1C, HIST1H2AC, HIST1H2BJ, HIST1H3D, TAF6L ↓
DNA metabolism	FOS, HIST1H1C, HIST1H2AC, HIST1H2BJ, HIST1H3D, NPAS2, SFPQ, SLC38A2, SMURF2, TAF6L, XRCC4 ↓	
Nucleic acid metabolism		HIST1H1C, HIST1H2AC, HIST1H2BJ, HIST1H3D, SLC38A2, SMURF2, TAF6L, TCF7L2, TEAD1 ↓
Regulation of transcription		HIST1H1C, HIST1H2BJ, SLC38A2, SMURF2, TAF6L, TCF7L2, TEAD1 ↓

[1]↑ up-regulated expression   ↓ down-regulated expression

TITLES AND LEGENDS TO FIGURES

Figure 1. Hierarchical cluster analysis of HCT116 cells exposed to BaP and BPDE.

Hierarchical clustering was performed on all experimental conditions using genes modulated in at least one of the cell lines after (A) BaP exposure (38 genes) and (B) BPDE exposure (108 genes).

Figure 2. Western blot analysis of p53 and CDKN1A protein expression in cells differing in p53 status exposed to BaP or BPDE.

(A) HCT116 control cells and those with knock-out of p53 by homologous recombination, (B) HCT116 control mock transfected cells and those transfected with p53-targeting siRNA molecule, (C) A549 control cells expressing empty vector or cells stably expressing p53-targeting shRNA plasmid and (D) A2780 control cells expressing empty vector or cells stably expressing either of two E6 clones were exposed to 2.5 µM BaP or 0.5 µM BPDE, and protein extracted for Western blot analysis of p53 and CDKN1A protein levels. GAPDH protein expression, was used as loading control.

Figure 3. DNA adduct levels detected in HCT116 p53-WT and p53-null cells after BaP or BPDE exposure.

Cells were exposed to (A) up to 5 µM BaP or (B) up to 1 µM BPDE and harvested after the times indicated for DNA adduct analysis by ^{32}P-postlabelling. The values are the mean ± range of duplicate cell incubations; each DNA sample was determined by two post-labelled analyses.

Statistical significance of differences between the adduct levels of p53-WT and p53-null cells was determined by a Student's t-test.

* $p < 0.01$, # $p < 0.05$.

Figure 4. Effects of BaP and BPDE on cell cycle in HCT116 p53-WT and p53-null cells.

The stacked bar-chart shows the percentage distribution of cell cycle parameters after exposure of HCT116 cells to 2.5 µM BaP, 0.5 µM BPDE or DMSO as control for the times indicated. The histograms are representative of two independent experiments for which the standard deviation was never greater than 5%.

Figure 5. Caspase 3/7 activities in HCT116 p53-WT and p53-null cells exposed to BPDE.

The activities of caspase-3 and caspase-7 were measured by luminescence detection in cells after exposure to BPDE (0.5 and 1 µM) for 6 and 24 h. The number of relative light units (RLU) is proportional to the amount of caspase activity. The values are the mean ± S.D. of three independent experiments.

Figure 6. DNA adduct analysis of cell lines pairs with complete or partial knock-down of p53.

DNA adduct levels were measured in (A) HCT116 control cells and those with knock-out of p53 by homologous recombination, (B) HCT116 control mock transfected cells and those transfected with p53-targeting siRNA molecule, (C) A549 control cells expressing empty vector or cells stably expressing p53-targeting shRNA and (D) A2780 control cells expressing empty vector or cells stably expressing either of two E6 clones after exposure to 2.5 µM BaP, 0.5 µM BPDE or 5 µM 3-NBA for 24 and 48 h. The values are the mean ± range of duplicate cell incubations; each DNA sample was determined by two postlabelled analyses.

Statistical significance of differences between the adduct levels of control and p53 knock-down cells was determined by a Student's t-test.
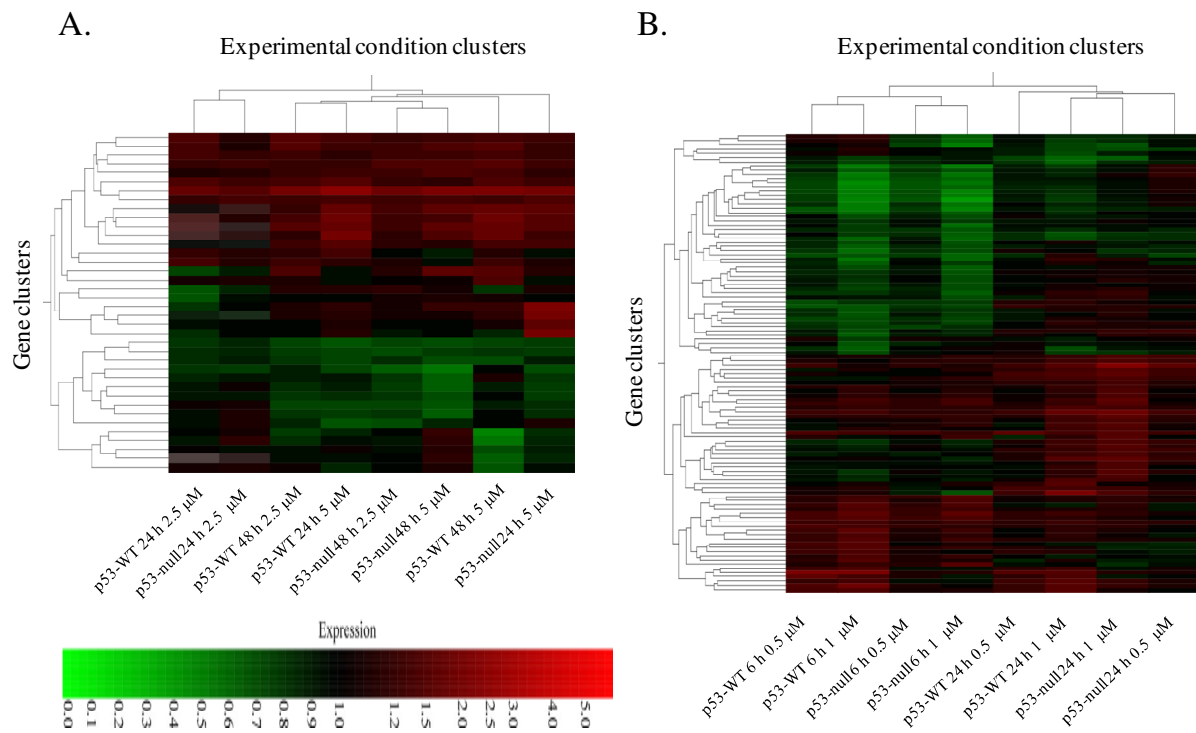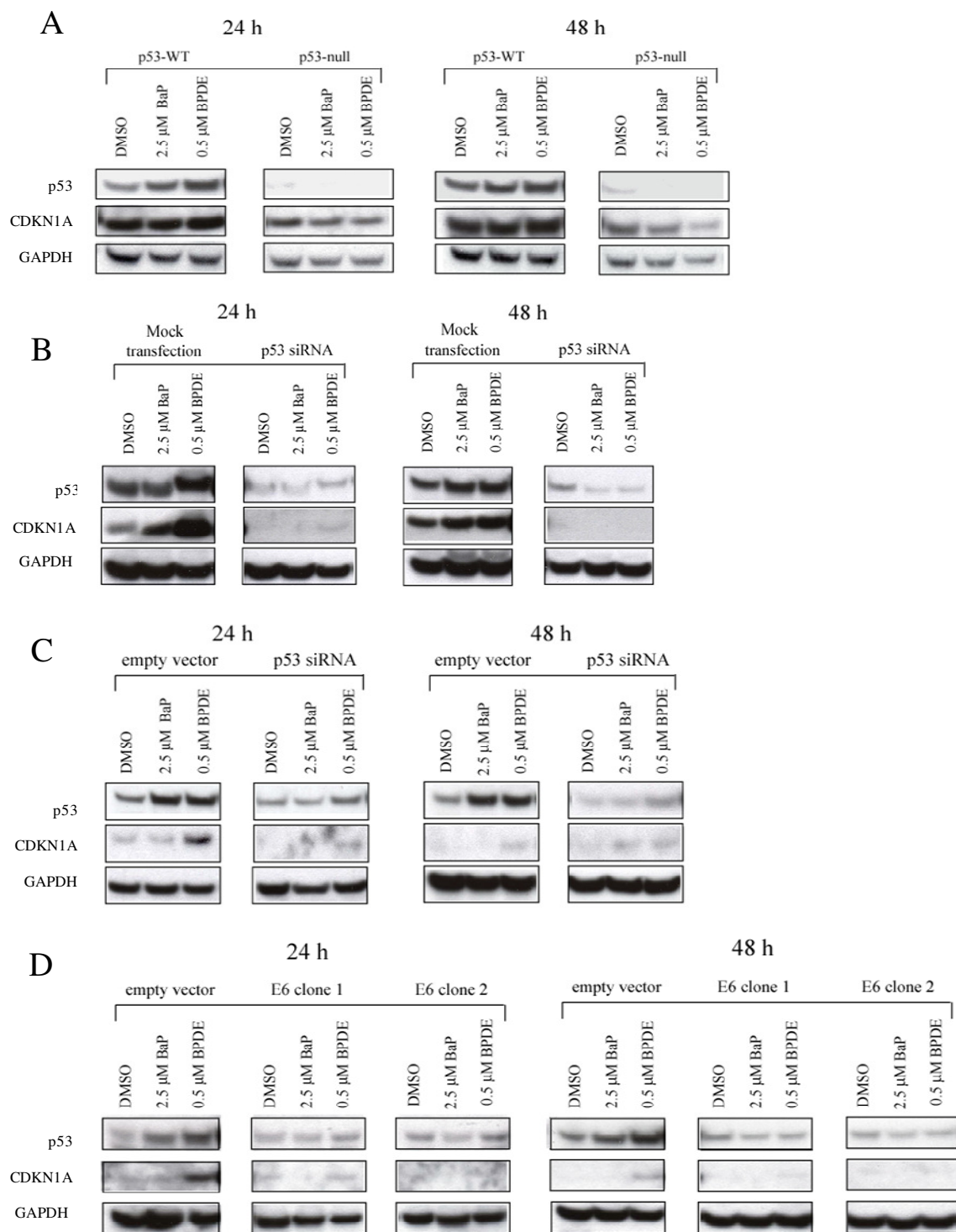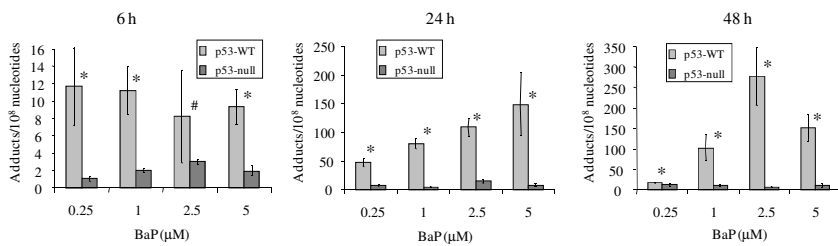
* $p < 0.01$, # $p < 0.05$.

A. Experimental condition clusters
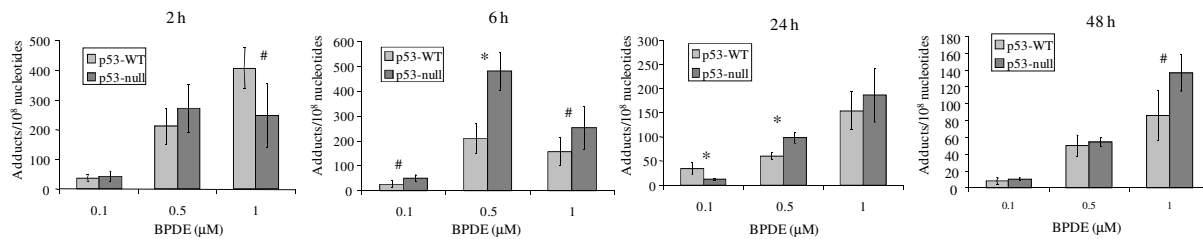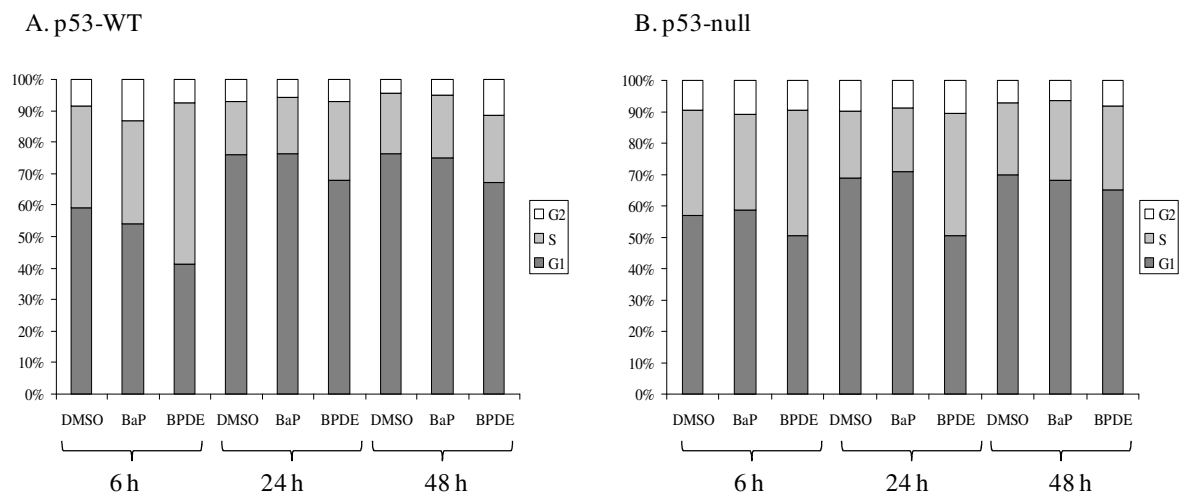

B. Experimental condition clusters

Figure 1

Figure 2

Figure 3

A. p53-WT

B. p53-null

Figure 4

Figure 5

Figure 6