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Full Length Article

Identification of essential transcription factors for adequate DNA damage response after benzo(a)pyrene and aflatoxin B1 exposure by combining transcriptomics with functional genomics

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ABSTRACT

DNA damage mediates widespread changes in transcription through activation or repression of transcription factors (TFs). However, the consequences of regulating specific TFs for the outcome of the DNA repair process remain incompletely understood. Here, we combined transcriptomics and TF binding prediction with functional genomics to identify TFs essential for adequate DNA repair in HepG2 liver cells after a non-cytotoxic dose of carcinogens benzo(a)pyrene (BaP) (2 μM) and aflatoxin B1 (AFB1) (5 μM). BaP and AFB1 induced a largely common transcriptional response, mediated by similar TFs. A lentiviral shRNA screen knocking down the top31 identified TFs, was performed to determine their effect on DNA repair by assessing phosphorylation of H2AX (γ-H2AX). In addition to the top candidate p53, we identified several other interesting TFs that modulated γ-H2AX after BaP and AFB1 treatment. Validation studies confirmed the role of p53 in reducing γ-H2AX formation and DNA breaks measured by COMET assay after BaP and AFB1 exposure. Expression of the cell cycle inhibitor p21 was profoundly impaired upon p53 knock-down. In addition, the expression of 2 genes involved in nucleotide exchange repair, DDB2 and XPC was significantly reduced in p53 knock-down cells. Although p63 knock-down affected DNA damage upon BaP treatment this was not associated with altered expression of DDB2 or XPC. Finally, knock-down of ARNT reduced γ-H2AX in response to BaP, which was associated with reduced CYP1A1 expression. Importantly, our results suggest a new role for ARNT and its dimerization partner AHR in the occurrence of H2AX phosphorylation after AFB1 treatment.

These data show that modulation of TF activity impacts on the repair of BaP- and AFB1-induced DNA damage. Our study also demonstrates the potential of combining functional genomics with genome-wide expression analysis to identify yet unknown causal relationships, thereby aiding in the interpretation of complex biological systems.

1. Introduction

DNA damage may occur after exposure to exogenous agents such as chemical carcinogens as well as endogenous agents like reactive oxygen species-generating factors. Unrepaired DNA damage may result in errors during DNA replication leading to mutations or large scale genomic rearrangements, ultimately contributing to cancer development. Mammalian cells have evolved a multitude of mechanisms for detecting DNA damage, coordinating repair, regulating cell-cycle progression and activating transcriptional programs (Jackson and Bartek, 2009).

The global transcriptional response to genotoxic agents has been extensively studied in order to design transcriptome-based assays for predicting genotoxicity and carcinogenicity in several model systems (Dickinson et al., 2004; Doktorova et al., 2013; Ellinger-Ziegelbauer et al., 2005; Harris et al., 2004; Hu et al., 2004; Magkoufopoulou et al., 2012; van Delft et al., 2004). Typically these transcriptome-based assays outperform the traditional test battery for predicting genotoxicity and carcinogenicity. Nevertheless a major challenge lies in the biological interpretation of the underlying mode of action for these identified classifying genes. Efforts to improve the toxicological interpretation of classifying genes include the analysis of complete biological pathways rather than single genes by performing network modeling and to correlate expression changes to phenotypic endpoints, a concept referred to as phenotypic anchoring (Jennen et al., 2015; Magkoufopoulou et al., 2011).

Phosphorylation of histone H2A variant H2AX on Ser139 (γ-H2AX),

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a mark associated with DNA double strand breaks (DSBs) (Rogakou et al., 1998) is regarded as an important phenotypic endpoint for assessing genotoxicity. Notably, extensive studies have shown that classical genotoxic carcinogens such as benzo(a)pyrene (BaP) and aflatoxin B1 (AFB1) are indeed capable of inducing γ-H2AX accumulation (Magkoufopoulou et al., 2011; Niziolek-Kierecka et al., 2012; Tsamou et al., 2012; Zhou et al., 2006). This confirms that the initial DNA damage consists of bulky DNA adducts that are repaired by nucleotide excision repair (NER) (Hoeijmakers, 2009). DSBs represent the most detrimental form of DNA damage to the cell. A single DSB can be lethal when left unrepaired or misrepaired. In the scenario in which a damaged cell is not eliminated, the DSB may result in mutations, chromosomal rearrangements and genomic instability, thereby contributing to carcinogenesis. Yet the mechanisms underlying the DSB formation after BaP and AFB1 exposure are poorly understood.

Consequently, to obtain new insights in molecular mechanisms inducing functional genotoxicity, we aimed to identify essential TFs that play causal roles in the formation of DSBs after BaP and AFB1 exposure. Both in house as well as publically available transcriptomic data were used to identify TFs that represent key nodes in the transcriptional response to BaP and AFB1. Candidate TFs were subsequently tested for their ability to alter the DNA damage response after exposure to Bap and AFB1 in terms of γ-H2AX accumulation and DNA damage measured using COMET assay, by applying an RNA interference strategy.

2. Material and methods

2.1. Data processing and selection of candidate transcription factors

To identify relevant transcription factors during genotoxic exposure, we first analyzed publicly available microarray data. Transcriptomic measurements from HepG2 cells exposed to 1 μM AFB1 and 2 μM BaP (Sigma-Aldrich, Zwijndrecht, Netherlands) after 24 h were retrieved from ArrayExpress (ebi.ac.uk/arrayexpress/, study accession E-GEOD-28878). Raw files (generated on Affymetrix Genome U133 Plus 2.0 platform) were assessed for quality control using ArrayAnalysis (arrayanalysis.org) and preprocessed (background correction, probe reannotation, data filtering and log2-transformation) using the R package affy. LIMMA (linear model for microarrays) (Gautier et al., 2004) was applied to each compound set to identify differentially expressed genes (DEGs). Since activity of a transcription factor (TF) can be predicted by the expression levels of its target genes, we first obtained a list of known, curated transcriptional interactions by retrieving target genes from 1544 human TF/TF complexes available from the MetaCore™ database (Essaghir et al., 2010). The resulting catalogue (comprised of ~40,000 interactions) was then used to estimate TF activation/repression in the DEGs induced by AFB1 or BaP exposure (FDR < 0.05) using right-sided Fisher's exact test. TFs were deemed significant when FDR < 0.01.

2.2. Cell culture

HepG2 and HEK293T (ATCC) cells were maintained in MEM and DMEM plus Glutamax supplemented with 10% FBS, 1% NEAA and 1% sodium pyruvate (Gibco, Breda, the Netherlands). HEK293T cells were solely used for the production of the lentiviral supernatants. For experiments HepG2 cells were cultured within a collagen sandwich like is done for primary cells as described previously (Mathijs et al., 2009). Briefly, after adhesion of HepG2 cells to the first collagen layer, medium was removed, cells were washed with HBSS and a second layer of collagen was added. This collagen mix for the second layer of collagen contains 1 mg/ml Collagen I rat tail (BD Biosciences, Breda, the Netherlands), 1× DMEM, and 5.8 mM NaOH.

2.3. RNA interference screen

Lentiviral shRNA constructs directed against selected TFs were selected from the Sigma MISSION TRC lentiviral shRNA library. Plasmid DNA for each selected shRNA, empty pLKO.1 and scrambled shRNA was isolated using the clean plasmid DNA kit (GC Biotech, Alphen aan de Rijn, the Netherlands). Lentiviral supernatants were produced by transfecting the plasmids together with psPAX2 and pMD2.G into HEK293T cells using transit reagent (Mirus, Ochten, the Netherlands). Twenty-four and 48 h post-transfection lentivirus-containing supernatant was harvested.

For the shRNA screen 2000 HepG2 cells were plated per well in 96-well collagen I coated plates (Gibco, Breda, the Netherlands). Overnight cells were attached to the surface and subsequently a second layer of collagen was added to the cells. Next, HepG2 cells were infected using 10 μl lentiviral supernatant in the presence of 8 μg/ml polybrene. Upon overnight incubation with virus, medium was replaced with normal MEM. One plate was treated with MEM containing 2 μg/ml puromycin to check infection efficiency. After 48 h the non-puromycin-treated cells were exposed for 24 h with 0.1% DMSO (control), 2 μM BaP and 5 μM AFB1. The concentrations BaP and AFB1 were selected based on the ability to robustly induce γ-H2AX foci in HepG2.

2.4. Cell viability assay

The sensitivity of HepG2 cells to AFB1- and BaP-induced cytotoxicity was evaluated by Alamar blue staining (Invitrogen, Breda, the Netherlands). Alamar blue reagent (10 μl/well) was added 19 h after the start of the treatment and incubated for 4 h at 37 °C. Medium was collected and transferred to a new 96-well plate to measure the fluorescent signal with the Fluorstar Optima platereader (BMG Labtech) using 560 nm extinction/590 nm emission filter settings.

2.5. γ-H2AX staining

After 24 h of treatment with 2 μM BaP or 5 μM AFB1 cells were fixed with 2% formaldehyde, 0.2% Triton X-100 in PBS, pH 8.2 for 20 min at RT. Cells were washed with PBS and treated with 0.5% NP-40 for 20 min at RT. Subsequently, cells were washed with PBS and blocked with 2% BSA in PBS for 1 h at RT. Anti-phospho-histone γ-H2AX clone JBW301 (Upstate) antibody was diluted in 3% BSA in PBS (1:800) and incubated overnight at 4 °C. Cells were washed with 0.5% BSA, 0.175% Tween20 in PBS and incubated for 45 min at RT with Alexa 488 goat-anti-mouse antibody 1:500 (Invitrogen, Breda, the Netherlands) diluted in 3% BSA in PBS. Cells were washed with PBS and incubated with 250 nM YOYO-3 for 0.5 h at RT. Cells were washed with PBS and γ-H2AX staining was detected with the IncuCyte Zoom (Essen BioScience, MI USA) at a magnification of 10×. With the IncuCyte software masks were made to identify nuclei and positive γ-H2AX staining. For the validation studies we used the Tox microscope or slide scanner to make photos of the cells. For the analysis of the γ-H2AX staining we used the CellProfiler software (Carpenter et al., 2006). We made masks for the nucleus based on the DAPI signal and used the integrated intensity (sum of pixels) of the $\gamma - H2AX$ staining.

2.6. RNA interference candidate TFs

The following lentiviral shRNA constructs were used for knockdown: TRCN0000003755 (p53), TRCN0000245285 (AHR), TRCN0000003819 (ARNT), TRCN0000013799 (E2F2 #1), TRCN0000013800 (E2F2 #2), TRCN0000226468 (CREB1 #1), TRCN0000226466 (CREB1 #2) and TRCN0000006502 (p63). The plasmids were packaged into lentiviral particles by co-transfection of 293T cells with packaging plasmids psPAX2 and pMD2.G as previously described (Ramaekers et al., 2011). Virus supernatant was harvested 48 and 72 h post transfection. HepG2 cells were transduced overnight with