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Identification of cyclobutane pyrimidine dimer-responsive genes using UVB-irradiated human keratinocytes transfected with in vitro-synthesized photolyase mRNA
--Manuscript Draft--

Manuscript Number:	PONE-D-15-07621R1
Article Type:	Research Article
Full Title:	Identification of cyclobutane pyrimidine dimer-responsive genes using UVB-irradiated human keratinocytes transfected with in vitro-synthesized photolyase mRNA
Short Title:	Characterization of CPD-responsive transcriptional changes
Corresponding Author:	Gábor Boros University of Debrecen Debrecen, HUNGARY
Keywords:	pseudouridine-modified mRNA; cyclobutane pyrimidine dimers; photolyase; UVB; transcriptional changes; human keratinocytes
Abstract:	Major biological effects of UVB are attributed to cyclobutane pyrimidine dimers (CPDs), the most common photolesions formed on DNA. To investigate the contribution of CPDs to UVB-induced changes of gene expression, a model system was established by transfecting keratinocytes with pseudouridine-modified mRNA (Ψ-mRNA) encoding CPD-photolyase. Microarray analyses of this model system demonstrated that more than 50% of the gene expression altered by UVB was mediated by CPD photolesions. Functional classification of the gene targets revealed strong effects of CPDs on the regulation of the cell cycle and transcriptional machineries. To confirm the microarray data, cell cycle-regulatory genes, CCNE1 and CDKN2B that were induced exclusively by CPDs were selected for further investigation. Following UVB irradiation, expression of these genes increased significantly at both mRNA and protein levels, but not in cells transfected with CPD-photolyase Ψ-mRNA and exposed to photoreactivating light. Treatment of cells with inhibitors of c-Jun N-terminal kinase (JNK) blocked the UVB-dependent upregulation of both genes suggesting a role for JNK in relaying the signal of UVB-induced CPDs into transcriptional responses. Thus, photolyase mRNA-based experimental platform demonstrates CPD-dependent and -independent events of UVB-induced cellular responses, and, as such, has the potential to identify novel molecular targets for treatment of UVB-mediated skin diseases.
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Response to Reviewers:	Responses to the comments of Reviewer 1 Dear Reviewer, Thank you for your thoughtful and constructive comments. We addressed all your comments and further experimentation was performed, according to your advice. Specifically: Comment 1.The paper would be much strengthened by further experiments directly addressing JNK in mediating CPD effects (e.g. inhibit JNK and then determine photolyase effect with and without UV), without this critical experiment, the manuscript is, in my opinion, too correlative. We agree with your assertion and carried out the proposed experiment. The results are included into the revised Figure 4 (see lines 330-340, on page 15 of the revised manuscript). Comment 2.Is it possible that certain genes (e.g. their promoters or enhancers) may be particularly vulnerable to UV-induced CPD formation? If so, then these genes may be down-regulated directly by UV rather than through a damage-response pathway such as JNK. (alternatively a gene's expression might be upregulated if an inhibitory gene's expression were CPD-prone). We agree that regulatory regions of selected genes might be directly vulnerable to UVB, therefore we included this potential in the manuscript and added background material to the revised manuscript and propounded an experimental procedure to appropriately support this issue (see lines 483-491, on pages 21-22 of the revised manuscript). Comment 3.It seems odd that the figure legends are embedded right in the text rather than as a separate section. We corrected this error. The figure legends are now in a separate section at the end of the revised manuscript. Comment 4.Do the data exclude a role for gene regulation by 6,4-photoproducts? We have added a new section to the revised manuscript in which we provide additional information related to the contribution of 6-4 photoproducts to UVB-induced changes of gene expression (see lines 368-379, on page 17 of the revised manuscript). Comment 5.The role of p53 should also be examined in CPD-induced gene expression changes. We agree, so we have inserted background material to provide additional information about the role of p53 in UVB-induced cellular stress responses (see lines 395-404, on page 18 of the revised manuscript). Response to the comment of Reviewer 2 Dear Reviewer, We appreciate your comment and following your suggestion we prepared a separate conclusion section. Comment 1.The major short-coming, which is mostly editorial, is coming from the lack of a separate conclusion section. The last paragraph, which effectively serves as a conclusion, needs to be expanded and be placed in line with the abstract. We have added a separate conclusion section to provide additional context to the revised manuscript (see lines 493-511, on page 22 of the revised manuscript).
Additional Information:	
Question	**Response**
Financial Disclosure Please describe all sources of funding	This work was supported by National Institutes of Health (grant number R01NS029331 and R42HL87688 to K.K.; R01AI50484 and R21DE019059 to D.W.), the Hungarian Scientific Research Fund (OTKA K105872 to É.R., OTKA K108421 to A.SZ.) and National Research Grant (TÁMOP-4.2.2-A-11/1/KONV-2012-0031 to É.R.). The funders had no role in study design, data collection and analysis, decision to

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I have read the journal's policy and the authors of this manuscript have the following competing interests: Katalin Karikó and Drew Weissman hold patents for the use of nucleoside-modified mRNA as a therapeutic protein delivery platform. They have also formed a small biotech company, RNARx, that received funding from the National Institutes of Health (R42HL87688) to explore the use of nucleoside-modified mRNA for transient gene therapy.

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Additional data availability information:

Dr. Mauro Picardo
The Editor
PLOS ONE

19/05/2014

Dear Professor Picardo,

Thank you for reviewing our manuscript "*Identification of cyclobutane pyrimidine dimer-responsive genes using UVB-irradiated human keratinocytes transfected with in vitro-synthesized photolyase mRNA*" (Manuscript ID PONE-D-15-07621) and for the opportunity to submit a revised manuscript for further peer review.

We are very grateful for your evaluating of our paper. We addressed all the comments of the reviewers and performed additional experiments. A separate conclusion section has been prepared as requested by Reviewer 2. The new text is underlined and highlighted in yellow while the crossed-out text refers to the deleted original text.

Hereby, we submit the revised manuscript and looking forward to your response.

We hope that the revised manuscript will meet the expectations of the reviewers and the high standards of PLOS ONE.

We approve Competing Interests statement proposed by editorial manager of PLOS ONE.

We confirm that Katalin Kariko and Hiromi Muramatsu moved to Biontech RNA Pharmaceuticals GmbH, Mainz, Germany after they performed their major role in the study at the U. Pennsylvania. Their contribution subsequently was to help to write the manuscript. They performed this task during their spare time off from work.

"This work was supported by National Institutes of Health (grant number R01NS029331 and R42HL87688 to K.K.; R01AI50484 and R21DE019059 to D.W.), the Hungarian Scientific Research Fund (OTKA K105872 to É.R., OTKA K108421 to A.S.) and National Research Grant (TÁMOP-4.2.2-A-11/1/KONV-2012-0031 to É.R.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the 'author contributions' section."

Yours sincerely,
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1 **Identification of cyclobutane pyrimidine dimer-responsive genes using UVB-irradiated**

2 **human keratinocytes transfected with *in vitro*-synthesized photolyase mRNA**

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20

Abstract

Major biological effects of UVB are attributed to cyclobutane pyrimidine dimers (CPDs), the most common photolesions formed on DNA. To investigate the contribution of CPDs to UVB-induced changes of gene expression, a model system was established by transfecting keratinocytes with pseudouridine-modified mRNA (Ψ-mRNA) encoding CPD-photolyase. Microarray analyses of this model system demonstrated that more than 50% of the gene expression altered by UVB was mediated by CPD photolesions. Functional classification of the gene targets revealed strong effects of CPDs on the regulation of the cell cycle and transcriptional machineries. To confirm the microarray data, cell cycle-regulatory genes, *CCNE1* and *CDKN2B* that were induced exclusively by CPDs were selected for further investigation. Following UVB irradiation, expression of these genes increased significantly at both mRNA and protein levels, but not in cells transfected with CPD-photolyase Ψ-mRNA and exposed to photoreactivating light. Treatment of cells with inhibitors of c-Jun N-terminal kinase (JNK) blocked the UVB-dependent upregulation of both genes suggesting a role for JNK in relaying the signal of UVB-induced CPDs into transcriptional responses. Thus, photolyase mRNA-based experimental platform demonstrates CPD-dependent and -independent events of UVB-induced cellular responses, and, as such, has the potential to identify novel molecular targets for treatment of UVB-mediated skin diseases.

Introduction

The incidence of keratinocyte-derived skin cancer, which is the most common human malignancy, continues to increase worldwide, thus presenting a serious challenge to healthcare systems [1]. Ultraviolet B (UVB) (290-320 nm) radiation is the main environmental risk factor for sunburn, skin carcinogenesis and premature skin ageing [2,3]. Cyclobutane pyrimidine dimers (CPDs) are the predominant photolesions caused by UVB radiation, and primarily they are responsible for these adverse effects [4]. CPDs are the most deleterious and premutagenic photolesions, due to their ability to distort the structure of the DNA, leading to disturbance of DNA replication and transcription [5,6]. The pathogenetic role of CPDs is further substantiated by presence of CPD-related signature mutations in genes involved in the formation of skin cancers [7], as well as, by the correlation between the action spectrum value for the induction of CPD photolesions and development of UV-induced skin cancer in animal models [8,9]. In addition, CPDs have been shown to mediate UVB-induced erythema [10] and immunosuppression [11,12]. Naturally, DNA lesions, including CPDs are excised by the nucleotide excision repair (NER) system of human keratinocytes [13]. However, the rate and accuracy of DNA repair by NER are suboptimal [14].

CPD-photolyase is a structure-specific DNA repair enzyme that specifically binds and cleaves CPDs using the energy of visible light ("photoreactivation"), thereby simply and rapidly restoring DNA integrity [15]. This enzyme functions in diverse organisms from bacteria to vertebrates but is absent in placental mammals, including humans, that must rely solely on the less potent NER to repair UV-induced DNA lesions [16]. Sunscreen lotions containing liposomal-encapsulated bacterial photolyase or CPD-specific endonuclease have been marketed for preventing UV-induced skin damages [17], especially in patients with NER-deficiency [18].

63   In a previous study, we applied a novel mRNA-based gene delivery method, and

64 demonstrated that transfection of pseudouridine-modified mRNA (Ψ-mRNA) encoding

65 *Potorous tridactylus* CPD-photolyase (CPD-PL) into human keratinocytes leads to rapid

66 repair of DNA-damage [19]. Pseudouridine modifications increase mRNA stability [20],

67 make it highly translatable [21,22] and abolish immunogenicity of the RNA [23]. It is well

68 documented that CPD lesions are considered to be the principal mediator of UV-induced

69 mutagenesis and DNA double-strand break (DSB) signalling [7,9]. However, so far, it has

70 been unclear how CPDs change gene expression and cell activities. To gain insight, we

71 performed a global analysis (microarray) of molecular networks. Most dermatological studies,

72 in which microarray technology was used, analysed differential expression of genes

73 comparing normal and pathologic skin samples in order to identify genes associated with a

74 specific skin condition or with tumor progression [24-28]. Microarray platforms were also

75 used to identify UV-regulated genes and have uncovered that significant change in the

76 expression profiles of hundreds of genes are induced by UV. Altered expression of genes in

77 response to UV irradiation have been determined in epidermal keratinocytes [29], fibroblasts

78 [30] and melanocytes [31]. Microarray experiments have demonstrated that UVB exposure

79 affects several biological processes indicating the complexity of UV-induced cellular

80 activities. Studies performed on human keratinocytes identified UVB-induced genes that were

81 involved in proteasome-mediated pathways, cytoskeleton organization, cell cycle and

82 apoptosis networks, and control of basal transcription and translation leading to inhibition of

83 cell growth [29,32-34]. Furthermore, it has been shown that the repair rate of DNA lesions

84 alters the UV-induced transcription profile, thus suggesting that adequate removal of the

85 photoproducts could avoid UV-related cutaneous pathologies [35]. However, until now, there

86 was no suitable experimental platform to identify directly CPD-responsive genes in human

87 cells, thus distinguish CPD-regulated cellular mechanisms from those mediated by other

88   UVB-induced derivatives, including diverse photoproducts, reactive oxygen species, cross-

89   linked protein-DNA and other damaged macromolecules.

90   Here, we present data obtained by using human keratinocytes transfected with *in vitro*-

91   synthesized nucleoside-modified mRNA encoding a marsupial-specific CPD-photolyase. This

92   model system enabled us to distinguish between CPD-dependent and -independent cellular

93   processes. We determined the UVB-induced transcriptional responses in human keratinocytes

94   using the combination of a CPD-specific photolyase Ψ-mRNA delivery with a genomics

95   approach. Pathway analysis revealed a strong effect of CPDs on the expression levels of

96   genes involved in the control of the cell cycle and transcriptional machineries. Our findings

97   demonstrate that the c-Jun N-terminal kinase (JNK) signalling pathway plays a role in

98   conversion of the cues generated by UVB-induced CPDs into a transcriptional response.

99   # Materials and Methods

100   ## RNA synthesis

101   Messenger RNAs were generated as previously described [19], using linearized plasmids

102   (pTEV-CPD-PL-A101 and pTEVeGFP-A101) encoding codon-optimized *Potorous* CPD-

103   photolyase (CPD-PL Ψ-mRNA) and enhanced green fluorescent protein (eGFP Ψ-mRNA).

104   The CPD-photolyase gene from *Potorous tridactylus* (rat kangaroo) was synthesized by

105   Entelechon (Bad Abbach, Germany). The Megascript T7 RNA polymerase kit (Ambion,

106   Austin, TX) was used for transcription, and UTP was replaced with pseudouridine

107   triphosphate (TriLink, San Diego, CA) [21]. To remove the template DNA Turbo DNase

108   (Ambion) was added to the reaction mix. Pseudouridine-modified mRNAs were HPLC-

109   purified as described [36] and provided with cap1 generated by using the m7G capping

110   enzyme and 2′-*O*-methyltransferase according to the manufacturer (CellScript, Madison, WI).

111 The mRNAs were transcribed to contain 101 nt-long 3' poly(A) tail. Small aliquots of RNA

112 samples were stored in siliconized tubes at −20°C.

Keratinocyte cell line

114 An established HaCaT cell line [37] was maintained in high glucose DMEM (Lonza,

115 Verviers, Belgium) supplemented with 2 mM L-glutamine (Lonza), 10% fetal bovine serum

116 (Lonza) and 0.5% antibiotic/antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA) at

117 37°C with 5% CO_2.

Transient transfection and treatments

119 Transient transfection of Ψ-mRNA into HaCaT cells, UVB exposure and photoreactivation

120 experiments were carried out, as previously described [19], except that a ratio of mRNA (0.25

121 µg) and Lipofectamine LTX-PLUS (Life Technologies) reagent (1.0 µl) in a final volume of

122 100 µl EpiLife keratinocyte growth medium (Life Technologies, Carlsbad, CA, USA) was

123 used. Following a two-hour transfection, the lipofectamine-RNA complex was replaced with

124 100 µl fresh culture medium. At 12 h post transfection, the cell monolayer in a well of a 96-

125 well plate, was covered with 50 µl Dulbecco's phosphate buffer saline (Life Technologies)

126 and subjected to 20 mJ/cm^2 UVB using two TL-20W/12 tubes (Philips). Proper dosage of

127 UVB was determined by a UVX Digital Radiometer (UVP Inc., San Gabriel, CA, USA).

128 Immediately after UVB treatment, cells were exposed to visible light ("photoreactivation")

129 using F18W Daylight fluorescent tubes (Sylvania) or kept in the dark for one hour. At 5 and

130 23 h following photoreactivation, total RNA and genomic DNA were isolated from the cells.

Enzyme-linked immunosorbent assay (ELISA)

132 Genomic DNA was extracted from HaCaT cell line using the Qiagen Blood and Cell Culture

133 kit (Qiagen, Hilden, Germany), according to the manufacturer. A direct ELISA was applied

134 for detection of CPD, as previously described [19]. Anti-CPD monoclonal antibody (TDM-2,

135 Cosmo Bio, Tokyo, Japan) diluted in PBS (1:1000) was used as primary antibody.

Microarray analysis

137 Total RNA was isolated from HaCaT cells using Trizol reagent (MRC, Cincinnati, OH,

138 USA), according to the manufacturer's instructions. RNA samples were treated by DNase I

139 (Fermentas, St. Leon-Rot, Germany) and their quality were analyzed using an Agilent 2100

140 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Gene expression profiling of

141 HaCaT cells transfected with CPD-photolyase mRNA was performed using a service provider

142 (ChromoScience, Budapest, Hungary, http://www.chromoscience.hu). Briefly, cyanine 3-

143 labeled cRNA was synthesized from 200 ng total RNA by the QuickAmp Labeling Kit

144 (Agilent Technologies), according to the manufacturer. For each sample, 1650 ng of one-

145 color labeled cRNA was hybridized to an Agilent 4x44 K Whole Human Genome Oligo

146 (Agilent Technologies) microarray platform at 65°C for 17 h. All further steps were carried

147 out according to the manufacturer (Agilent Technologies). The slides were scanned with the

148 Agilent Microarray Scanner. Data were then normalized by the Feature Extraction software

149 version 9.5 (Agilent Technologies) with default settings for one-color oligonucleotide

150 microarrays and then transferred to GeneSpringGX program (Agilent Technologies) for

151 further statistical evaluation. In GeneSpring, the normalization and data transformation steps

152 recommended by Agilent Technologies for one-color data were applied at each time point.

153 Microarray data have been submitted to Gene Expression Omnibus (GEO) [38] database and

154 can be retrieved at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE65034 (GEO

155 Series accession number: GSE65034). Three replicates of the following 3 types of CPD-PL

156 Ψ-mRNA transfected samples were studied by the microarray: non-irradiated, UVB-irradiated

157 without photoreactivating light (inactive CPD-photolyase), UVB-irradiated plus

158 photoreactivated (active CPD-photolyase). Genes showing statistically significant 2-fold

159 change were selected for further study by Ingenuity Pathway Analysis (IPA, Qiagen;

160 http://www.ingenuity.com/products/ipa) and Database for Annotation, Visualization and

161 Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov) gene functional classification

162 tool.

Real-time quantitative RT-PCR analysis

164 To validate the microarray data, real-time quantitative reverse transcription (RT)-PCR was

165 used. RT was performed using the High-Capacity cDNA Reverse Transcription Kit (Life

166 Technologies), according to the manufacturer. RT reactions contained 500-500 ng of RNA.

167 To quantitate the expression of candidate genes, the following TaqMan Gene Expression

168 assays (Life Technologies) were used: *ATF3* (Hs00231069_m1), *CCNE1* (Hs01026536_m1),

169 *CDKN2B* (p15INK4b) (Hs00793225_m1), *EGR1* (Hs00152928_m1), *ID2* (Hs04187239_m1),

170 *PTGS2 (COX-2)* (Hs00153133_m1), *RUNX1* (Hs00231079_m1), *SNAI2* (Hs00950344_m1),

171 the sequences are proprietary and not released by the company. To determine mRNA

172 expression of *SNAI1* the following custom-designed primers and probe set were used:

173 Forward primer: 5'-ACT ATG CCG CGC TCT TTC-3'; Reverse primer: 5'-GCT GGA AGG

174 TAA ACT CTG GAT-3'; and the probe sequence is: 5'-[6-carboxyfluorescein (FAM)] AAT

175 CGG AAG CCT AAC TAC AGC GAG C [tetramethylrhodamine (TAMRA)]-3'. The

176 composition of RT mixes, the PCR reactions and the RT-PCR protocols were carried out as

177 previously described [19] using the ABI 7900 HT Sequence Detection System (Life

178 Technologies). Relative RNA expression values were calculated using the $2^{-\Delta\Delta Ct}$ method [39]

179 in which expression levels in samples containing active CPD-photolyase were compared to

180 those containing inactive one. *SDHA* ((Hs00188166_m1) and *PGK1* (Hs00943178_g1)

181 mRNA levels that showed the smallest variation upon UVB exposure in keratinocytes [40]

182 were used for normalization.

Western blot analysis

184 For Western blot analyses, whole cell extracts were prepared, as previously described [19].

185 Proteins (10 μg) were separated on 12% polyacrylamide gels and transferred to nitrocellulose

186 membranes (BioRad, Berkeley, CA, USA). After blocking the membranes in 5% non-fat dry

187 milk, the membranes were incubated with primary antibodies diluted (1:750) in 5% skimmed

188 milk at 4°C overnight, then with the appropriate secondary antibodies (1:3000) at room

189 temperature for 1 hour. The following primary antibodies were used: anti-CCNE1 (4129),

190 anti-ß-actin (3700) (Cell Signaling Technology, Danvers, MA, USA) and anti-CDKN2B

191 (p15INK4b) (MA1-12294, Thermo Fisher Scientific, Rockford, IL, USA). Goat anti-mouse or

192 anti-rabbit IgG conjugated with horseradish peroxidase (HRP) was used as a secondary

193 antibody (BioRad). The visualization of proteins was achieved with ECL Prime Western

194 blotting detection system (GE Healthcare, Little Chalfont, UK) and densitometry was

195 performed using ImageJ public software (NIH, Bethesda, MD, USA).

196 **Inhibitor treatment**

197 Keratinocytes at 85% confluency were transfected with lipofectamine-complexed CPD-PL Ψ-

198 mRNA. Twelve hours later, cells were pretreated with specific inhibitors of JNK (SP600125),

199 p38 MAPK (SB203580) or AKT (MK-2206) at a final concentration of 40 μM, 10 μM and 10

200 μM, respectively for 1 hour before treatment with UVB and subsequent photoreactivation. All

201 chemical agents were purchased from SelleckChem (Selleck Chemicals, Houston, TX, USA)

202 and were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich). Stock solutions were

203 prepared according to the manufacturer and stored at −20°C.

204 **Statistical evaluation**

205 Identification of genes differentially expressed in microarray experiments was carried out by

206 the unpaired, Student's *t*-test followed by Benjamini-Hochberg correction. Statistical analysis

207 of qRT-PCR data was performed using GraphPad Prism 5 software (GraphPad Software Inc.,

208 San Diego, CA, USA). The significance of differences in terms of mRNA expression

209 comparing UVB-irradiated samples to non-irradiated ones, or UVB-irradiated plus

210 photoreactivated (active CPD-photolyase) samples to non-photoreactivated (inactive CPD-

211 photolyase) samples, respectively, was determined by the two-tailed, unpaired *t*-test. A *p*

212 value of equal to or less than 0.05 was considered statistically significant.

Results

Accelerated removal of CPD lesions in keratinocytes transfected with CPD-photolyase mRNA

216 To test the enzymatic activity of CPD-photolyase translated in HaCaT cells from the

217 transfected mRNA (CPD-PL Ψ-mRNA), cells were subjected to a physiological dose of UVB

218 (20 mJ/cm^2) 12 h after transfection of lipofectamine-complexed CPD-PL Ψ-mRNA.

219 Immediately thereafter, cells were either exposed to photoreactivating light, or left in the dark

220 for 1 hour. Cells were harvested 5 and 23 hours later, and CPD-specific ELISA was

221 performed to measure the amount of CPDs formed in genomic DNA. The amount of CPDs in

222 CPD-PL Ψ-mRNA transfected cells was reduced by 90% in those receiving photoreactivating

223 light (photoreactivated), as compared to cells kept in the dark (non-photoreactivated) (Fig. 1).

224 Cells transfected with control eGFP Ψ-mRNA contained the highest amount of CPD lesions

225 at both 6 and 24 h after UVB irradiation (Fig. 1). A reduced amount of CPD lesions were

226 measured in all irradiated samples at 24 h vs. at 6 h due to slower endogenous NER-mediated

227 DNA repair.

CPD-dependent changes were distinguished within the UVB-induced alterations of gene expression in human keratinocytes

230 To determine the CPD-dependent gene expression profile of human keratinocytes exposed to

231 UVB irradiation, an oligonucleotide-based DNA microarray analysis was performed in which

232 HaCaT cells transfected with CPD-PL Ψ-mRNA were used. Total RNA was isolated at 6 and

233 24 h after UVB exposure and subsequent photoreactivation (or not). Gene expression values

234 of UVB-irradiated plus photoreactivated samples (active CPD-photolyase) were compared to

235 non-irradiated samples and to those that were UVB irradiated, but left without

236 photoreactivation (inactive CPD-photolyase). UVB irradiated, but non-photoreactivated

237 samples were also compared to non-irradiated samples. Cut-off values for changes in gene

238 expression were set at $\pm$ 2-fold, and according to this criterion, expression levels of 2,370 out

239 of 41,000 genes were changed at 6 or 24 h after UVB exposure. Altered expression of 1,334

240 (56%) of UVB-regulated genes was restored in cells that contained active CPD-photolyase

241 indicating that the changes in the expression of these genes were CPD-dependent (S1 Fig.).

242 Importantly, more CPD-dependent gene expression changes (1,008 out of 1,334 genes) were

243 observed at 6 h vs. 24 h, potentially due to the higher difference in CPD levels at 6 h

244 comparing cells with active CPD-photolyase to those with inactive one (S1 Fig., S1 and S2

245 Tables). There are other reasons, such as transient expression of induced genes. On the other

246 hand, CPD-dependent genes represented more than half of the UVB-regulated genes (1008

247 out of 1743 genes and 326 out of 627 genes at 6 and 24 h after UVB irradiation, respectively)

248 at both time points after the exposure (S1 Fig., S1 and S2 Tables). These data suggest that

249 UVB-induced CPDs mediate a significant portion of the transcriptional alterations after 6 and

250 24 hours in response to UVB irradiation of human keratinocytes.

251 **UVB-induced, CPD-dependent genes are associated with cellular**

252 **stress responses**

253 To determine possible network interactions and associated biological functions of CPD-

254 regulated genes, datasets representing all the differentially expressed genes derived from

255   microarray analyses were imported into IPA and DAVID software. Using these applications,

256   we determined that a majority of CPD-dependent genes at 6 h after UVB exposure belong to

257   the regulatory network of *Cell Cycle and Gene Expression*, while those at the 24 h belong to

258   the network of *Cellular Development, Growth and Proliferation* (S2A Fig., S3A-S3G Fig.).

259   The top-rated network of CPD-regulated genes at 6 h after UVB irradiation was centered

260   around a proto-oncogene *(c-Jun)*; the prostaglandin-endoperoxide synthase 2 *(PTGS2)*, which

261   is a major mediator of inflammation, and the glucocorticoid receptor *(NR3C1)*, which mainly

262   functions as a transcription factor (S2B Fig.). In addition, c-Jun and interleukin 6 *(IL6)*, the

263   transcriptional regulator nuclear factor kappa B *(NF-κB)*, *p38* and the protein kinase *ERK*

264   were central nodes in the top network of CPD-dependent genes at 24 h after UVB exposure

265   (S2C Fig.). 122 out of the 1008 CPD-regulated genes belonged to the best-scored IPA

266   network at 6 h after UVB and 60 out of the 326 CPD-regulated genes at 24 h after the

267   exposure (S2A Fig.). Functional classification of CPD-regulated genes within networks (S2B-

268   S2C Fig.) suggested that a wide range of cell cycle-regulatory genes, positive or negative

269   regulators of the transcriptional and cell recovery machineries, proto-oncogenes, apoptosis

270   and protein kinase related genes were uniquely induced or repressed by CPD photolesions

271   (S3A-S3G Fig.). Nine genes regulated by CPDs were selected for further investigation based

272   on the microarray data and their involvement in cell cycle regulation *(CCNE1, CDKN2B)*,

273   transcriptional regulation *(ATF3, EGR1, ID2, RUNX1)*, epithelial to mesenchymal transition

274   *(SNAI1, SNAI2)* and inflammation *(PTGS2)* (Table 1).

Table 1. Summarized fold change values by microarray results of the selected 9 CPD-dependent genes.

GenBank ID	Gene	Description	6 h after UVB irradiation		24 h after UVB irradiation	
			vs non-irradiated	active PL vs inactive PL	vs non-irradiated	active PL vs inactive PL
NM_001040619	ATF3	activating transcription factor 3	5.65	-3.30	1.85	-1.90
NM_001238	CCNE1	cyclin E1	2.87	-2.14	1.17	1.13
NM_004936	CDKN2B	cyclin-dependent kinase inhibitor 2B	7.76	-3.36	2.05	-2.06
NM_001964	EGR1	early growth response 1	2.51	-3.95	3.38	-2.39
NM_002166	ID2	inhibitor of DNA binding 2	4.35	-3.89	-2.09	1.45
NM_000963	PTGS2	prostaglandin-endoperoxide synthase 2	6.75	-3.72	4.66	-3.04
NM_001001890	RUNX1	runt-related transcription factor 1	-4.45	4.36	1.05	1.09
NM_005985	SNAI1	snail family zinc finger 1	6.44	-3.44	2.76	-2.59
NM_003068	SNAI2	snail family zinc finger 2	2.39	-2.15	1.59	-1.24

active PL: samples containing active CPD-photolyase

inactive PL: samples containing inactive CPD-photolyase

CPD-dependency of transcriptional changes of 9 genes selected on the basis of microarray data were confirmed by RT-qPCR

To validate the microarray data, we employed real time quantitative RT-PCR analyses. Regarding *ATF3*[41], *CCNE1*[45], *EGR1*[41], *ID2*[42], *PTGS2*[44], *SNAI1*[43] and *SNAI2*[43], UV-induced upregulation of these genes has been previously described [41-45], and our results were in agreement with the literature. We found significant upregulation of the expression of *CDKN2B* (p15INK4b), and downregulation of the expression of *RUNX1* in response to UVB (Fig. 2). Furthermore, presence of active CPD-photolyase significantly prevented the changes in the expression of all tested genes (Fig. 2), thereby confirming the results of the microarray analysis.

Overexpression of cyclin E1 *(CCNE1)* and p15INK4b *(CDKN2B)* proteins in response to UVB is dependent on the generation of CPD and activation of JNK pathway

For further investigation, we selected two cell cycle-regulatory genes that were less characterized, related to their involvement in UVB-induced stress response. We have found that the expression of cyclin E1 and p15INK4b proteins were significantly increased at 24 h after UVB irradiation, but this increase was prevented by active CPD-photolyase (Fig. 3A-3B).

UVB can activate various protein kinases to regulate cell proliferation and survival pathways in human keratinocytes [46]. To investigate the signalling pathways involved in modulation of cyclin E1 and p15INK4b expression upon UVB exposure, HaCaT cells transfected with CPD-PL Ψ-mRNA were first exposed to specific inhibitors of JNK (SP600125), p38 MAPK (SB203580) or AKT (MK-2206) for 1 h, then immediately subjected to 20 mJ/cm^2 of UVB and subsequent photoreactivating light (active CPD-photolyase) or not (inactive CPD-photolyase). After photoreactivation, cells were cultured in keratinocyte medium containing inhibitor until harvested at 6 and 24 h. We determined the expression of cyclin E1 and p15INK4b proteins by western blot analysis. While suppression of p38 MAPK or AKT had no effect on UVB-induced increase in the levels of these proteins (data not shown), we found that the treatment of cells with the JNK inhibitor SP600125 abolished the induction of cyclin E1 and p15INK4b protein expression at 24 h after UVB (Fig. 4). Taken together, these results indicate that activation of JNK could be responsible for transducing the signal from CPD lesions towards the induction of *CCNE1* and *CDKN2B* gene expression in UVB-exposed human keratinocytes.

Discussion

The use of *in vitro*-transcribed messenger RNA for delivery of genetic material into mammalian cells has grown into a novel and alternative tool to oust DNA-based gene transfer in the last decade [47]. It is well documented that cells transfected with mRNA can synthesize the encoded protein [47]. We have previously shown that human cultured keratinocytes expressing CPD-photolyase from transfected *in vitro*-synthesized mRNA are suitable for investigating the cellular and molecular effects of UVB-induced CPDs [19]. Optimization of the transfection procedure resulted in 90% removal of CPDs within the first hour of photoreactivation, making this platform highly suitable for identification of CPD-dependent gene expression changes after UVB exposure. UV-induced mutagenesis is attributed to CPDs, which under normal conditions are repaired by the slow NER system [14]. Mutations in NER genes result in a less effective and error-prone DNA repair, which increases the probability of developing cancer [48]. Identification of the genes regulated by CPD photolesions might have clinical relevance, as it will facilitate defining novel targets for diagnosis or treatment of UVB-mediated skin diseases. We identified 1334 CPD-responsive genes, which represent more than 50% of the genes regulated by UVB at 6 and 24 h after the exposure, indicating that CPDs are major contributors to altered gene expression induced by UVB irradiation, as well as, to the harmful effects of UVC exposure [49]. These data also indicate that transcriptional change of a significant number of genes remained the same at 6 or 24 h after UVB exposure and subsequent photoreactivation in cells transfected with Ψ-mRNA encoding CPD-specific photolyase. This confirms that other UVB-related factors also play a role in gene regulation, but are independent from CPDs. Such factors include 6-4 photoproduct (6-4PP), which is the second most abundant UV-induced photolesion formed in DNA [50]. However, the removal of these photoproducts from the mammalian genome is very fast [14], thus 6-4PPs could potentially contribute to the early wave of UV-induced transcriptional

333   response [49]. In addition to 6-4PPs, other UVB-induced products, including reactive oxygen

334   species, oxidized nucleobases (e.g. 8-oxo-7,8-dihydroxyguanine), crosslinked protein-DNA

335   are likely to be responsible for CPD-independent transcriptional changes in human

336   keratinocytes. A significant number of CPD-responsive genes (738 and 250 genes at 6 h and

337   24 h after the exposure, respectively) were downregulated, most likely due to the stalling of

338   RNA polymerase II and inhibition of transcription elongation caused by UVB-induced DNA

339   damage [51]. Furthermore, we found a markedly higher number of CPD-dependent genes to

340   show altered expression levels at 6 h, as compared to 24 h.

341   Herein, we mainly focused on analysis of genes regulated by UVB-induced CPD lesions at

342   6 h after the exposure. Top functional categories were determined among these genes, and we

343   found that CPD-related gene expression changes were mainly associated with regulation of

344   the cell cycle and transcriptional machineries in response to UVB irradiation of human

345   keratinocytes. Molecular studies have provided evidence that recognition of DNA damage,

346   including UVB-induced photolesions, can temporarily halt cell cycle progression, allowing

347   time for cells to repair damages prior to replication [52,53]. Unsuccessful repair of these

348   photoproducts initiates intracellular apoptotic signalling in order to prevent the multiplication

349   of mutated chromosomes, but may lead to a permanent cell cycle block, genomic instability or

350   skin carcinogenesis [3,9,12,54]. The importance of cell cycle arrest is supported by studies in

351   which the function of genes encoding cell cycle inhibitors (INK4 and Cip/Kip family) and cell

352   cycle regulators (p53, Rb) were analysed [55-58]. A strong correlation has been demonstrated

353   between the occurrence of CPD-caused "UV fingerprint" mutations in p53 gene and the

354   development of skin cancer [59]. In addition, p53 was also shown to be regulated by UVB at

355   a posttranslational rather than at the transcriptional level [60]. And that might be the reason,

356   why we did not detect a change in the expression of p53 in cells exposed to UVB (GEO

357   database - GSE65034), but observed CPD-dependent changes in mRNA levels of several p53

358   target genes, including MDM2, ATF3, CCNE1, SNAI1, CDK7, WWOX (Fig. 2, S1 and S2

359   Tables). These data demonstrated that germline mutations, chemical inactivation or alteration

360   of cell cycle key regulators, including p53, result in perturbations of cell cycle checkpoint

361   control leading to the transformation of the cells. Therefore, adequate regulation of cell cycle

362   arrest is critical to eliminate mutated and potentially malignant cells. According to our

363   microarray data, CPDs are the primary cause of UVB-induced cell cycle arrest. This finding is

364   further supported by data demonstrating that photolyase-dependent removal of CPDs prevents

365   UVB-induced upregulation of negative regulators of cell cycle, including *BTG2*, *CDKN2B*

366   (p15), *CDKN1C* (p57), *GADD45B*, and *RGS2* (S3A Fig.). Our microarray experiment

367   provides new data that CPDs are the primary cause of UVB-induced cell cycle arrest [52].

368   Among the regulated cell cycle-related genes, *CDKN2B*, which is involved in G1/S

369   checkpoint control of cell cycle as well as gain and loss events for p15INK4b have been

370   extensively studied in recent years [61,62] with less established roles in UVB-mediated

371   cellular damage in keratinocyte biology. Results of our RT-qPCR and western blotting

372   analyses showed that the CPD-photolyase mediated repair of UVB-induced CPDs prevented

373   increased expression of *CDKN2B* (p15INK4b) mRNA and protein. Loss of p15INK4b

374   function and *CDKN2B* mutations has been described in several types of tumors [57,61-63]

375   confirming that the inactivation of p15INK4b significantly contributes to the transformation

376   of normal cells into cancer cells. Mutations and deletions in p15INK4b have been

377   demonstrated in melanoma tumorigenesis [64], and considered to be a heightened risk of

378   melanoma according to several wide-range examinations [65,66]. Altered expression of

379   *CDKN2B* (p15INK4b) mRNA level and new mutations have been detected in patients with

380   actinic keratosis (AK) suggesting its possible role in AK development [67]. Taken together,

381   our data strongly suggest the high relevance of CPDs in disruption of cell cycle progression.

382   In addition to cell cycle arrest, UVB exposure may affect the cell cycle machinery and cell

383   proliferation via regulation of target genes involved in apoptosis or inflammation. The most

384   studied gene associated with UVB-mediated cellular processes is cyclooxygenase 2 *(COX-2*

385   or *PTGS2).* Its upregulation is described as a diagnostic marker and predictive factor in most

386   tumors and other diseases related to chronic inflammation [68,69]. The role of *COX-2*

387   inhibition is paramount important in prevention and treatment of different types of cancer

388   [70]. *In vivo* studies have demonstrated that the use of specific inhibitors of *COX-2* leads to

389   accumulation of cells in G0/G1 phase of cell cycle and reduced proliferation of cancer cells

390   [69]. Our study confirmed upregulation of *COX-2* in UVB-irradiated keratinocytes, and here

391   we also demonstrated that its increased expression was CPD-dependent (Fig. 2, S3E Fig.).

392   Furthermore, we have identified another gene, *CCNE1,* which similarly to *COX-2* related

393   to enhanced cell proliferation and upregulated in a CPD-dependent manner. A study using a

394   mouse model has shown that the cell cycle disturbance in epidermal keratinocytes subjected

395   to an erythematogenic dose of UVB irradiation is at least in part caused by increased

396   expression of cyclins, including cyclin E1 [71]. Their data suggests that UVB-induced

397   hyperplasia and tumorigenesis is partly mediated by the upregulation of cyclin E1 [71], it

398   however has not been investigated in humans. Several studies suggested that *CCNE1* may

399   represent a useful prognostic marker and has the potential to be a target for therapy in breast

400   and ovarian cancer [72,73]. This has not yet been investigated in skin cancer.

401   The second best scored gene network was related to transcriptional machinery. We

402   validated the microarray results for six transcriptional regulators, *ATF3*[71]*, EGR1*[72]*, ID2*[73]*,*

403   *RUNX1*[74]*, SNAI1*[75] and *SNAI2*[75]. Mutations or altered expression of these genes has been

404   reported in various types of cancer, including skin cancer [74-78]. Upregulation of *ATF3*[41]*,*

405   *EGR1*[41]*, ID2*[42]*, SNAI1*[43] and *SNAI2*[43] has also been implicated in UV-induced cellular

406   stress responses [41-43]. Here, we have demonstrated that expression of these transcription

407 regulators are CPD-dependent, which could serve as novel biomarkers for evaluation of the

408 involvement of UVB in various photosensitive skin disorders or skin cancers. Our findings

409 underscore that UVB-induced CPD photolesions play a critical role in tumor development,

410 including skin carcinogenesis and not only by induction of mutations but also by changing the

411 expression of physiologically important genes.

412   The question of how CPD photolesions activate intracellular signalling and how they

413 modulate the expression of genes in cellular mechanisms (e.g. cell cycle arrest) in response to

414 UVB irradiation has not been answered. Using CPD-photolyase expressing transgenic mice, it

415 has been shown previously that unrepaired CPD photolesions are the major mediator of UV-

416 induced transcriptional responses and induce S-phase cell cycle arrest [49]. UV radiation

417 activates different signal transduction pathways in a wavelength and dose-dependent manner

418 [79]. It is well established that these signal pathways are regulated by specific protein kinases

419 including p38 kinase, PKC, AKT and JNK, which play a crucial role in the response network

420 to skin damage caused by UV exposure [46]. We examined how specific inhibitors of protein

421 kinases, such as p38 kinase, AKT and JNK, affect gene expression changes initiated by CPD

422 photolesions. We found that only the JNK inhibitor influenced UVB-induced overexpression

423 of cyclin E1 and p15INK4b proteins mainly at 24 hours after UVB irradiation. Reviewing the

424 literature [80] and comparing the data to our microarray findings (S1 and S2 Tables) forty-

425 two CPD-dependent genes could be identified that might be regulated through the activation

426 of JNK. These findings indicate an important role for JNK kinases in the control of gene

427 expression modulated by CPD photolesions. These genes are involved in cell adhesion,

428 apoptosis, regulation of the cell cycle and transcriptional machineries, cytoskeletal

429 remodelling, cellular development, growth and proliferation. Activation of *NF-κB* and *AP-1*

430 transcription factors (the major downstream targets of the JNK cascade to regulate the

431 expression of several genes involved in proliferation and survival pathways or inflammation)

432 are candidates to mediate the effect of JNK upon UVB irradiation [46]. Increased levels of the

433 activated form of JNK have been shown in UV exposed keratinocytes [81,82]. It has been

434 reported that active JNK is mainly associated with keratinocyte proliferation and

435 differentiation [80,83]. Some studies demonstrated enhanced JNK activity in psoriatic and

436 wound-healing epidermal cells [84,85], as well as in cylindromas and other hair follicle

437 derived tumors [86]. Moreover, experiments using animal or human tissue samples suggested

438 that the JNK-Ap1 signalling pathway has an important role in development of squamous cell

439 carcinoma and melanoma [86]. It is also possible that CPDs (or other UV-induced DNA-

440 lesions) formed in the promoter or enhancer region of genes alter binding potentials of

441 transcription factor leading to change in gene expression [87,88]. These published data raise

442 the question whether expression of certain genes can be changed directly by UV-induced

443 CPDs formed in their promoter region rather than through a damage-response signalling

444 pathway. The answer to this question should come from studies with systematic investigation

445 using specific inhibitors of signalling pathways to prove that indeed the observed

446 transcriptional changes is mediated through a CPD-dependent signalling event or caused by a

447 change in the promoter. Studies are ongoing to discern the mechanisms of CPD photolesion-

448 mediated activation of JNK and its downstream effectors.

449 ## Conclusion

450 *In vitro*-transcribed mRNA can be easily generated and it is a new platform for the delivery of

451 We demonstrated that *in vitro*-transcribed mRNA encoding non-human CPD-photolyase can

452 efficiently be translated into a functional protein in cultured human keratinocytes. Using

453 pseudouridine-modified mRNA encoding CPD-specific photolyase, which can specifically

454 remove UVB-induced CPD lesions after exposure to photoreactivating light, we found that

455 the CPD lesions represents the major contributors to the transcriptional response to UVB

456 irradiation. Network analysis of CPD-regulated genes revealed that CPDs are principal

457 mediators of biological processes related to cell cycle signalling and transcriptional control.

458 Consequently, the presence of CPDs modulated the transcription profile of many genes that

459 are involved in the regulation of cell cycle (e.g. CCNE1, CDKN2B) or function as

460 transcription factors (e.g. ATF3, ID2, RUNX1). Taken together, the results presented here

461 demonstrates that an approach based on transfection of *in vitro*-transcribed mRNA is suitable

462 for distinguishing UVB-induced CPD-dependent and -independent cellular mechanisms, and

463 explore the molecular details of the involved signalling pathways.

464 therapeutic proteins opening wide perspectives for dermatological or other medical

465 utilizations. This novel mRNA-based model provides an opportunity to identify additional

466 UV-specific molecular targets and achieve a better understanding of UVB-mediated skin

467 diseases.

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Figure legends

Figure 1. Accelerated photorepair of CPDs in HaCaT cells transfected with CPD-PL Ψ-mRNA. HaCaT cells were transfected with lipofectamine-complexed Ψ-mRNA encoding CPD-photolyase. Twelve hours later, cells were subjected to 20 mJ/cm^2 UVB and immediately exposed to photoreactivating light (photoreactivated) or left in the dark (non-photoreactivated) for 1 h and then maintained at 37°C for 5 and 23 hs. After incubation at 5 and 23 h, genomic DNA was isolated at the indicated times after UVB irradiation and the amount of CPDs was measured by ELISA. The values were calculated relative to those obtained with cells that were not UVB-irradiated. Significance was assessed by unpaired, two-sample *t*-test, p<0.05. Error bars represent the standard error of the mean from three experiments performed independently.

Figure 2. Experimental verification of microarray results for 9 selected genes. HaCaT cells were exposed to 20 mJ/cm^2 UVB at 12 h after delivery of CPD-PL Ψ-mRNA. Immediately thereafter, cells were either subjected to photoreactivating light (active CPD-photolyase) or left in the dark (inactive CPD-photolyase) for 1 h. Following incubation total RNA was extracted at 5 and 23 h, then real-time RT-qPCR was performed to validate the CPD-dependent expression of ATF3, CCNE1, CDKN2B, EGR1, ID2, PTGS2, RUNX1,

705 SNAI1 and SNAI2. Values measured in UVB irradiated cells with or without

706 photoreactivation were related to those measured in non-UVB irradiated cells that were

707 transfected control Ψ-mRNA, (pecked lines). Asterisks indicate significant differences (two-

708 tailed, unpaired *t*-test; p<0.05) between photoreactivated (active CPD-photolyase) and non-

709 photoreactivated (inactive CPD-photolyase) samples. The results of RT-qPCR are means ±

710 SEM from three independent experiments in triplicate.

711 **Figure 3. Photorepair of CPDs prevents altered expression of cyclin E1 and p15INK4b**

712 **protein in UVB irradiated HaCaT cells.** Cells were transfected with lipofectamine-

713 complexed CPD-PL Ψ-mRNA, 12 hs later irradiated with 20 mJ/cm^2 UVB and immediately

714 exposed to photoreactivating light (active CPD-photolyase) or kept in the dark (inactive CPD-

715 photolyase) for 1 h. Subsequently, cells were cultured at 37°C until harvested at the indicated

716 time after UVB irradiation. (A) The expression of cyclin E1 and p15INK4b were analyzed by

717 Western blot. (B) Quantitation of western blots displays relative changes in protein expression

718 normalized to β-actin. Pixel densities were calculated relative to those obtained with cells that

719 were not UVB irradiated (pecked lines). Significance was assessed by two-tailed, unpaired *t*-

720 test (asterisk, p<0.05) showing differences between photoreactivated and non-

721 photoreactivated samples. Error bars represent the standard error of the mean. The results are

722 means of three independent experiments.

723 **Figure 4. Induction of cyclin E1 and p15INK4b protein expression upon UVB exposure**

724 **is regulated through the JNK signalling pathway in HaCaT cells.** Keratinocytes

725 transfected with CPD-PL Ψ-mRNA were incubated in serum-free medium supplemented with

726 JNK inhibitor (SP600125) for 1 h. Immediately thereafter, cells were irradiated with a

727 physiological dose of UVB or left untreated followed by exposure to photoreactivating light

728 (or not) for 1 h. The cells were cultured further in serum-free medium supplemented with the

729 inhibitor. Cells were harvested for western blot assay at the indicated time. Protein levels of

730   cyclin E1, p15INK4b, and β-actin are noted. The figure shows representative results from

731   three independent experiments.

Supporting information

733   **S1 Table. List of all up and downregulated genes related to CPD lesions determined in**

734   **CPD-PL Ψ-mRNA transfected human keratinocytes at 6 h after UVB exposure by**

735   **microarray analyses.**

736   **S2 Table. List of all up and downregulated genes related to CPD lesions determined in**

737   **CPD-PL Ψ-mRNA transfected human keratinocytes at 24 h after UVB exposure by**

738   **microarray analyses.**

739   **S1 Fig. Divergence of UVB-mediated gene expression changes using CPD-specific**

740   **photolyase encoded by *in vitro*-transcribed mRNA.** To characterize the expression profile

741   of CPD-related genes, oligonucleotide microarray was carried out as described in Materials

742   and Methods. Bar graph represents the total number of UVB-responsive genes determined 6

743   and 24 h after the exposure. Bioset was divided into CPD-independent (the presence of active

744   photolyase had no effect on the expression level of genes modified by UVB irradiation) and

745   CPD-dependent (the presence of active photolyase has restored the expression level of genes

746   modified by UVB irradiation) genes. Cut-off values for changes in gene expression were set

747   at $\pm$ 2-fold.

748   **S2 Fig. Top network interactions among CPD-dependent genes determined at 6 and 24**

749   **h after UVB irradiation in CPD-PL Ψ-mRNA transfected keratinocytes.** To analyze

750   network interactions of CPD-dependent genes, datasets representing differentially regulated

751   genes derived from microarray were imported into the Ingenuity Pathway Analysis (IPA)

752   application. The list of the top three networks and associated cellular functions of all CPD-

753   related gene datasets are shown with their respective scores and *p*-values (p < 0.05) obtained

754 from IPA (panel A). The score is derived from a *p*-value and indicates the likelihood of the

755 focus genes in a network being found together due to random chance (defined as: - log10 (p-

756 value)). The most highly rated networks of genes, determined 6 (panel B) and 24 h (panel C)

757 after UVB irradiation, are illustrated with the significantly up- (red shaded) and

758 downregulated (green shaded) genes modulated in a CPD-dependent manner. Genes in empty

759 nodes were not identified as differentially expressed in our experiment and were generated

760 automatically by IPA Knowledge Base indicating a relevance to this network. The genes

761 marked with blue circle have been validated by RT-qPCR.

762 **S3 Fig. Functional classification of CPD-related genes belonging to top rated networks**

763 **determined by IPA.** To analyze functional classification of CPD-responsive genes belonging

764 to top networks, datasets derived from the results of network analysis were imported into the

765 Database for Annotation, Visualization and Integrated Discovery (DAVID) tool. The list of

766 these CPD-dependent genes, determined at 6 and 24 h after UVB irradiation is shown

767 according to their cellular functions (panel A-G). Gene expression values measured in

768 photolyase mRNA transfected and UVB irradiated cells (PL+UVB) were compared to those

769 measured in non-UVB irradiated control cells, while photolyase mRNA transfected, UVB-

770 irradiated and photoreactivated samples (active CPD-photolyase) were compared to those that

771 were photolyase mRNA transfected and UVB irradiated, but left without photoreactivation

772 (inactive CPD-photolyase). Cut-off values for changes in gene expression were set at ± 2-fold.

773 To evaluate statistical analysis unpaired, Student's *t*-test followed by Benjamini-Hochberg

774 corrections were used. The genes marked with red boxes were selected for further

775 investigation. The blue box in (F) represents a gene (IL6) that was confirmed in previous
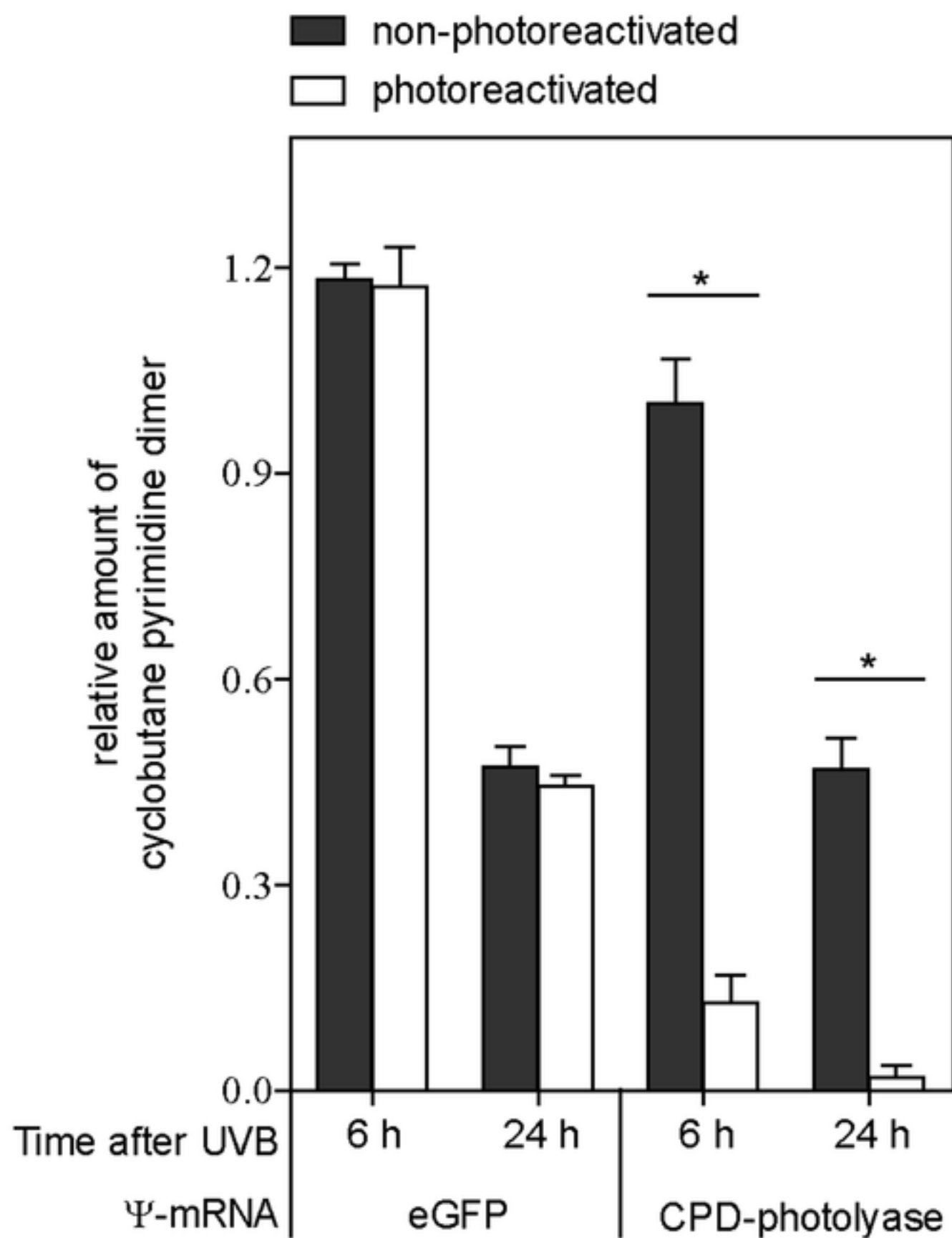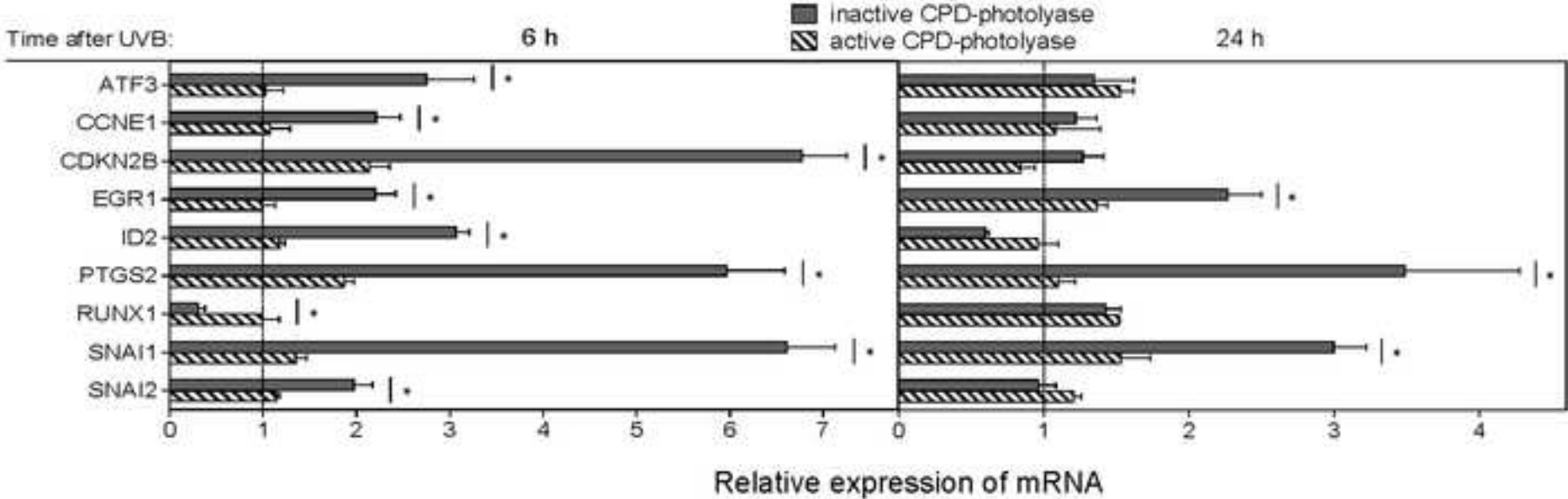
776 work [19] to have CPD-dependent changes in expression.

Relative expression of mRNA

(A)

Time after UVB:	6 h			24 h		
UVB	−	+	+	−	+	+
Photoreactivation	−	−	+	−	−	+

Cyclin E1 ——————————— —48kDa

β-actin ——————————— —43kDa

p15INK4b ——————————— —15kDa

β-actin ——————————— —43kDa

(B)

Cyclin E1   ▣ inactive CPD-photolyase   p15INK4b
Time after UVB   ▨ active CPD-photolyase

6 h

24 h

0.0 0.5 1.0 1.5 2.0 2.5   0.0 0.5 1.0 1.5 2.0 2.5 3.0

Relative levels of protein

Time after UVB: 6 h   24 h

Inhibitor of JNK	−	+	−	+	+		−	+	−	+	+
UVB	−	−	+	+	+		−	−	+	+	+
Photoreactivation	−	−	−	−	+		−	−	−	−	+

Cyclin E1

β-actin

p15INK4b

β-actin

1 **Identification of cyclobutane pyrimidine dimer-responsive genes using UVB-irradiated**

2 **human keratinocytes transfected with *in vitro*-synthesized photolyase mRNA**

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20

Abstract

Major biological effects of UVB are attributed to cyclobutane pyrimidine dimers (CPDs), the most common photolesions formed on DNA. To investigate the contribution of CPDs to UVB-induced changes of gene expression, a model system was established by transfecting keratinocytes with pseudouridine-modified mRNA (Ψ-mRNA) encoding CPD-photolyase. Microarray analyses of this model system demonstrated that more than 50% of the gene expression altered by UVB was mediated by CPD photolesions. Functional classification of the gene targets revealed strong effects of CPDs on the regulation of the cell cycle and transcriptional machineries. To confirm the microarray data, cell cycle-regulatory genes, *CCNE1* and *CDKN2B* that were induced exclusively by CPDs were selected for further investigation. Following UVB irradiation, expression of these genes increased significantly at both mRNA and protein levels, but not in cells transfected with CPD-photolyase Ψ-mRNA and exposed to photoreactivating light. Treatment of cells with inhibitors of c-Jun N-terminal kinase (JNK) ~~hindered~~blocked the UVB-dependent upregulation of both genes suggesting a role for JNK in relaying the signal of UVB-induced CPDs into transcriptional responses. Thus, photolyase mRNA-based experimental platform demonstrates CPD-dependent and -independent events of UVB-induced cellular responses, and, as such, has the potential to identify novel molecular targets for treatment of UVB-mediated skin diseases.

Introduction

The incidence of keratinocyte-derived skin cancer, which is the most common human malignancy, continues to increase worldwide, thus presenting a serious challenge to healthcare systems [1]. Ultraviolet B (UVB) (290-320 nm) radiation is the main environmental risk factor for sunburn, skin carcinogenesis and premature skin ageing [2,3]. Cyclobutane pyrimidine dimers (CPDs) are the predominant photolesions caused by UVB radiation, and primarily they are responsible for these adverse effects [4]. CPDs are the most deleterious and premutagenic photolesions, due to their ability to distort the structure of the DNA, leading to disturbance of DNA replication and transcription [5,6]. The pathogenetic role of CPDs is further substantiated by presence of CPD-related signature mutations in genes involved in the formation of skin cancers [7], as well as, by the correlation between the action spectrum value for the induction of CPD photolesions and development of UV-induced skin cancer in animal models [8,9]. In addition, CPDs have been shown to mediate UVB-induced erythema [10] and immunosuppression [11,12]. Naturally, DNA lesions, including CPDs are excised by the nucleotide excision repair (NER) system of human keratinocytes [13]. However, the rate and accuracy of DNA repair by NER are suboptimal [14].

CPD-photolyase is a structure-specific DNA repair enzyme that specifically binds and cleaves CPDs using the energy of visible light ("photoreactivation"), thereby simply and rapidly restoring DNA integrity [15]. This enzyme functions in diverse organisms from bacteria to vertebrates but is absent in placental mammals, including humans, that must rely solely on the less potent NER to repair UV-induced DNA lesions [16]. Sunscreen lotions containing liposomal-encapsulated bacterial photolyase or CPD-specific endonuclease have been marketed for preventing UV-induced skin damages [17], especially in patients with NER-deficiency [18].

63   In a previous study, we applied a novel mRNA-based gene delivery method, and

64   demonstrated that transfection of pseudouridine-modified mRNA (Ψ-mRNA) encoding

65   *Potorous tridactylus* CPD-photolyase (CPD-PL) into human keratinocytes leads to rapid

66   repair of DNA-damage [19]. Pseudouridine modifications increase mRNA stability [20],

67   make it highly translatable [21,22] and abolish immunogenicity of the RNA [23]. It is well

68   documented that CPD lesions are considered to be the principal mediator of UV-induced

69   mutagenesis and DNA double-strand break (DSB) signalling [7,9]. However, so far, it has

70   been unclear how CPDs change gene expression and cell activities. To gain insight, we

71   performed a global analysis (microarray) of molecular networks. Most dermatological studies,

72   in which microarray technology was used, analysed differential expression of genes

73   comparing normal and pathologic skin samples in order to identify genes associated with a

74   specific skin condition or with tumor progression [24-28]. Microarray platforms were also

75   used to identify UV-regulated genes and have uncovered that significant change in the

76   expression profiles of hundreds of genes are induced by UV. Altered expression of genes in

77   response to UV irradiation have been determined in epidermal keratinocytes [29], fibroblasts

78   [30] and melanocytes [31]. Microarray experiments have demonstrated that UVB exposure

79   affects several biological processes indicating the complexity of UV-induced cellular

80   activities. Studies performed on human keratinocytes identified UVB-induced genes that were

81   involved in proteasome-mediated pathways, cytoskeleton organization, cell cycle and

82   apoptosis networks, and control of basal transcription and translation leading to inhibition of

83   cell growth [29,32-34]. Furthermore, it has been shown that the repair rate of DNA lesions

84   alters the UV-induced transcription profile, thus suggesting that adequate removal of the

85   photoproducts could avoid UV-related cutaneous pathologies [35]. However, until now, there

86   was no suitable experimental platform to identify directly CPD-responsive genes in human

87   cells, thus distinguish CPD-regulated cellular mechanisms from those mediated by other

88 UVB-induced derivatives, including diverse photoproducts, reactive oxygen species, cross-

89 linked protein-DNA and other damaged macromolecules.

90 Here, we present data obtained by using human keratinocytes transfected with *in vitro*-

91 synthesized nucleoside-modified mRNA encoding a marsupial-specific CPD-photolyase. This

92 model system enabled us to distinguish between CPD-dependent and -independent cellular

93 processes. We determined the UVB-induced transcriptional responses in human keratinocytes

94 using the combination of a CPD-specific photolyase Ψ-mRNA delivery with a genomics

95 approach. Pathway analysis revealed a strong effect of CPDs on the expression levels of

96 genes involved in the control of the cell cycle and transcriptional machineries. Our findings

97 demonstrate that the c-Jun N-terminal kinase (JNK) signalling pathway plays a role in

98 conversion of the cues generated by UVB-induced CPDs into a transcriptional response.

Materials and Methods

RNA synthesis

101 Messenger RNAs were generated as previously described [19], using linearized plasmids

102 (pTEV-CPD-PL-A101 and pTEVeGFP-A101) encoding codon-optimized *Potorous* CPD-

103 photolyase (CPD-PL Ψ-mRNA) and enhanced green fluorescent protein (eGFP Ψ-mRNA).

104 The CPD-photolyase gene from *Potorous tridactylus* (rat kangaroo) was synthesized by

105 Entelechon (Bad Abbach, Germany). The Megascript T7 RNA polymerase kit (Ambion,

106 Austin, TX) was used for transcription, and UTP was replaced with pseudouridine

107 triphosphate (TriLink, San Diego, CA) [21]. To remove the template DNA Turbo DNase

108 (Ambion) was added to the reaction mix. Pseudouridine-modified mRNAs were HPLC-

109 purified as described [36] and provided with cap1 generated by using the m7G capping

110 enzyme and 2′-*O*-methyltransferase according to the manufacturer (CellScript, Madison, WI).

111 The mRNAs were transcribed to contain 101 nt-long 3' poly(A) tail. Small aliquots of RNA

112 samples were stored in siliconized tubes at −20°C.

Keratinocyte cell line

114 An established HaCaT cell line [37] was maintained in high glucose DMEM (Lonza,

115 Verviers, Belgium) supplemented with 2 mM L-glutamine (Lonza), 10% fetal bovine serum

116 (Lonza) and 0.5% antibiotic/antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA) at

117 37°C with 5% CO_2.

Transient transfection and treatments

119 Transient transfection of Ψ-mRNA into HaCaT cells, UVB exposure and photoreactivation

120 experiments were carried out, as previously described [19], except that a ratio of mRNA (0.25

121 µg) and Lipofectamine LTX-PLUS (Life Technologies) reagent (1.0 µl) in a final volume of

122 100 µl EpiLife keratinocyte growth medium (Life Technologies, Carlsbad, CA, USA) was

123 used. Following a two-hour transfection, the lipofectamine-RNA complex was replaced with

124 100 µl fresh culture medium. At 12 h post transfection, the cell monolayer in a well of a 96-

125 well plate, was covered with 50 µl Dulbecco's phosphate buffer saline (Life Technologies)

126 and subjected to 20 mJ/cm^2 UVB using two TL-20W/12 ~~bulbs~~tubes (Philips). Proper dosage

127 of UVB was determined by a UVX Digital Radiometer (UVP Inc., San Gabriel, CA, USA).

128 Immediately after UVB treatment, cells were exposed to visible light ("photoreactivation")

129 using F18W Daylight fluorescent ~~bulbs~~tubes (Sylvania) or kept in the dark for one hour. At 5

130 and 23 h following photoreactivation, total RNA and genomic DNA were isolated from the

131 cells.

Enzyme-linked immunosorbent assay (ELISA)

133 Genomic DNA was extracted from HaCaT cell line using the Qiagen Blood and Cell Culture

134 kit (Qiagen, Hilden, Germany), according to the manufacturer. A direct ELISA was applied

135   for detection of CPD, as previously described [19]. Anti-CPD monoclonal antibody (TDM-2,

136   Cosmo Bio, Tokyo, Japan) diluted in PBS (1:1000) was used as primary antibody.

Microarray analysis

138   Total RNA was isolated from HaCaT cells using Trizol reagent (MRC, Cincinnati, OH,

139   USA), according to the manufacturer's instructions. RNA samples were treated by DNase I

140   (Fermentas, St. Leon-Rot, Germany) and their quality were analyzed using an Agilent 2100

141   Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Gene expression profiling of

142   HaCaT cells transfected with CPD-photolyase mRNA was performed using a service provider

143   (ChromoScience, Budapest, Hungary, http://www.chromoscience.hu). Briefly, cyanine 3-

144   labeled cRNA was synthesized from 200 ng total RNA by the QuickAmp Labeling Kit

145   (Agilent Technologies), according to the manufacturer. For each sample, 1650 ng of one-

146   color labeled cRNA was hybridized to an Agilent 4x44 K Whole Human Genome Oligo

147   (Agilent Technologies) microarray platform at 65°C for 17 h. All further steps were carried

148   out according to the manufacturer (Agilent Technologies). The slides were scanned with the

149   Agilent Microarray Scanner. Data were then normalized by the Feature Extraction software

150   version 9.5 (Agilent Technologies) with default settings for one-color oligonucleotide

151   microarrays and then transferred to GeneSpringGX program (Agilent Technologies) for

152   further statistical evaluation. In GeneSpring, the normalization and data transformation steps

153   recommended by Agilent Technologies for one-color data were applied at each time point.

154   Microarray data have been submitted to Gene Expression Omnibus (GEO) [38] database and

155   can be retrieved at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE65034 (GEO

156   Series accession number: GSE65034). Three replicates of the following 3 types of CPD-PL

157   Ψ-mRNA transfected samples were studied by the microarray: non-irradiated, UVB-irradiated

158   without photoreactivating light (inactive CPD-photolyase), UVB-irradiated plus

159   photoreactivated (active CPD-photolyase). Genes showing statistically significant 2-fold

160   change were selected for further study by Ingenuity Pathway Analysis (IPA, Qiagen;

161   http://www.ingenuity.com/products/ipa) and Database for Annotation, Visualization and

162   Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov) gene functional classification

163   tool.

Real-time quantitative RT-PCR analysis

165   To validate the microarray data, real-time quantitative reverse transcription (RT)-PCR was

166   used. RT was performed using the High-Capacity cDNA Reverse Transcription Kit (Life

167   Technologies), according to the manufacturer. RT reactions contained 500-500 ng of RNA.

168   To quantitate the expression of candidate genes, the following TaqMan Gene Expression

169   assays (Life Technologies) were used: *ATF3* (Hs00231069_m1), *CCNE1* (Hs01026536_m1),

170   *CDKN2B* (p15INK4b) (Hs00793225_m1), *EGR1* (Hs00152928_m1), *ID2* (Hs04187239_m1),

171   *PTGS2 (COX-2)* (Hs00153133_m1), *RUNX1* (Hs00231079_m1), *SNAI2* (Hs00950344_m1),

172   the sequences are proprietary and not released by the company. To determine mRNA

173   expression of *SNAI1* the following custom-designed primers and probe set were used:

174   Forward primer: 5'-ACT ATG CCG CGC TCT TTC-3'; Reverse primer: 5'-GCT GGA AGG

175   TAA ACT CTG GAT-3'; and the probe sequence is: 5'-[6-carboxyfluorescein (FAM)] AAT

176   CGG AAG CCT AAC TAC AGC GAG C [tetramethylrhodamine (TAMRA)]-3'. The

177   composition of RT mixes, the PCR reactions and the RT-PCR protocols were carried out as

178   previously described [19] using the ABI 7900 HT Sequence Detection System (Life

179   Technologies). Relative RNA expression values were calculated using the $2^{-\Delta\Delta Ct}$ method [39]

180   in which expression levels in samples containing active CPD-photolyase were compared to

181   those containing inactive one. *SDHA* ((Hs00188166_m1) and *PGK1* (Hs00943178_g1)

182   mRNA levels that showed the smallest variation upon UVB exposure in keratinocytes [40]

183   were used for normalization.

Western blot analysis

185 For Western blot analyses, whole cell extracts were prepared, as previously described [19].

186 Proteins (10 μg) were separated on 12% polyacrylamide gels and transferred to nitrocellulose

187 membranes (BioRad, Berkeley, CA, USA). After blocking the membranes in 5% non-fat dry

188 milk, the membranes were incubated with primary antibodies diluted (1:750) in 5% skimmed

189 milk at 4°C overnight, then with the appropriate secondary antibodies (1:3000) at room

190 temperature for 1 hour. The following primary antibodies were used: anti-CCNE1 (4129),

191 anti-ß-actin (3700) (Cell Signaling Technology, Danvers, MA, USA) and anti-CDKN2B

192 (p15INK4b) (MA1-12294, Thermo Fisher Scientific, Rockford, IL, USA). Goat anti-mouse or

193 anti-rabbit IgG conjugated with horseradish peroxidase (HRP) was used as a secondary

194 antibody (BioRad). The visualization of proteins was achieved with ECL Prime Western

195 blotting detection system (GE Healthcare, Little Chalfont, UK) and densitometry was

196 performed using ImageJ public software (NIH, Bethesda, MD, USA).

Inhibitor treatment

198 Keratinocytes at 85% confluency were transfected with lipofectamine-complexed CPD-PL Ψ-

199 mRNA. cultured until reaching approximately 85-95% confluency andTwelve hours later,

200 cells were pretreated with specific inhibitors of JNK (SP600125), p38 MAPK (SB203580) or

201 AKT (MK-2206 2HCl) for 1 hour before UVB irradiation at a final concentration of 40 μM,

202 10 μM and 10 μM, respectively for 1 hour before treatment with UVB and subsequent

203 photoreactivation. All chemical agents were purchased from SelleckChem (Selleck

204 Chemicals, Houston, TX, USA) and were dissolved in dimethyl sulfoxide (DMSO) (Sigma-

205 Aldrich). Stock solutions were prepared according to the manufacturer and stored at −20°C.

Statistical evaluation

207 Identification of genes differentially expressed in microarray experiments was carried out by

208 the unpaired, Student's *t*-test followed by Benjamini-Hochberg correction. Statistical analysis

209   of qRT-PCR data was performed using GraphPad Prism 5 software (GraphPad Software Inc.,

210   San Diego, CA, USA). The significance of differences in terms of mRNA expression

211   comparing UVB-irradiated samples to non-irradiated ones, or UVB-irradiated plus

212   photoreactivated (active CPD-photolyase) samples to non-photoreactivated (inactive CPD-

213   photolyase) samples, respectively, was determined by the two-tailed, unpaired t-test. A p

214   value of equal to or less than 0.05 was considered statistically significant.

215   Results

216   Accelerated removal of CPD lesions in keratinocytes transfected
217   with CPD-photolyase mRNA

218   To test the enzymatic activity of CPD-photolyase translated in HaCaT cells from the

219   transfected mRNA (CPD-PL Ψ-mRNA), cells were subjected to a physiological dose of UVB

220   (20 mJ/cm^2) 12 h after transfection of lipofectamine-complexed CPD-PL Ψ-mRNA.

221   Immediately thereafter, cells were either exposed to photoreactivating light, or left in the dark

222   for 1 hour. Cells were harvested ~~at~~ 5 and 23 hours later, and CPD-specific ELISA was

223   performed to measure the amount of CPDs formed in genomic DNA. The amount of CPDs in

224   CPD-PL Ψ-mRNA transfected cells was reduced by 90% in those receiving photoreactivating

225   light (photoreactivated), as compared to cells kept in the dark (non-photoreactivated) (Fig. 1).

226   Cells transfected with control eGFP Ψ-mRNA contained the highest amount of CPD lesions

227   at both 6 and 24 h after UVB irradiation (Fig. 1). A reduced amount of CPD lesions were

228   measured in all irradiated samples at 24 h vs. at 6 h due to slower endogenous NER-mediated

229   DNA repair.

230   ~~**Figure 1. Accelerated photorepair of CPDs in HaCaT cells transfected with CPD-PL Ψ-**~~

231   ~~**mRNA.** HaCaT cells were transfected with lipofectamine-complexed Ψ-mRNA encoding~~

232   ~~CPD-photolyase. Twelve hours later, cells were subjected to 20 mJ/cm^2 UVB and~~

233 ~~immediately exposed to photoreactivating light (photoreactivated) or left in the dark (non-~~
234 ~~photoreactivated) for 1 h and then maintained at 37°C for 5 and 23 h. After incubation 5 and~~
235 ~~23 h, genomic DNA was isolated at the indicated times after UVB irradiation and the amount~~
236 ~~of CPDs was measured by ELISA. The values were calculated relative to those obtained with~~
237 ~~cells that were not UVB irradiated. Significance was assessed by unpaired, two-sample *t* test,~~
238 ~~p<0.05. Error bars represent the standard error of the mean from three experiments performed~~
239 ~~independently.~~

CPD-dependent changes were distinguished within the UVB-induced alterations of gene expression in human keratinocytes

242 To determine the CPD-dependent gene expression profile of human keratinocytes exposed to
243 UVB irradiation, an oligonucleotide-based DNA microarray analysis was performed in which
244 HaCaT cells transfected with CPD-PL Ψ-mRNA were used. Total RNA was isolated at 6 and
245 24 h after UVB exposure and subsequent photoreactivation (or not). Gene expression values
246 of UVB-irradiated plus photoreactivated samples (active CPD-photolyase) were compared to
247 non-irradiated samples and to those that were UVB irradiated, but left without
248 photoreactivation (inactive CPD-photolyase). UVB irradiated, but non-photoreactivated
249 samples were also compared to non-irradiated samples. Cut-off values for changes in gene
250 expression were set at ± 2-fold, and according to this criterion, expression levels of 2,370 out
251 of 41,000 genes were changed at 6 or 24 h after UVB exposure. Altered expression of 1,334
252 (56%) of UVB-regulated genes was restored in cells that contained active CPD-photolyase
253 indicating that the changes in the expression of these genes were CPD-dependent (S1 Fig.).
254 Importantly, more CPD-dependent gene expression changes (1,008 out of 1,334 genes) were
255 observed at 6 h vs. 24 h, potentially due to the higher difference in CPD levels at 6 h
256 comparing cells with active CPD-photolyase to those with inactive one (S1 Fig., S1 and S2

257 Tables). There are other reasons, such as transient expression of induced genes. On the other

258 hand, CPD-dependent genes represented more than half of the UVB-regulated genes (1008

259 out of 1743 genes and 326 out of 627 genes at 6 and 24 h after UVB irradiation, respectively)

260 at both time points after the exposure (S1 Fig., S1 and S2 Tables). These data suggest that

261 UVB-induced CPDs mediate a significant portion of the transcriptional alterations after 6 and

262 24 hours in response to UVB irradiation of human keratinocytes.

UVB-induced, CPD-dependent genes are associated with cellular stress responses

265 To determine possible network interactions and associated biological functions of CPD-

266 regulated genes, datasets representing all the differentially expressed genes derived from

267 microarray analyses were imported into IPA and DAVID software. Using these applications,

268 we determined that a majority of CPD-dependent genes at 6 h after UVB exposure belong to

269 the regulatory network of *Cell Cycle and Gene Expression*, while those at the 24 h belong to

270 the network of *Cellular Development, Growth and Proliferation* (S2A Fig., S3A-S3G Fig.).

271 The top-rated network of CPD-regulated genes at 6 h after UVB irradiation was centered

272 around a proto-oncogene *(c-Jun)*; the prostaglandin-endoperoxide synthase 2 *(PTGS2)*, which

273 is a major mediator of inflammation, and the glucocorticoid receptor *(NR3C1)*, which mainly

274 functions as a transcription factor (S2B Fig.). In addition, c-Jun and interleukin 6 *(IL6)*, the

275 transcriptional regulator nuclear factor kappa B *(NF-κB)*, *p38* and the protein kinase *ERK*

276 were central nodes in the top network of CPD-dependent genes at 24 h after UVB exposure

277 (S2C Fig.). 122 out of the ~~966~~1008 CPD-regulated genes belonged to the best-scored IPA

278 network at 6 h after UVB and 60 out of the ~~175~~326 CPD-regulated genes at 24 h after the

279 exposure (S2A Fig.). Functional classification of CPD-regulated genes within networks (S2B-

280 S2C Fig.) suggested that a wide range of cell cycle-regulatory genes, positive or negative

281   regulators of the transcriptional and cell recovery machineries, proto-oncogenes, apoptosis

282   and protein kinase related genes were uniquely induced or repressed by CPD photolesions

283   (S3A-S3G Fig.). Nine genes regulated by CPDs were selected for further investigation based

284   on the microarray data and their involvement in cell cycle regulation *(CCNE1, CDKN2B)*,

285   transcriptional regulation *(ATF3, EGR1, ID2, RUNX1)*, epithelial to mesenchymal transition

286   *(SNAI1, SNAI2)* and inflammation *(PTGS2)* (Table 1).

Table 1. Summarized fold change values by microarray results of the selected 9 CPD-dependent genes.

GenBank ID	Gene	Description	6 h after UVB irradiation		24 h after UVB irradiation	
			vs non-irradiated	active PL vs inactive PL	vs non-irradiated	active PL vs inactive PL
NM_001040619	ATF3	activating transcription factor 3	5.65	-3.30	1.85	-1.90
NM_001238	CCNE1	cyclin E1	2.87	-2.14	1.17	1.13
NM_004936	CDKN2B	cyclin-dependent kinase inhibitor 2B	7.76	-3.36	2.05	-2.06
NM_001964	EGR1	early growth response 1	2.51	-3.95	3.38	-2.39
NM_002166	ID2	inhibitor of DNA binding 2	4.35	-3.89	-2.09	1.45
NM_000963	PTGS2	prostaglandin-endoperoxide synthase 2	6.75	-3.72	4.66	-3.04
NM_001001890	RUNX1	runt-related transcription factor 1	-4.45	4.36	1.05	1.09
NM_005985	SNAI1	snail family zinc finger 1	6.44	-3.44	2.76	-2.59
NM_003068	SNAI2	snail family zinc finger 2	2.39	-2.15	1.59	-1.24

active PL: samples containing active CPD-photolyase

inactive PL: samples containing inactive CPD-photolyase

287 **CPD-dependency of transcriptional changes of 9 genes selected on**

288 **the basis of microarray data were confirmed by RT-qPCR**

289   To validate the microarray data, we employed real time quantitative RT-PCR analyses.

290   Regarding *ATF3*[41]*, CCNE1*[45]*, EGR1*[41]*, ID2*[42]*, PTGS2*[44]*, SNAI1*[43] and *SNAI2*[43], UV-

291   induced upregulation of these genes has been previously described [41-45], and our results

were in agreement with the literature. We found significant upregulation of the expression of *CDKN2B* (p15INK4b), and downregulation of the expression of *RUNX1* in response to UVB (Fig. 2). Furthermore, presence of active CPD-photolyase significantly prevented the changes in the expression of all tested genes (Fig. 2), thereby confirming the results of the microarray analysis.

Figure 2. Experimental verification of microarray results for 9 selected genes. HaCaT cells were exposed to 20 mJ/cm^2 UVB 12 h after delivery of CPD-PL Ψ-mRNA. Immediately thereafter, cells were either subjected to photoreactivating light (active CPD-photolyase) or left in the dark (inactive CPD-photolyase) for 1 h. Following incubation total RNA was extracted at 5 and 23 h, then real-time RT-qPCR was performed to validate the CPD-dependent expression of ATF3, CCNE1, CDKN2B, EGR1, ID2, PTGS2, RUNX1, SNAI1 and SNAI2. Values measured in UVB irradiated cells with or without photoreactivation were related to those measured in non-UVB irradiated cells that were transfected control Ψ-mRNA, (pecked lines). Asterisks indicate significant differences (two-tailed, unpaired *t*-test; p<0.05) between photoreactivated (active CPD-photolyase) and non-photoreactivated (inactive CPD-photolyase) samples. The results of RT-qPCR are means ± SEM from three independent experiments in triplicate.

Overexpression of cyclin E1 *(CCNE1)* and p15INK4b *(CDKN2B)* proteins in response to UVB is dependent on the generation of CPD and activation of JNK pathway

For further investigation, we selected two cell cycle-regulatory genes that were less characterized, related to their involvement in UVB-induced stress response. We have found that the expression of cyclin E1 and p15INK4b proteins were significantly increased at 24 h after UVB irradiation, but this increase was prevented by active CPD-photolyase (Fig. 3A-

316   3B).

317   ~~Figure 3. Photorepair of CPDs prevents altered expression of cyclin E1 and p15INK4b~~

318   ~~protein in UVB irradiated HaCaT cells. Cells were transfected with lipofectamine-~~

319   ~~complexed CPD-PL Ψ-mRNA, 12 h later irradiated with 20 mJ/cm² UVB and immediately~~

320   ~~exposed to photoreactivating light (active CPD-photolyase) or kept in the dark (inactive CPD-~~

321   ~~photolyase) for 1 h. Subsequently, cells were cultured at 37°C until harvested at the indicated~~

322   ~~time after UVB irradiation. (A) The expression of cyclin E1 and p15INK4b were analyzed by~~

323   ~~Western blot. (B) Quantitation of western blots displays relative changes in protein expression~~

324   ~~normalized to β-actin. Pixel densities were calculated relative to those obtained with cells that~~

325   ~~were not UVB irradiated (pecked lines). Significance was assessed by two-tailed, unpaired *t*-~~

326   ~~test (asterisk, p<0.05) showing differences between photoreactivated and non-~~

327   ~~photoreactivated samples. Error bars represent the standard error of the mean. The results are~~

328   ~~means of three independent experiments.~~

329   UVB can activate various protein kinases to regulate cell proliferation and survival pathways

330   in human keratinocytes [46]. To investigate the signalling pathways involved in modulation

331   of cyclin E1 and p15INK4b expression upon UVB exposure, HaCaT cells transfected with

332   CPD-PL Ψ-mRNA were first exposed to specific inhibitors of JNK (SP600125), p38 MAPK

333   (SB203580) or AKT (MK-2206 ~~2HCl~~) for 1 h, then immediately subjected to 20 mJ/cm² of

334   UVB and subsequent photoreactivating light (active CPD-photolyase) or not (inactive CPD-

335   photolyase). After photoreactivation, ~~Subsequently,~~ cells were cultured in keratinocyte

336   medium containing inhibitor until harvested at 6 and 24 h. We determined the expression of

337   cyclin E1 and p15INK4b proteins by western blot analysis. While suppression of p38 MAPK

338   or AKT had no effect on UVB-induced increase in the levels of these proteins (data not

339   shown), we found that the treatment of cells with the JNK inhibitor SP600125 abolished the

340   induction of cyclin E1 and p15INK4b protein expression at 24 h after UVB (Fig. 4). Taken

341 together, these results indicate that activation of JNK could be responsible for transducing the

342 signal from CPD lesions towards the induction of *CCNE1* and *CDKN2B* gene expression in

343 UVB-exposed human keratinocytes.

344 **Figure 4. Induction of cyclin E1 and p15INK4b protein expression upon UVB exposure**

345 **is regulated through the JNK signalling pathway in HaCaT cells.** Keratinocytes were

346 incubated in serum-free medium supplemented with JNK inhibitor (SP600125) for 1 h, then

347 irradiated with a physiological dose of UVB or left untreated. The cells were cultured further

348 in serum free medium supplemented with the inhibitor. Cells were harvested for western blot

349 assay at the indicated time. Protein levels of cyclin E1, p15INK4b, and β-actin are noted. The

350 figure shows representative results from three independent experiments.

Discussion

352   The use of *in vitro*-transcribed messenger RNA for delivery of genetic material into

353 mammalian cells has grown into a novel and alternative tool to oust DNA-based gene transfer

354 in the last decade [47]. It is well documented that cells transfected with mRNA can synthesize

355 the encoded protein [47]. We have previously shown that human cultured keratinocytes

356 expressing CPD-photolyase from transfected *in vitro*-synthesized mRNA are suitable for

357 investigating the cellular and molecular effects of UVB-induced CPDs [19]. Optimization of

358 the transfection procedure resulted in 90% removal of CPDs within the first hour of

359 photoreactivation, making this platform highly suitable for identification of CPD-dependent

360 gene expression changes after UVB exposure. UV-induced mutagenesis is attributed to CPDs,

361 which under normal conditions are repaired by the slow NER system [14]. Mutations in NER

362 genes result in a less effective and error-prone DNA repair, which increases the probability of

363 developing cancer [48]. Identification of the genes regulated by CPD photolesions might have

364 clinical relevance, as it will facilitate defining novel targets for diagnosis or treatment of

365 UVB-mediated skin diseases. We identified 1334 CPD-responsive genes, which represent

more than 50% of the genes regulated by UVB at 6 and 24 h after the exposure, indicating

that CPDs are major contributors to altered gene expression induced by UVB irradiation, as

well as, to the harmful effects of UVC exposure [49]. These data also indicate that

transcriptional change of a significant number of genes remained the same at 6 or 24 h after

UVB exposure and subsequent photoreactivation in cells transfected with Ψ-mRNA encoding

CPD-specific photolyase. This confirms that other UVB-related factors also play a role in

gene regulation, but are independent from CPDs. Such factors include 6-4 photoproduct (6-

4PP), which is the second most abundant UV-induced photolesion formed in DNA [50].

However, the removal of these photoproducts from the mammalian genome is very fast [14],

thus 6-4PPs could potentially contribute to the early wave of UV-induced transcriptional

response [49]. In addition to 6-4PPs, other UVB-induced products, including reactive oxygen

species, oxidized nucleobases (e.g. 8-oxo-7,8-dihydroxyguanine), crosslinked protein-DNA

are likely to be responsible for CPD-independent transcriptional changes in human

keratinocytes. A significant number of theseCPD-responsive genes (738 and 250 genes at 6 h

and 24 h after the exposure, respectively) were downregulated, most likely due to the stalling

of RNA polymerase II and inhibition of transcription elongation caused by UVB-induced

DNA damage [51]. Furthermore, we found a markedly higher number of CPD-dependent

genes to show altered expression levels at 6 h, as compared to 24 h.

Herein, we mainly focused on analysis of genes regulated by UVB-induced CPD lesions at

6 h after the exposure. Top functional categories were determined among these genes, and we

found that CPD-related gene expression changes were mainly associated with regulation of

the cell cycle and transcriptional machineries in response to UVB irradiation of human

keratinocytes. Molecular studies have provided evidence that recognition of DNA damage,

including UVB-induced photolesions, can temporarily halt cell cycle progression, allowing

time for cells to repair damages prior to replication [52,53]. Unsuccessful repair of these

391 photoproducts initiates intracellular apoptotic signalling in order to prevent the multiplication

392 of mutated chromosomes, but may lead to a permanent cell cycle block, genomic instability or

393 skin carcinogenesis [3,9,12,54]. The importance of cell cycle arrest is supported by studies in

394 which the function of genes encoding cell cycle inhibitors (INK4 and Cip/Kip family) and cell

395 cycle regulators (p53, Rb) were analysed [55-58]. A strong correlation has been demonstrated

396 between the occurrence of CPD-caused "UV fingerprint" mutations in p53 gene and the

397 development of skin cancer [59]. In addition, p53 was also shown to be regulated by UVB at

398 a posttranslational rather than at the transcriptional level [60]. And that might be the reason,

399 why we did not detect a change in the expression of p53 in cells exposed to UVB (GEO

400 database - GSE65034), but observed CPD-dependent changes in mRNA levels of several p53

401 target genes, including MDM2, ATF3, CCNE1, SNAI1, CDK7, WWOX (Fig. 2, S1 and S2

402 Tables). These data demonstrated that germline mutations ~~or~~, chemical inactivation or

403 alteration of ~~these~~ cell cycle key regulators, including p53, ~~could~~ result in perturbations of cell

404 cycle checkpoint control leading to the transformation of the cells. Therefore, adequate

405 regulation of cell cycle arrest is critical to eliminate mutated and potentially malignant cells.

406 According to our microarray data, CPDs are the primary cause of UVB-induced cell cycle

407 arrest. This finding is further supported by data demonstrating that photolyase-dependent

408 removal of CPDs prevents UVB-induced upregulation of negative regulators of cell cycle,

409 including *BTG2*, *CDKN2B* (p15), *CDKN1C* (p57), *GADD45B*, and *RGS2* (S3A Fig.). Our

410 microarray experiment provides new data that CPDs are the primary cause of UVB-induced

411 cell cycle arrest [52].

412 Among the regulated cell cycle-related genes, *CDKN2B*, which is involved in G1/S

413 checkpoint control of cell cycle as well as gain and loss events for p15INK4b have been

414 extensively studied in recent years [61,62] with less established roles in UVB-mediated

415 cellular damage in keratinocyte biology. Results of our RT-qPCR and western blotting

416   analyses showed that the CPD-photolyase mediated repair of UVB-induced CPDs prevented

417   increased expression of *CDKN2B* (p15INK4b) mRNA and protein. Loss of p15INK4b

418   function and *CDKN2B* mutations has been described in several types of tumors [57,61-63]

419   confirming that the inactivation of p15INK4b significantly contributes to the transformation

420   of normal cells into cancer cells. Mutations and deletions in p15INK4b have been

421   demonstrated in melanoma tumorigenesis [64], and considered to be a heightened risk of

422   melanoma according to several wide-range examinations [65,66]. Altered expression of

423   *CDKN2B* (p15INK4b) mRNA level and new mutations have been detected in patients with

424   actinic keratosis (AK) suggesting its possible role in AK development [67]. Taken together,

425   our data strongly suggest the high relevance of CPDs in disruption of cell cycle progression.

426   In addition to cell cycle arrest, UVB exposure may affect the cell cycle machinery and cell

427   proliferation via regulation of target genes involved in apoptosis or inflammation. The most

428   studied gene associated with UVB-mediated cellular processes is cyclooxygenase 2 *(COX-2*

429   or *PTGS2).* Its upregulation is described as a diagnostic marker and predictive factor in most

430   tumors and other diseases related to chronic inflammation [68,69]. The role of *COX-2*

431   inhibition is paramount important in prevention and treatment of different types of cancer

432   [70]. *In vivo* studies have demonstrated that the use of specific inhibitors of *COX-2* leads to

433   accumulation of cells in G0/G1 phase of cell cycle and reduced proliferation of cancer cells

434   [69]. Our study confirmed upregulation of *COX-2* in UVB-irradiated keratinocytes, and here

435   we also demonstrated that its increased expression was CPD-dependent (Fig. 2, S3E Fig.).

436   Furthermore, we have identified another gene, *CCNE1,* which similarly to *COX-2* related

437   to enhanced cell proliferation and upregulated in a CPD-dependent manner. A study using a

438   mouse model has shown that the cell cycle disturbance in epidermal keratinocytes subjected

439   to an erythematogenic dose of UVB irradiation is at least in part caused by increased

440   expression of cyclins, including cyclin E1 [71]. Their data suggests that UVB-induced

441 hyperplasia and tumorigenesis is partly mediated by the upregulation of cyclin E1 [71], it

442 however has not been investigated in humans. Several studies suggested that *CCNE1* may

443 represent a useful prognostic marker and has the potential to be a target for therapy in breast

444 and ovarian cancer [72,73]. This has not yet been investigated in skin cancer.

445   The second best scored gene network was related to transcriptional machinery. We

446 validated the microarray results for six transcriptional regulators, *ATF3*[71], *EGR1*[72], *ID2*[73],

447 *RUNX1*[74], *SNAI1*[75] and *SNAI2*[75]. Mutations or altered expression of these genes has been

448 reported in various types of cancer, including skin cancer [74-78]. Upregulation of *ATF3*[41],

449 *EGR1*[41], *ID2*[42], *SNAI1*[43] and *SNAI2*[43] has also been implicated in UV-induced cellular

450 stress responses [41-43]. Here, we have demonstrated that expression of these transcription

451 regulators are CPD-dependent, which could serve as novel biomarkers for evaluation of the

452 involvement of UVB in various photosensitive skin disorders or skin cancers. Our findings

453 underscore that UVB-induced CPD photolesions play a critical role in tumor development,

454 including skin carcinogenesis and not only by induction of mutations but also by changing the

455 expression of physiologically important genes.

456   The question of how CPD photolesions activate intracellular signalling and how they

457 modulate the expression of genes in cellular mechanisms (e.g. cell cycle arrest) in response to

458 UVB irradiation has not been answered. Using CPD-photolyase expressing transgenic mice, it

459 has been shown previously that unrepaired CPD photolesions are the major mediator of UV-

460 induced transcriptional responses and induce S-phase cell cycle arrest [49]. UV radiation

461 activates different signal transduction pathways in a wavelength and dose-dependent manner

462 [79]. It is well established that these signal pathways are regulated by specific protein kinases

463 including p38 kinase, PKC, AKT and JNK, which play a crucial role in the response network

464 to skin damage caused by UV exposure [46]. We examined how specific inhibitors of protein

465 kinases, such as p38 kinase, AKT and JNK, affect gene expression changes initiated by CPD

466   photolesions. We found that only the JNK inhibitor influenced UVB-induced overexpression

467   of Ccyclin E1 and p15INK4b proteins mainly at 24 hours after UVB irradiation. Reviewing

468   the literature [80] and comparing the data to our microarray findings (S1 and S2 Tables)

469   forty-two CPD-dependent genes could be identified that might be regulated through the

470   activation of JNK. These findings indicate an important role for JNK kinases in the control of

471   gene expression modulated by CPD photolesions. These genes are involved in cell adhesion,

472   apoptosis, regulation of the cell cycle and transcriptional machineries, cytoskeletal

473   remodelling, cellular development, growth and proliferation. Activation of *NF-κB* and *AP-1*

474   transcription factors (the major downstream targets of the JNK cascade to regulate the

475   expression of several genes involved in proliferation and survival pathways or inflammation)

476   are candidates to mediate the effect of JNK upon UVB irradiation [46]. Increased levels of the

477   activated form of JNK have been shown in UV exposed keratinocytes [81,82]. It has been

478   reported that active JNK is mainly associated with keratinocyte proliferation and

479   differentiation [80,83]. Some studies demonstrated enhanced JNK activity in psoriatic and

480   wound-healing epidermal cells [84,85], as well as in cylindromas and other hair follicle

481   derived tumors [86]. Moreover, experiments using animal or human tissue samples suggested

482   that the JNK-Ap1 signalling pathway has an important role in development of squamous cell

483   carcinoma and melanoma [86]. It is also possible that CPDs (or other UV-induced DNA-

484   lesions) formed in the promoter or enhancer region of genes alter binding potentials of

485   transcription factor leading to change in gene expression [87,88]. These published data raise

486   the question whether expression of certain genes can be changed directly by UV-induced

487   CPDs formed in their promoter region rather than through a damage-response signalling

488   pathway. The answer to this question should come from studies with systematic investigation

489   using specific inhibitors of signalling pathways to prove that indeed the observed

490   transcriptional changes is mediated through a CPD-dependent signalling event or caused by a

491 change in the promoter. Studies are ongoing to discern the mechanisms of CPD photolesion-

492 mediated activation of JNK and its downstream effectors.

Conclusion

494 *In vitro*-transcribed mRNA can be easily generated and it is a new platform for the delivery of

495 We demonstrated that *in vitro*-transcribed mRNA encoding non-human CPD-photolyase can

496 efficiently be translated into a functional protein in cultured human keratinocytes. Using

497 pseudouridine-modified mRNA encoding CPD-specific photolyase, which can specifically

498 remove UVB-induced CPD lesions after exposure to photoreactivating light, we found that

499 the CPD lesions represents the major contributors to the transcriptional response to UVB

500 irradiation. Network analysis of CPD-regulated genes revealed that CPDs are principal

501 mediators of biological processes related to cell cycle signalling and transcriptional control.

502 Consequently, the presence of CPDs modulated the transcription profile of many genes that

503 are involved in the regulation of cell cycle (e.g. CCNE1, CDKN2B) or function as

504 transcription factors (e.g. ATF3, ID2, RUNX1). Taken together, the results presented here

505 demonstrates that an approach based on transfection of *in vitro*-transcribed mRNA is suitable

506 for distinguishing UVB-induced CPD-dependent and -independent cellular mechanisms, and

507 explore the molecular details of the involved signalling pathways.

508 therapeutic proteins opening wide perspectives for dermatological or other medical

509 utilizations. This novel mRNA-based model provides an opportunity to identify additional

510 UV-specific molecular targets and achieve a better understanding of UVB-mediated skin

511 diseases.

512 We demonstrated that nucleoside-modified mRNA encoding non-human CPD-photolyase

513 can efficiently be translated into a functional protein in cultured human keratinocytes. *In*

514 *vitro*-transcribed mRNA is commercially available and is a new platform for the delivery of

515 therapeutic proteins opening wide perspectives for dermatological or other medical

516 ~~utilizations. We also demonstrated that our mRNA-based model is suitable for investigating~~

517 ~~CPD-dependent cellular functions and provides an opportunity to identify novel UV-specific~~

518 ~~molecular targets and achieve a better understanding of UVB-mediated skin diseases.~~

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Figure legends

Figure 1. Accelerated photorepair of CPDs in HaCaT cells transfected with CPD-PL Ψ-mRNA. HaCaT cells were transfected with lipofectamine-complexed Ψ-mRNA encoding CPD-photolyase. Twelve hours later, cells were subjected to 20 mJ/cm^2 UVB and immediately exposed to photoreactivating light (photoreactivated) or left in the dark (non-photoreactivated) for 1 h and then maintained at 37°C for 5 and 23 hs. After incubation at 5 and 23 h, genomic DNA was isolated at the indicated times after UVB irradiation and the amount of CPDs was measured by ELISA. The values were calculated relative to those obtained with cells that were not UVB-irradiated. Significance was assessed by unpaired, two-sample *t*-test, p<0.05. Error bars represent the standard error of the mean from three experiments performed independently.

Figure 2. Experimental verification of microarray results for 9 selected genes. HaCaT cells were exposed to 20 mJ/cm^2 UVB at 12 h after delivery of CPD-PL Ψ-mRNA. Immediately thereafter, cells were either subjected to photoreactivating light (active CPD-photolyase) or left in the dark (inactive CPD-photolyase) for 1 h. Following incubation total RNA was extracted at 5 and 23 h, then real-time RT-qPCR was performed to validate the CPD-dependent expression of ATF3, CCNE1, CDKN2B, EGR1, ID2, PTGS2, RUNX1, SNAI1 and SNAI2. Values measured in UVB irradiated cells with or without photoreactivation were related to those measured in non-UVB irradiated cells that were transfected control Ψ-mRNA, (pecked lines). Asterisks indicate significant differences (two-tailed, unpaired *t*-test; p<0.05) between photoreactivated (active CPD-photolyase) and non-photoreactivated (inactive CPD-photolyase) samples. The results of RT-qPCR are means ± SEM from three independent experiments in triplicate.

Figure 3. Photorepair of CPDs prevents altered expression of cyclin E1 and p15INK4b protein in UVB irradiated HaCaT cells. Cells were transfected with lipofectamine-

complexed CPD-PL Ψ-mRNA, 12 hs later irradiated with 20 mJ/cm^2 UVB and immediately exposed to photoreactivating light (active CPD-photolyase) or kept in the dark (inactive CPD-photolyase) for 1 h. Subsequently, cells were cultured at 37°C until harvested at the indicated time after UVB irradiation. (A) The expression of cyclin E1 and p15INK4b were analyzed by Western blot. (B) Quantitation of western blots displays relative changes in protein expression normalized to β-actin. Pixel densities were calculated relative to those obtained with cells that were not UVB irradiated (pecked lines). Significance was assessed by two-tailed, unpaired *t*-test (asterisk, p<0.05) showing differences between photoreactivated and non-photoreactivated samples. Error bars represent the standard error of the mean. The results are means of three independent experiments.

Figure 4. Induction of cyclin E1 and p15INK4b protein expression upon UVB exposure is regulated through the JNK signalling pathway in HaCaT cells. Keratinocytes transfected with CPD-PL Ψ-mRNA were incubated in serum-free medium supplemented with JNK inhibitor (SP600125) for 1 h. Immediately thereafter, cells were irradiated with a physiological dose of UVB or left untreated followed by exposure to photoreactivating light (or not) for 1 h. The cells were cultured further in serum-free medium supplemented with the inhibitor. Cells were harvested for western blot assay at the indicated time. Protein levels of cyclin E1, p15INK4b, and β-actin are noted. The figure shows representative results from three independent experiments.

Supporting information

S1 Table. List of all up and downregulated genes related to CPD lesions determined in CPD-PL Ψ-mRNA transfected human keratinocytes at 6 h after UVB exposure by microarray analyses.

787 **S2 Table. List of all up and downregulated genes related to CPD lesions determined in**

788 **CPD-PL Ψ-mRNA transfected human keratinocytes at 24 h after UVB exposure by**

789 **microarray analyses.**

790 **S1 Fig. Divergence of UVB-mediated gene expression changes using CPD-specific**

791 **photolyase encoded by *in vitro*-transcribed mRNA.** To characterize the expression profile

792 of CPD-related genes, oligonucleotide microarray was carried out as described in Materials

793 and Methods. Bar graph represents the total number of UVB-responsive genes determined 6

794 and 24 h after the exposure. Bioset was divided into CPD-independent (the presence of active

795 photolyase had no effect on the expression level of genes modified by UVB irradiation) and

796 CPD-dependent (the presence of active photolyase has restored the expression level of genes

797 modified by UVB irradiation) genes. Cut-off values for changes in gene expression were set

798 at $\pm$ 2-fold.

799 **S2 Fig. Top network interactions among CPD-dependent genes determined at 6 and 24**

800 **h after UVB irradiation in CPD-PL Ψ-mRNA transfected keratinocytes.** To analyze

801 network interactions of CPD-dependent genes, datasets representing differentially regulated

802 genes derived from microarray were imported into the Ingenuity Pathway Analysis (IPA)

803 application. The list of the top three networks and associated cellular functions of all CPD-

804 related gene datasets are shown with their respective scores and *p*-values (p < 0.05) obtained

805 from IPA (panel A). The score is derived from a *p*-value and indicates the likelihood of the

806 focus genes in a network being found together due to random chance (defined as: - log10 (p-

807 value)). The most highly rated networks of genes, determined 6 (panel B) and 24 h (panel C)

808 after UVB irradiation, are illustrated with the significantly up- (red shaded) and

809 downregulated (green shaded) genes modulated in a CPD-dependent manner. Genes in empty

810 nodes were not identified as differentially expressed in our experiment and were generated

811   automatically by IPA Knowledge Base indicating a relevance to this network. The genes

812   marked with blue circle have been validated by RT-qPCR.

813   **S3 Fig. Functional classification of CPD-related genes belonging to top rated networks**

814   **determined by IPA.** To analyze functional classification of CPD-responsive genes belonging

815   to top networks, datasets derived from the results of network analysis were imported into the

816   Database for Annotation, Visualization and Integrated Discovery (DAVID) tool. The list of

817   these CPD-dependent genes, determined at 6 and 24 h after UVB irradiation is shown

818   according to their cellular functions (panel A-G). Gene expression values measured in

819   photolyase mRNA transfected and UVB irradiated cells (PL+UVB) were compared to those

820   measured in non-UVB irradiated control cells, while photolyase mRNA transfected, UVB-

821   irradiated and photoreactivated samples (active CPD-photolyase) were compared to those that

822   were photolyase mRNA transfected and UVB irradiated, but left without photoreactivation

823   (inactive CPD-photolyase). Cut-off values for changes in gene expression were set at $\pm$ 2-fold.

824   To evaluate statistical analysis unpaired, Student's *t*-test followed by Benjamini-Hochberg

825   corrections were used. The genes marked with red boxes were selected for further

826   investigation. The blue box in (F) represents a gene (IL6) that was confirmed in previous

827   work [19] to have CPD-dependent changes in expression.

Responses to the comments of Reviewer 1

Dear Reviewer,

Thank you for your thoughtful and constructive comments.

We addressed all your comments and further experimentation was performed, according to your advice.

Specifically:

Comment 1. The paper would be much strengthened by further experiments directly addressing JNK in mediating CPD effects (e.g. inhibit JNK and then determine photolyase effect with and without UV), without this critical experiment, the manuscript is, in my opinion, too correlative.

We agree with your assertion and carried out the proposed experiment. The results are included into the revised Figure 4 (see lines 330-340, on page 15 of the revised manuscript).

Comment 2. Is it possible that certain genes (e.g. their promoters or enhancers) may be particularly vulnerable to UV-induced CPD formation? If so, then these genes may be down-regulated directly by UV rather than through a damage-response pathway such as JNK. (alternatively a gene's expression might be upregulated if an inhibitory gene's expression were CPD-prone).

We agree that regulatory regions of selected genes might be directly vulnarable to UVB, therefore we included this potential in the manuscript and added background material to the revised manuscript and propounded an experimental procedure to appropriately support this issue (see lines 483-491, on pages 21-22 of the revised manuscript).

Comment 3. It seems odd that the figure legends are embedded right in the text rather than as a separate section.

We corrected this error. The figure legends are now in a separate section at the end of the revised manuscript.

Comment 4.   Do the data exclude a role for gene regulation by 6,4-photoproducts?

We have added a new section to the revised manuscript in which we provide additional information related to the contribution of 6-4 photoproducts to UVB-induced changes of gene expression (see lines 368-379, on page 17 of the revised manuscript).

Comment 5.   The role of p53 should also be examined in CPD-induced gene expression changes.

We agree, so we have inserted background material to provide additional information about the role of p53 in UVB-induced cellular stress responses (see lines 395-404, on page 18 of the revised manuscript).

Response to the comment of Reviewer 2

Dear Reviewer,

We appreciate your comment and following your suggestion we prepared a separate conclusion section.

Comment 1.   The major short-coming, which is mostly editorial, is coming from the lack of a separate conclusion section. The last paragraph, which effectively serves as a conclusion, needs to be expanded and be placed in line with the abstract.

We have added a separate conclusion section to provide additional context to the revised manuscript (see lines 493-511, on page 22 of the revised manuscript).