Effect of Low-Dose Cadmium Exposure on DNA Methylation in the Endangered European Eel

ARTICLE *in* ENVIRONMENTAL SCIENCE & TECHNOLOGY · DECEMBER 2013

Impact Factor: 5.33 · DOI: 10.1021/es4048347 · Source: PubMed

5 AUTHORS, INCLUDING:

Fabien Pierron
French National Centre for Scientific Research
29 PUBLICATIONS **327** CITATIONS
SEE PROFILE

Lucie Baillon
Université de Montréal
5 PUBLICATIONS **25** CITATIONS
SEE PROFILE

Patrice Gonzalez
French National Centre for Scientific Research
79 PUBLICATIONS **1,475** CITATIONS
SEE PROFILE

Environmental Science & Technology

Article

pubs.acs.org/est

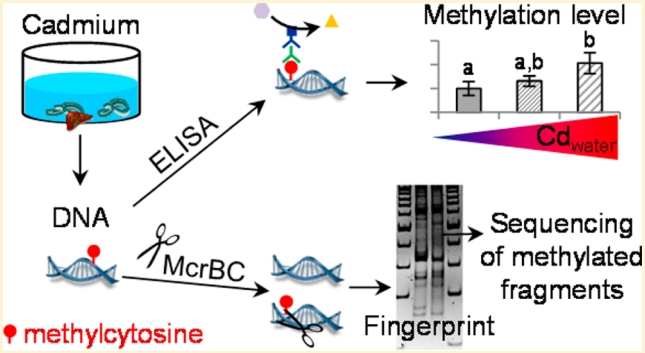
Effect of Low-Dose Cadmium Exposure on DNA Methylation in the Endangered European Eel

Fabien Pierron,*[,†,‡] Lucie Baillon,[†,‡] Mohamedou Sow,[†,‡] Salomé Gotreau,[†,‡] and Patrice Gonzalez[†,‡]

[†]Université de Bordeaux, EPOC, UMR 5805, F-33400 Talence, France

[‡]CNRS, EPOC, UMR 5805, F-33400 Talence, France

ABSTRACT: There is increasing evidence that epigenetics can play a key role in the etiology of diseases engendered by chronic pollutant exposure. Although epigenetics has received significant attention in the field of biomedicine during the last years, epigenetics research is surprisingly very limited in ecotoxicology. The aim of the present study was to investigate the possible effects of low-dose cadmium exposure on the DNA methylation profile in a critically endangered fish species, the European eel. Eels were exposed to environmentally realistic concentrations of cadmium (0.4 and 4 $\mu g \cdot L^{-1}$) during 45 days. The global CpG methylation status of eel liver was determined by means of a homemade ELISA assay. We then used a methylation-sensitive arbitrarily primed PCR method to identify genes that are differentially methylated between control and Cd-exposed eels. Our results show that cadmium exposure is associated with DNA hypermethylation and with a decrease in total RNA synthesis. Among hypermethylated sequences identified, several fragments presented high homologies with genes encoding for proteins involved in intracellular trafficking, lipid biosynthesis, and phosphatidic acid signaling pathway. In addition, few fragments presented high homologies with retrotransposon-like sequences. Our study illustrates how DNA methylation can be involved in the chronic stress response to Cd in fish.



1. INTRODUCTION

Epigenetics is the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA code.[1−3] One of the most studied epigenetic processes is DNA methylation. DNA methylation, which refers to the addition of a methyl group to cytosine to form the 5-methylcytosine base, is commonly associated with "gene silencing", i.e. transcriptional repression (with few exceptions; see Vandegehuchte and Janssen, 2011[1]). In mammals and plants, for which significant research was carried out, DNA methylation was found to be involved in fundamental biological functions such as development, cell differentiation and chromosome stability. In recent years, the role of epigenetics in the etiology of human diseases, especially carcinogenesis, has received increasing attention. Recent discoveries have shown that environmental factors, such as nutrition quality or exposure to pollutants, can strongly affect the epigenetic status of mammalian cells. These defects or unintended changes in the DNA methylation status can be associated with negative health outcomes such as embryonic lethality, tumor genesis, diabetes, and infertility.[2,4−6] There is thus increasing evidence that adverse effects engendered by chronic exposure to pollutants, even at very low concentrations, are linked to epigenetic modifications in mammals.[3] Despite such evidence, epigenetics research is surprisingly very limited in ecotoxicology. As a major aim of ecotoxicology is to measure and predict the effects of contaminants on natural populations, communities, and ecosystems, the study of epigenetics in an ecotoxicological context appears to be highly relevant.

Therefore, the aim of the present study was to investigate the potential impacts of cadmium (Cd) exposure on the DNA methylation profile in an endangered fish species, the European eel *Anguilla anguilla*. Cd is a widespread nonessential and highly toxic trace metal. Cd is known to be a mutagenic metal and to induce apoptosis and oxidative stress.[7] Anthropogenic activities such as mining or ore treatment have greatly increased the global flux of Cd in the Earth's surficial environment leading to elevated concentrations in the environment, notably in aquatic systems. Historically abundant and widespread in Europe, European eel recruitment has suffered a sharp decline since early 1980s. European eel's recruitment would represent now only one tenth of what it was in the early 1980s.[8] In view of these data, the European eel is currently considered as critically endangered and is listed on the red list of threatened species. There is now some evidence that pollution, among other factors, could contribute to the dramatic decline of the species. Their unusual and complex life cycle makes them indeed

Received: October 30, 2013
Revised: December 12, 2013
Accepted: December 13, 2013
Published: December 13, 2013

particularly vulnerable to pollution.[9−12] Thus, the European eel can be considered as a sentinel species in ecotoxicology and could provide a model to explore recent hypotheses that human activities have led to a tremendous decrease in aquatic biodiversity in the last century.[13]

To test whether chronic Cd exposure could induce changes in the DNA methylation profile in a nonmodel but ecologically relevant species, European eels were exposed to dissolved Cd at environmentally realistic concentrations ($[Cd]_{water}$ = 0.4 and 4 $\mu g \cdot L^{-1}$) for 45 days.[14] Thereafter, the global CpG methylation status of liver of both control and Cd-exposed eels was determined by means of a homemade ELISA assay. In order to identify and characterize genes and, by extension, biological processes, that are differentially methylated between control and Cd-exposed eels, we used a methylation-sensitive arbitrarily primed PCR (msAP-PCR) method, the McrBC-msAP-PCR method.[15] Finally, the transcription level of genes identified by this method was determined by quantitative RT-PCR.

2. MATERIALS AND METHODS

2.1. Experimental Design. All procedures used in this experiment were approved by the Aquitaine fish-birds ethic committee. Immature yellow eels (*Anguillla anguilla*), averaging 37 cm in length and 70 g in weight (36.9 ± 2.5 cm and 70.4 ± 2.4 g, respectively, mean ± SE, n = 24) were captured in the Arcachon Bay (southwest of France) in May 2012. The animals were transferred to the laboratory (Marine Biology Station of Arcachon) and kept in running aerated brackish water (salinity 5‰, natural seawater dilution with aerated tap water) thermostatted at 23 °C over a 1-month maintenance period, prior to experimentation. Over this period, fish were fed every day with mussels, and no lethality was observed.

The experiment was performed by means of a flow-through system consisting of three separate 215 L experimental units (EU). Each tank was supplied with brackish water (salinity = 5‰) by three water flowmeters and contained eight organisms. Fish in the first EU were in uncontaminated water and constituted control animals. In the two other tanks, fish were exposed to Cd at a dissolved metal concentration of 0.4 and 4 $\mu g \cdot L^{-1}$. Metal exposure was initiated after one month of adaptation to the experimental conditions by adding Cd as $CdCl_2$ from a stock solution in water. To maintain constant Cd contamination over time, contaminated tanks were fitted with a peristaltic pump which added Cd at the desired concentration. During the experiment, the water column was permanently controlled for temperature (23 ± 1 °C), pH (7.7 ± 0.1), and salinity (4.85 ± 0.7). Moreover, water samples were collected three times a week. After acidification and dilution, they were checked in Cd concentration, and flows were adjusted if necessary. The average concentrations were 0.09 ± 0.01 $\mu g \cdot L^{-1}$, 0.43 ± 0.03 $\mu g \cdot L^{-1}$, and 4.00 ± 0.18 $\mu g \cdot L^{-1}$ (mean ± SE, n = 12) in control and contaminated tanks, respectively. During the 45 days of exposure, eels were fed every evening in excess (7.5% wet weight animal/day) with artificial food (fresh fish flesh mixed with oil (1.3%) and agar−agar (0.8%)).

At the end of the exposure period, 5 eels per EU were removed and dissected. Two samples of liver were immediately fixed in RNAlater solution and stored at −20 °C until needed for analyses.

2.2. DNA Purification. Genomic DNA from liver tissue was isolated by overnight digestion with proteinase K, followed by standard phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. The pellet was resuspended in 100 μL of TE solution and treated with RNase A (2 $mg \cdot mL^{-1}$) at 37 °C during 1 h. DNA was *de novo* treated with phenol/chloroform and precipitated with absolute ethanol. The pellet was resuspended in 100 μL of TE.

2.3. Global CpG Methylation Assay. In order to facilitate maximum binding of DNA, 96-well polystyrene microplates (Greiner-bio-one) were incubated overnight at 4 °C with 200 μL per well of a 0.1% aqueous solution of protamine sulfate (Sigma). The plates were then emptied by simple inversion and washed five times with 200 μL of ultrapure Milli-Q water. These coated plates were then dried at 37 °C for 10 min and stored at 4 °C in dark before use.

To establish a standard curve, DNA from several eels was treated with CpG methyltransferase M.SssI (New England Biolabs) according to manufacturer instructions. Briefly, 1 μL of DNA solution at 1 $\mu g/\mu L$ was incubated at 37 °C for 1 h 30 min with 2 μL of SAM at 32 mM, 2 μL of 10X NEBuffer, 1 μL of SssI methylase at 4 $U/\mu L$, and 14 μL of nuclease free water. Methylated DNA was then isolated and purified by phenol/chloroform extraction and ethanol precipitation. Methylated DNA was evaluated by spectrophotometry (Take3, Epoch, Biotek). DNA standard was diluted with TE to obtain a standard solution at 10 $ng/\mu L$. The solution was then aliquoted in 0.5 mL tubes and stored at −20 °C until needed.

Two hundred microliters of standard solutions containing 8, 20, 40, 100, and 200 ng of methylated-DNA as well as DNA samples containing 200 ng of DNA were heated at 94 °C for 2 min and immediately cooled in ice. Then, 50 μL of heat-treated DNA was added in the desired number of wells and incubated 1 h at 37 °C. After DNA attachment, wells were washed five times with 200 μL of PBS (NaCl 0.14 M, Na_2HPO_4 0.01M, pH 7.3). To diminish nonspecific antibody binding, each well was then filled with 200 μL of PBS-BSA solution (2% BSA), and the plate was incubated 1 h at 37 °C. The plate was then washed five times with PBS. Primary antibodies, i.e. anti-5-methyl-cytosine monoclonal antibody (Epigentek), were diluted with PTB-BSA solution (PBS solution with BSA 2% and Tween-20 0.02%) at a final concentration of 0.5 $ng/\mu L$. Wells were then filled with 50 μL of primary antibody solution, and the plate was incubated 1 h at 37 °C. After incubation, the plate was washed three-times with PTB solution (PBS solution with Tween-20 at 0.02%) followed by two-times with PBS. Secondary antibodies, i.e. Goat anti-Mouse IgG1 Antibody HRP Conjugated (Bethyl), were diluted with PTB-BSA solution at a final concentration of 0.2 $ng/\mu L$. Wells were then filled with 50 μL of secondary antibody solution, and the plate was incubated 30 min at 37 °C. After incubation, the plate was washed three-times with PTB solution followed by two-times with PBS. Then, 150 μL of TMB solution (Pierce) was added to each well, and the plate was incubated 2 min at room temperature. The reaction was stopped by the addition of 50 μL of H_2SO_4 at 2 N. The absorbance was then read at 450 nm.

2.4. McrBC-msAP-PCR. The protocol described by Tryndyak et al. (2006) was used. Briefly, 20 μg of genomic DNA was digested overnight at 37 °C with 5 $U \cdot \mu g^{-1}$ by *McrBC* endonuclease (New England Biolabs). McrBC-digested DNA fragments were then separated on a 0.8% agarose gel electrophoresis. Fragments larger than 1 Kbp were purified by means of the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's instructions. These fragments were then digested overnight at 25 °C with 20 $U \cdot \mu g^{-1}$ of methylation-sensitive restriction endonuclease *Sma*I endonuclease (New England Biolabs). The reaction was stopped by

heat treatment (65 °C, 25 min). Then, fragments were digested overnight at 37 °C with 40 U·μg^{-1} by *HpaII* (New England Biolabs). For all enzymatic reactions, reactions were stopped by heat treatment (65 °C, 25 min) and freezing (−20 °C). Digested DNA fragments were PCR-amplified ((95 °C 30 s, 40 °C 60 s, 72 °C 90 s) × 5 cycles; followed by (95 °C 15 s, 55 °C 15 s, 72 °C 60 s) × 30 cycles) using 50 pmol of a single primer; MLG2 (5′-AACCCTCACCCTAACCCCGGG-3′). PCR products were resolved on 5% polyacrylamide gel. Bands presenting different intensities among fish groups (Control and Cd-exposed eels) were excised. DNA was then extracted by a crush and soak method before to be PCR-amplified as described above. PCR products were purified from a 0.8% agarose gel electrophoresis by means of the QIAquick Gel Extraction Kit (Qiagen). Purified fragments were then cloned using a pGEM-T easy vector system (Promega) according to the manufacturer's instructions. Selected clones were sequenced (GATC Biotech). Sequence homologies were searched using the Blast+ program using the *Anguilla japonica* database.[16]

2.5. Quantitative RT-PCR. For each gene, specific primers were determined (Table 1) using the Primer3Plus software and

Table 1. Specific Primer Pairs Used in Quantitative Real Time PCR Analysis

gene name	primer sequences
β-actin	CAGCCTTCCTTCCTGGGT[a]
	AGTATTTGCGCTCGGGTG[b]
arf1	GCTGCTGGAAAAACAACCAT[a]
	CTAGTAGAACGGCGTCTCGG[b]
kif21a	CATCTGCTTCATCAGACGGA[a]
	GACTACGAGAAGCGGCTGAC[b]
agpat4	CATCTGCTTCATCAGACGGA[a]
	GACTACGAGAAGCGGCTGAC[b]
line-1	CGTCGCCTGTTCCATATTCT[a]
	AGCAGACAGTGGACAGCCTT[b]
18s	CATGGCCGTTCTTAGTTGGT[a]
	CTCTAAGAAGTTGGACGCCG[b]

[a]Forward primer. [b]Reverse primer.

EeelBase.[17] Total RNA was extracted from 20 mg of liver (20.41 ± 0.84 mg, mean ± SE, n = 15) and treated with DNaseI using the Absolutely RNA Miniprep Kit (Agilent), according to the manufacturer's instructions. For each sample, RNA quality was evaluated by electrophoresis on a 1% agarose gel, and RNA concentrations as well as purity were determined by spectrophotometry (Take3, Epoch, Biotek). First-strand cDNA was then synthesized from 1 μg of total RNA using the GoScript Reverse Transcription System (Promega), according to the manufacturer's instructions. Following the reverse transcriptase reaction, cDNA was diluted 10-fold. Real-time PCR reactions were then performed in an MX3000P (Stratagene; 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s and 72 °C for 30 s). Each 20 μL reaction contained 12.5 μL of GoTaq qPCR master mix (Promega), 5 μL of template, and the specific primer pairs at a final concentration of 250 nM each. Amplification efficiencies for all primer sets were calculated; all values proved to be sufficient to allow direct comparison of amplification plots according to the ΔΔCt method.[18] Relative quantification of gene expression was achieved by concurrent amplification of the *β-actin* endogenous control. Hence, during our experiment, total RNAs were quantified, and 1 μg was used to be reverse-transcribed. During the subsequent qPCR amplifications, the output cycle corresponding to the *β-actin* was examined. This output was always obtained around the same output cycle; i.e. 21.87 ± 0.17 (mean ± SE, n = 5) for control fish, 21.67 ± 0.36 (mean ± SE, n = 5) for Cd-exposed fish at 0.4 $\mu g \cdot L^{-1}$, and 21.27 ± 0.57 (mean ± SE, n = 5) for Cd-exposed fish at 4 $\mu g \cdot L^{-1}$, demonstrating the relevance of the *β-actin* as a reference gene in our conditions.

2.6. Statistical Analyses. Comparisons among fish groups were performed by analysis of variance (ANOVA), after checking assumptions of normality and homoscedasticity of the error terms. When the assumptions were not met as deduced graphically and from ad-hoc tests, we used log and box-cox data transformations. If significant effects were detected, the Least Square Deviation test (LSD) was used to determine whether means between pairs of samples were significantly different from one another. Computations were performed using STATISTICA version 6.1 software (StatSoft, USA). Numerical results are given as means ± SE (n = 5 per condition).

3. RESULTS

3.1. Effect of Cd Exposure on Global CpG Methylation Level and Total RNA Concentration. The calibration of our ELISA method is presented in Figure 1. A significant

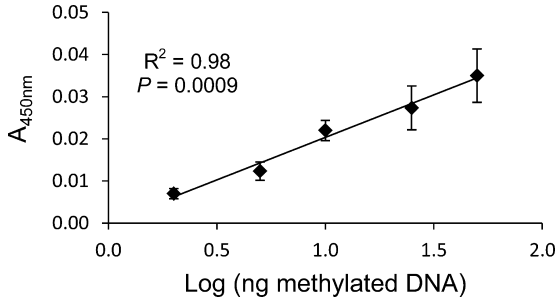


Figure 1. Linear regression of log of ng 5-methylcytosine DNA versus absorption at 450 nm (mean ± SE, n = 3 technical replicates, the coefficient (R^2), and the level of significance (P) of the correlation are reported on the graph).

correlation (r^2 = 0.98, P = 0.0009) was observed between the quantity of methylated DNA and the absorbance measured at 450 nm, allowing thus to investigate a possible effect of Cd exposure on this parameter in eels. The effect of Cd exposure on the global CpG methylation status of eel's liver is presented in Figure 2A. Increasing concentrations of Cd exposure were associated with an increase in the global CpG methylation level in eels. However, this increase was only significant in the case of eels exposed to the highest concentration (i.e., $[Cd]_{water}$ = 4 $\mu g \cdot L^{-1}$). After 45 days of exposure, a significant 2-fold increase in global CpG methylation level was detected in the liver of fish exposed to Cd at 4 $\mu g \cdot L^{-1}$ compared to nonexposed eels. This increase in methylation level was associated with a significant decrease in total RNA concentrations (Figure 2B). After 45 days of exposure, a significant 1.9-fold decrease in total RNA concentration was detected in the liver of fish exposed to Cd at 4 $\mu g \cdot L^{-1}$ compared to controls. No significant effect of Cd was observed on this parameter at the lowest Cd concentration tested, i.e. 0.4 $\mu g \cdot L^{-1}$.

3.2. Identification of Differentially Methylated Sites. The McrBC-msAP-PCR method uses three methylation-

dx.doi.org/10.1021/es4048347 | Environ. Sci. Technol. 2014, 48, 797−803

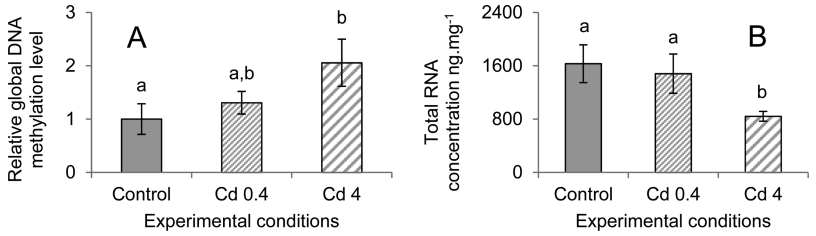


Figure 2. Change in relative global CpG methylation level (A) and in total RNA concentration (B) in liver of control and Cd exposed European eels ($[Cd]_w$ = 0.4 and 4 $\mu g \cdot L^{-1}$). Data are expressed as mean ± SE (n = 5). Bars sharing same-case letters do not differ significantly ($P > 0.05$).

specific restriction endonucleases which cleave or, on the contrary, do not cleave DNA containing 5-methylcystosine. This method was used on genomic DNA of eels in order to detect differentially methylated fragments among control and Cd-exposed eels. Using this method, hypermethylation and hypomethylation are visualized by the increase or the decrease in the band intensity of DNA fingerprints. Results are presented in Figure 3. Genomic DNA from liver of Cd-contaminated fish

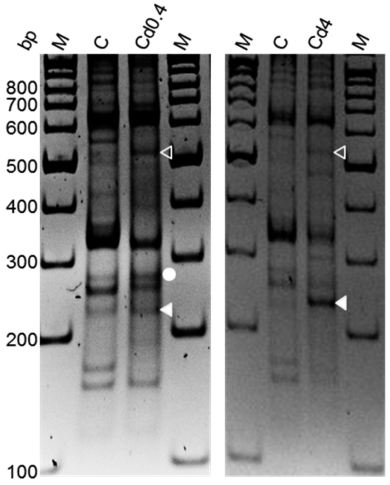


Figure 3. Representative McrBC-msAP-PCR fingerprints of control (C) and Cd-exposed eels ($[Cd]_w$ = 0.4 and 4 $\mu g \cdot L^{-1}$, Cd0.4 and Cd4, respectively). Bands that appeared in Cd-exposed eels and not in control eels and bands with increasing intensity of band in Cd-exposed eels compared to control eels were considered as hypermethylation. M refers to molecular marker. Open triangles refer to *kif21a*. Closed triangles refer to *agpat4*. Circle refers to *arf1*.

exhibited an emergence of novel sequences compared to control eels. A total of 24 bands that showed differential intensities were excised from the gels, reamplified, cloned, and sequenced. The resulting sequences were compared to the genome of *Anguilla japonica* using the Blast+ program on Linux.[19] For sequences that showed high homologies, a portion of the *Anguilla japonica* genome containing the sequence plus 1000 bp upstream and 1000 bp downstream was compared to Genbank database using the Blast program. Only 3 fragments showed high homologies with genes of known functions. These fragments were located near or in the genes encoding for ADP-ribosylation factor 1 (ARF1), kinesin-like protein KIF21A (KIF21A), and 1-acyl-sn-glycerol-3-phosphate acyltransferase delta (AGPAT4). All these sequences presented on the gel an increase in band intensity in response to Cd exposure and can be thus considered as hypermethylated in Cd contaminated fish. Whereas *kif21a* and *agpat4* were found in both groups of Cd exposed eels, *arf1* was found only in eels exposed to the lowest concentration tested; i.e. 0.4 $\mu g \cdot L^{-1}$. Using directly the Genbank database and the Blast program, within sequences, some fragments were found to show highly significant homologies with flanking regions (noncoding regions) of retrotransposon-like sequences. Interestingly, such elements were even observed in the fragments containing the sequences of *kif21a* and *agpat4* genes (Figure 4). All these sequences were found to be hypermethylated in Cd contaminated fish.

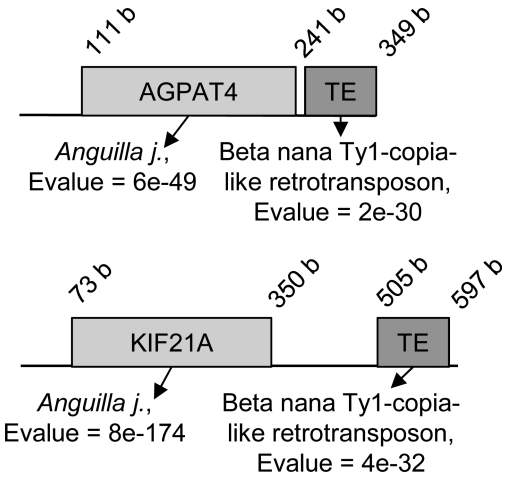


Figure 4. Schematic diagram detailing the fragments obtained by McrBC-msAP-PCR and that contain both sequences with high homology for *agpat4* and *kif21a* genes from *Anguilla japonica* and sequences with high homology for transposable elements (TE). Expect Evalue are indicated on the graph. These schematic diagrams were carried out using BLASTn program and MEME software.[20]

3.3. Gene Transcription Level. To test whether changes in the DNA methylation status of fragments identified by the McrBC-msAP-PCR method could induce changes in the transcription level of the corresponding genes, specific primer pairs were designed for *kif21a*, *agpat4*, and *arf1* genes. In order to substantiate a potential effect of Cd on the methylation status and, concomitantly, on the transcription level of TEs in eel, specific primer pairs were also designed for the retrotransposon *line-1*. Among TEs, *line-1* is indeed the most abundant family of nonlong terminal repeat retrotransposons found throughout the genome. In consequence, its methylation status is often used as a proxy to assess global DNA methylation.[21] The transcription level of these genes was then determined by quantitative RT-PCR. As a significant decrease in total RNA concentration was observed in response to Cd exposure, we also determined the transcription level of the gene encoding for 18s rRNA. Results are presented in the Figure 5A. Cd exposure at 0.4 $\mu g \cdot L^{-1}$ significantly triggered a down-regulation of the gene encoding for ARF1. A significant

dx.doi.org/10.1021/es4048347 | Environ. Sci. Technol. 2014, 48, 797−803

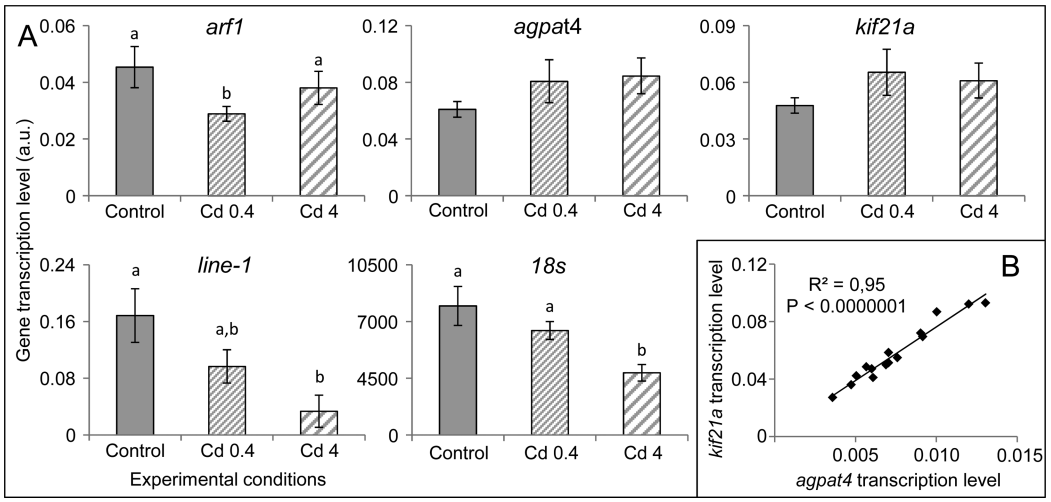


Figure 5. Effect of Cd exposure on gene transcription. (A) Change in gene transcription level (mean ± SE; $n = 5$) in liver of control and Cd exposed European eels ($[Cd]_w$ = 0.4 and 4 $\mu g \cdot L^{-1}$). Bars sharing same-case letters do not differ significantly ($P > 0.05$). (B) Relation between the transcription level of *kif21a* and *agpat4* ($n = 15$, the coefficient (R^2) and the level of significance (P) of the correlation are reported on the graph).

decrease in the transcription level of genes encoding for 18s and LINE-1 was also observed at the highest Cd concentration (4 $\mu g \cdot L^{-1}$). Despite a trend to increase in response to Cd exposure, no significant effect of Cd was observed on the transcription level of genes encoding for AGPAT4 and KIF21A. Interestingly, the transcription levels of these two genes were found to be highly correlated ($R^2 = 0.95$, $P < 0.0000001$, Figure 5B).

4. DISCUSSION

Our results show that low-dose exposure to Cd induced changes in the CpG methylation status of eel's hepatocytes. Cd exposure was associated with a significant increase in the global CpG methylation status of eels. Such results are consistent with previous studies carried out on human embryo lung fibroblast cells or in hens.[5,22] For both studies, subchronic Cd exposure triggered a concentration-related increase in the global DNA methylation level. As epigenetic is still in its infancy, it is unclear why and how the genomic DNA methylation of organisms can be modified by Cd exposure. Moreover, it is also unclear if changes in DNA methylation in response to pollutant exposure represent an adaptive response to alleviate pollutant toxicity or, on the contrary, a mechanism by which pollutants exert their toxicity. For example, aberrant DNA methylation status was found to be an important factor in tumorigenesis, including in the development of hepatocellular adenoma tumors in wild fish from contaminated environments.[23] However, it was shown in numerous models that global genomic levels of DNA methylation are lower in cancer cells than in noncancer tissues.[23] As Cd is known to induce DNA damages,[24] an increased in global DNA methylation could aim to protect DNA and, by extension, organisms against Cd toxicity. Indeed, recent research has shown that chromatin compaction, a well-known effect of DNA methylation, protects DNA from damage.[25,26]

Since DNA methylation is commonly associated with gene silencing,[1] we then quantified the amount of total RNA in eel liver. Our data indicate that Cd exposure induces a significant decrease in total RNA concentration. Given that rRNAs represent at least 95% of the total RNA in fish hepatocytes, such a decrease could be mainly explained by a decrease in ribosome biosynthesis. In direct support of this hypothesis, a significant decrease in the transcription level of the 18s rRNA gene was observed in Cd-exposed eels.

In order to obtain more information about genes and, by extension, biological processes that are differentially methylated between control and Cd-exposed eels we used the McrBC-msAP-PCR method. Our results indicate that Cd exposure can modify the methylation profile of the eel genome. Three genes encoding for proteins involved in intracellular trafficking (ARF1 and KIF21A) and phospholipid biosynthetic process (AGPAT4) were found to be hypermethylated in response to Cd exposure.

For *arf1*, this gene was found to be hypermethylated only in eels exposed to the lowest Cd concentration tested, i.e. 0.4 $\mu g \cdot L^{-1}$. In accordance to an increase in its methylation status, its transcription level was found to be significantly lower in eels exposed to Cd at 0.4 $\mu g \cdot L^{-1}$. Interestingly, ARF1 protein was recently found to be involved in intracellular Cd trafficking and more precisely in the transport of Cd-metallothionein complex.[27] Metallothioneins (MT) are cysteine-rich proteins involved in maintaining sufficient intracellular supplies of some essential metals and in detoxifying excess intracellular metals (including nonessential metals such as Cd). Using a dominant-negative mutant for *arf1* (DN-arf1), authors have shown that the transfer of the Cd-MT complex to lysosome can lead to cytotoxicity. Cd toxicity was indeed found to be significantly lower in DN-*arf1* than in wild type cells.[27] The hyper-methylation of the *arf1* gene coupled with a down-regulation of its transcription level in response to metal exposure could thus appear as a defense mechanism to alleviate Cd cytotoxicity.

Concerning *kif21a* and *agpat4* genes, despite these genes being found to be hypermethylated in response to Cd exposure, no significant effect of Cd on their gene transcription levels was observed. The gene *kif21a* encodes a microtubule-binding molecular protein involved in intracellular trafficking. The gene *agpat4* encodes an enzyme involved in lipid biosynthesis (i.e., triglycerides and phospholipids). It is interesting to note that *kif21a* and *agpat4* genes, despite seeming to be involved in different biological processes, were found to be highly coexpressed, suggesting a common regulatory mechanism. This could be explained at least in part by the presence at the

dx.doi.org/10.1021/es4048347 | Environ. Sci. Technol. 2014, 48, 797−803

genomic level of a transposable element (TE) at their proximity. TEs have long been considered as "selfish" or "parasitic" DNA elements since active TEs are known to be highly mutagenic and to impair the fitness of their host.[28] However, the importance of TEs in genome evolution and transcription regulation has received increasing attention in recent years. Recent discoveries have demonstrated that TEs are mostly inactive in host. Despite in most cases this being due to TE truncation, DNA methylation was found to play a key role in keeping full-length TEs transcriptionally silent. It is becoming increasingly clear that TEs play important roles in genome transcription regulation since few authors have demonstrated that the activation of TEs by stresses can change the transcriptionnal activity of neighboring genes.[21,29−31] In our case, in accordance with an increase in global CpG methylation and in the methylation level of TEs in Cd-exposed eels, the transcription level of *line-1* gene was found to be down-regulated in response to Cd exposure, suggesting an effect of chronic Cd exposure on the methylation level of TEs. It is thus intriguing to speculate that dynamic DNA methylation changes within TEs in response to Cd exposure have contributed among other factors to the (co)regulation of *agpat4* and *kif21a* transcription levels. Limited evidence exists in the literature about the relationships between the methylation status of TEs and pollutant exposure or diseases (especially for Cd and fish). Moreover, data are mainly restricted to epidemiologic studies and to the cancer field.[32,33] In most cases, TEs were found to be hypomethylated in response to pollutant (e.g., benzene, lead, traffic particles) or to biotic[29] exposures. The response appears thus to be different for Cd in our study. For a better understanding, it could be interesting to refer to the functions of AGPAT4 and KIF21A. The product of AGPAT4, i.e. phosphatidic acid (PA), is both a precursor of lipid biosynthesis and an emerging signaling lipid. PA is known to be involved in the regulation of diverse cellular functions in plants and animals notably intracellular trafficking but also microtubule organization, cytoskeleton reorganization, and survival signaling. In addition, it is interesting to note that proteomic investigations have shown that kinesin-like proteins (KIF proteins) are PA-binding proteins.[34] It could thus explain why *agpat4* and *kif21a* genes present a common pattern of gene transcription. Intracellular levels of PA are known to increase in response to abiotic stress. It has been proposed that PA is a second messenger in a broad range of stress signaling pathways and that PA mediates responses to various stresses.[35,36] To our knowledge, there is no data on a potential role of PA signaling pathway in response to Cd exposure. As DNA methylation is commonly associated with gene silencing (with few exceptions; see Vandegehuchte and Janssen, 2011[1]), our data could indicate that chronic Cd exposure *via* DNA methylation of TEs represses this stress pathway, bringing the transcription level of *agpat4* and *kif21a* in Cd-exposed eels close to control eels. This could impair the capacity of eels to respond to Cd stress. Alternatively, such a response could correspond to the setup of an adaptive response to chronic Cd exposure. Indeed, under chronic exposure conditions, it could be difficult to maintain over time increased levels of PA, i.e. an energy-consuming stress response. In this context, it is interesting to note that Takiguchi et al.[37] using rat liver cells showed that whereas prolonged exposure to Cd (10 weeks) triggers DNA hypermethylation, a short-term exposure (1 week) to the same conditions induces, on the contrary, DNA hypomethylation. Further kinetic studies are needed to test a potential effect of both acute and chronic Cd exposures on PA biosynthesis.

Our study illustrates how epigenetics and, more precisely, DNA methylation can be involved in the chronic stress response to Cd in a nonmodel fish species. Cd exposure was found to increase the global CpG methylation status of eel liver. The methylation level of three genes involved in intracellular trafficking and lipid biosynthesis was also increased in Cd-exposed eels. Two of them were associated with a TE sequence at the genomic level. This could reinforce the general hypothesis that DNA methylation of TEs could represent a widespread response to abiotic stress in eukaryotic organisms by regulating the transcription level of neighboring genes.

AUTHOR INFORMATION

Corresponding Author

*Phone: 33556223927. Fax: 33556549383. E-mail: f.pierron@epoc.u-bordeaux1.fr.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the Agence Nationale de la Recherche of France (ANR Immorteel ANR-10-INTB-1704) jointly funded by the Innovative Project of the UMR EPOC 5805.

REFERENCES

(1) Vandegehuchte, M.; Janssen, C. Epigenetics and its implications for ecotoxicology. *Ecotoxicology* **2011**, *20* (3), 607−624.

(2) Feil, R.; Fraga, M. F. Epigenetics and the environment: emerging patterns and implications. *Nat. Rev. Genet.* **2012**, *13* (2), 97−109.

(3) Head, J. A.; Dolinoy, D. C.; Niladri, B. Epigenetics for ecotoxicologists. *Environ. Toxicol. Chem.* **2012**, *31* (2), 221−227.

(4) Jaenisch, R.; Bird, A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat. Genet.* **2003**, *33*, 245−254.

(5) Jiang, G.; Xu, L.; Song, S.; Zhu, C.; Wu, Q.; Zhang, L.; Wu, L. Effects of long-term low-dose cadmium exposure on genomic DNA methylation in human embryo lung fibroblast cells. *Toxicology* **2008**, *244* (1), 49−55.

(6) Skinner, M. K.; Manikkam, M.; Guerrero-Bosagna, C. Epigenetic transgenerational actions of environmental factors in disease etiology. *Trends Endocrinol. Metab.* **2010**, *21* (4), 214−222.

(7) Nair, A. R.; Degheselle, O.; Smeets, K.; Van Kerkhove, E.; Cuypers, A. Cadmium-induced pathologies: where is the oxidative balance lost (or not)? *Int. J. Mol. Sci.* **2013**, *14*, 6116−6143.

(8) Stone, R. Freshwater eels are slip-sliding away. *Science* **2003**, *302* (5643), 221−22.

(9) Robinet, T. T.; Feunteun, E. E. Sublethal effects of exposure to chemical compounds: a cause for the decline in Atlantic eels? *Ecotoxicology* **2002**, *11* (4), 265−277.

(10) Pierron, F.; Baudrimont, M.; Bossy, A.; Bourdineaud, J.-P.; Brèthes, D.; Elie, P.; Massabuau, J.-C. Impairment of lipid storage by cadmium in the European eel (*Anguilla anguilla*). *Aquat. Toxicol.* **2007**, *81* (3), 304−311.

(11) Pierron, F.; Baudrimont, M.; Dufour, S.; Elie, P.; Bossy, A.; Baloche, S.; Mesmer-Dudons, N.; Gonzalez, P.; Bourdineaud, J.-P.; Massabuau, J.-C. How cadmium could compromise the completion of the European eel's reproductive migration. *Environ. Sci. Technol.* **2008**, *42*, 4607−4612.

(12) Geeraerts, C.; Belpaire, C. The effects of contaminants in European eel: a review. *Ecotoxicology* **2010**, *19* (2), 239−266.

(13) Wilson, E. O. *The Future of Life*; Knopf: New York, 2003; p 256.

(14) Couture, P.; Busby, P.; Gauthier, C.; Rajotte, J. W.; Pyle, G. G. Seasonal and regional variations of metal contamination and condition

indicators in yellow perch (*Perca flavescens*) along two polymetallic gradients. I. Factors influencing tissue metal concentrations. *Hum. Ecol. Risk Assess.* **2008**, *14* (1), 97−125.
(15) Tryndyak, V.; Kovalchuk, O.; Pogribny, I. P. Identification of differentially methylated sites within unmethylated DNA domains in normal and cancer cells. *Anal. Biochem.* **2006**, *356* (2), 202−207.
(16) Henkel, C. V.; Dirks, R. P.; De Wijze, D. L.; Minegishi, Y.; Aoyama, J.; Jansen, H. J.; Turner, B.; Knudsen, B.; Bundgaard, M.; Hvam, K. L.; Boetzer, M.; Pirovano, W.; Weltzien, F.-A.; Dufour, S.; Tsukamoto, K.; Spaink, H. P.; Van Den Thillart, G.E. First draft genome sequence of the Japanese eel, *Anguilla japonica*. *Gene* **2012**, *511* (2), 195−201.
(17) Pujolar, J.; Marino, I.; Milan, M.; Coppe, A.; Maes, G.; Capoccioni, F.; Ciccotti, E.; Bervoets, L.; Covaci, A.; Belpaire, C.; Cramb, G.; Patarnello, T.; Bargelloni, L.; Bortoluzzi, S.; Zane, L. Surviving in a toxic world: transcriptomics and gene expression profiling in response to environmental pollution in the critically endangered European eel. *BMC Genomics* **2012**, *13*, 507.
(18) Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2ΔCT method. *Methods* **2001**, *25* (4), 402−408.
(19) Zhang, Z.; Schwartz, S.; Wagner, L.; Miller, W. A greedy algorithm for aligning DNA sequences. *J. Comput. Biol.* **2000**, *7* (1−2), 203−14.
(20) Bailey, T. L.; Bodén, M.; Buske, F. A.; Frith, M.; Grant, C. E.; Clementi, L.; Ren, J.; Li, W. W.; Noble, W. S. MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res.* **2009**, *37*, 202−208.
(21) Pearce, M. S.; Mcconnell, J. C.; Potter, C.; Barrett, L. M.; Parker, L.; Mathers, J. C.; Relton, C. L. Global LINE-1 DNA methylation is associated with blood glycaemic and lipid profiles. *Int. J. Epidemiol.* **2012**, *41*, 210−217.
(22) Zhang, J.; Fu, Y.; Li, J.; Wang, J.; He, B.; Xu, S. Effects of subchronic cadmium poisoning on DNA methylation in hens. *Environ. Toxicol. Pharmacol.* **2009**, *27* (3), 345−349.
(23) Mirbahai, L.; Yin, G.; Bignell, J. P.; Li, N.; Williams, T. D.; Chipman, J. K. DNA methylation in liver tumorigenesis in fish from the environment. *Epigenetics* **2011**, *6* (11), 1319−1333.
(24) Badisa, V. L. D.; Latinwo, L. M.; Odewumi, C. O.; Ikediobi, C. O.; Badisa, R. B.; Ayuk-Takem, L. T.; Nwoga, J.; West, J. Mechanism of DNA damage by cadmium and interplay of antioxidant enzymes and agents. *Environ. Toxicol.* **2007**, *22* (2), 144−151.
(25) Choy, J. S.; Wei, S.; Lee, J. Y.; Tan, S.; Chu, S.; Lee, T.-H. DNA methylation increases nucleosome compaction and rigidity. *J. Am. Chem. Soc.* **2010**, *132* (6), 1782−1783.
(26) Cann, K. L.; Dellaire, G. Heterochromatin and the DNA damage response: the need to relax. *Biochem. Cell Biol.* **2011**, *89*, 45−60.
(27) Wolff, N. A.; Lee, W.-K.; Thévenod, F. Role of Arf1 in endosomal trafficking of protein-metal complexes and cadmium-metallothionein-1 toxicity in kidney proximal tubule cells. *Toxicol. Lett.* **2011**, *203* (3), 210−218.
(28) Slotkin, R. K.; Martienssen, R. Transposable elements and the epigenetic regulation of the genome. *Nat. Rev. Genet.* **2007**, *8* (4), 272−285.
(29) Dowen, R. H.; Pelizola, M.; Schmitz, R. J.; Lister, R.; Dowen, J. M.; Nery, J. R.; Dixon, J. E.; Ecker, J. R. Widespread dynamic DNA methylation in response to biotic stress. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109* (32), 2183−2191.
(30) Kashkush, K.; Feldman, M.; Levy, A. A. Transcriptional activation of retrotransposons alters the expression of adjacent genes in wheat. *Nat. Genet.* **2003**, *33*, 102−106.
(31) Ito, H.; Yoshida, T.; Tsukahara, S.; Kawabe, A. Evolution of the ONSEN retrotransposon family activated upon heat stress in Brassicaceae. *Gene* **2013**, *518* (2), 256−261.
(32) Baccarelli, A.; Bollati, V. Epigenetics and environmental chemicals. *Curr. Opin. Pediatr.* **2009**, *21* (2), 243−251.
(33) Terry, M. B.; Delgado-Cruzata, L.; Vin-Raviv, N.; Wu, H. C.; Santella, R. M. DNA methylation in white blood cells: Association with risk factors in epidemiologic studies. *Epigenetics* **2011**, *6* (7), 828−837.
(34) Manifava, M.; Thuring, J. W. J. F.; Lim, Z.-Y.; Packman, L.; Holmes, A. B.; Ktistakis, N. T. Differential binding of traffic-related proteins to phosphatidic acid- or phosphatidylinositol (4,5)- bisphosphate-coupled affinity reagents. *J. Biol. Chem.* **2001**, *276*, 8987−8994.
(35) Wang, X.; Devaiah, S. P.; Zhang, W.; Welti, R. Signaling functions of phosphatidic acid. *Prog. Lipid Res.* **2006**, *45* (3), 250−278.
(36) Shin, J.; Loewen, C. Putting the pH into phosphatidic acid signaling. *BMC Biol.* **2011**, *9*, 85.
(37) Takiguchi, M.; Achanzar, W. E.; Qu, W.; Li, G.; Waalkes, M. P. Effects of cadmium on DNA-(cytosine-5) methyltransferase activity and DNA methylation status during cadmium-induced cellular transformation. *Exp. Cell Res.* **2003**, *286* (2), 355−365.