



National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

**The potential of two models to screen
for epigenome modifying effects of food
contaminants**

Studies in zebrafish embryos and mesenchymal stem cells

RIVM Letter report 2014-0023
L. van der Ven



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Colophon

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Tijdens de vroege ontwikkeling van de mens worden verschillende typen weefsels en organen gevormd. De informatie hiervoor ligt in het DNA opgeslagen. Per weefseltype worden specifieke delen van het DNA afgelezen, en die afleesbaarheid wordt gereguleerd door de zogenoemde methylering van het DNA. Wetenschappers gaan er steeds meer van uit dat de omgeving van invloed is op de wijze waarop het DNA precies wordt afgelezen. Geopperd wordt dat de toename van chronische ziekten zoals kanker, diabetes en overgewicht zijn oorsprong kan hebben in een verkeerde inregeling van de methylering van het DNA tijdens de vroege ontwikkeling, bijvoorbeeld door chemische verontreiniging in voedsel.

Om meer zicht te krijgen op deze processen is het van belang in kaart te brengen of en in welke mate chemische stoffen de methylering van het DNA beïnvloeden. Het RIVM heeft hiervoor een testmodel ontwikkeld in embryo's van de zebravis. Inderdaad zijn veranderingen in de DNA-methylering vastgesteld nadat de embryo's aan meerdere teststoffen zijn blootgesteld. Vervolgonderzoek moet bevestigen of deze veranderingen gekoppeld zijn aan bepaalde typen chemische stoffen. Ook moet nader worden onderzocht of de geconstateerde veranderingen in de methylering effecten op de gezondheid hebben. Halverwege 2015 worden de resultaten van dit vervolgonderzoek verwacht.

Het zebravis-model blijkt dus toereikend te zijn om metingen in uit te voeren. Voor het onderzoek is ook een stamcelmodel getoetst maar dit bleek minder geschikt.

Trefwoorden: prenatale programmering, chronische ziekten, epigenetica, zebravis embryo's

Abstract

Epigenetic modifications have been hypothesized as a mode of action in programmed responses, i.e. responses upon toxic exposure early in life that are only expressed later in life, e.g. as increased sensitivity to develop chronic disease. Factors implicated in such toxic exposures included consumer goods such as maternal food substances and additives, food contaminants, cosmetic ingredients, and drugs. All such maternal factors may reach the embryo/fetus/lactating neonate, and thus affect the developing organism. There are presently no tests that address epigenetic effects, and therefore we here explored two models that have been suggested as suitable in the scientific literature for that purpose.

The first model was the zebrafish embryo. In this model, we tested a set of control compounds, including 5-azacytidine (5AC, a known DNA methylation inhibitor); 3 metals, nickel, arsenic, and cadmium; 3 estrogenic compounds ethynylestradiol (EE2), diethylstilbestrol (DES), bisphenol-A (BPA); and PFOA, a non-estrogenic endocrine active compound. After exposure to these compounds, starting within 2h after fertilization, epigenetic effects were evaluated after 72 hours of exposure in whole embryo DNA extracts. This was done through analysis of 1) global DNA methylation after DNA digestion and single nucleotide HPLC chromatography, and 2) methylation at three specific loci with shown (*vasa*, *vtgI*) or suggested (*cyp19a2*) DNA methylation plasticity, through pyrosequencing of defined DNA fragments.

In this analysis, there were compound specific responses in a locus specific way, with *vasa* as the most responsive target, showing responses with 7 out of 8 tested compounds. Global methylation was only affected by 5AC, and therefore not an informative parameter. Arsenite did not affect any of the methylation parameters, whereas the other two metals induced methylation effects at concentrations that also produced developmental (morphological) effects. 5AC and the endocrine active compounds, on the other hand, induced methylation responses at subtoxic concentrations. Such epigenetic effects at subtoxic concentrations are of particular interest because these could be hypothesized to predict adverse health effects later in life. Our results confirm that compound induced epigenetic effects can be analyzed in the zebrafish embryo model.

The second model was the primary human umbilical cord derived mesenchymal stem cells, which can be differentiated into adipocytes. Epigenetic changes play an important role in cell differentiation. Therefore, this model could perhaps serve to causally relate epigenetic changes in differentiation-related genes to morphological effects on cell differentiation. We tested a set of 5 compounds that have known effects on adipocyte differentiation. Although we observed patterns suggesting compound specific effects on adipocyte differentiation, there was considerable variation of responses between and within the explored cell lines. Overall, we conclude that this model is not sufficiently robust to serve a phenotype controlled evaluation of epigenetic effects.

Key words: prenatal programming, chronic diseases, epigenetics, zebrafish embryos

Contents

1	Introduction – 11
2	part 1 – Zebrafish embryos – 13
2.1	methods – 13
2.2	Results – 15
2.3	Conclusions and Recommendations – 18
3	part 2 - human umbilical cord mesenchymal stem cells (huMSC) – 19
3.1	methods – 19
3.2	Results and Discussion – 20
3.3	Table 3-1 – Effects on adipogenic differentiation of human umbilical cord mesenchymal stem cells – 21
3.4	Conclusions and Recommendations – 22

Summary

Epigenetic modifications have been hypothesized as a mode of action in programmed responses, i.e. responses upon toxic exposure early in life that are only expressed later in life, e.g. as increased sensitivity to develop chronic disease. Factors implicated in such toxic exposures included consumer goods such as maternal food substances and additives, food contaminants, cosmetic ingredients, drugs, and also non-chemical stressors like circadian disruption. All such maternal factors may reach the embryo/fetus/lactating neonate, and thus affect the developing organism. There are presently no tests that address epigenetic effects, and therefore we here explored two models that have been suggested as suitable in the scientific literature for that purpose.

The first model was the zebrafish embryo, which has the advantage of potential detection of effects in all tissues in the whole organism, overcoming lineage specificity of cultured cell models. Furthermore, the zebrafish embryo enables study of functional consequences of epigenetic modifications, such as a changed behaviour, changed metabolism, or decreased capacity to withstand oxidative stress.

In this model, we tested a set of control compounds, including 5-azacytidine (5AC, a known DNA methylation inhibitor); 3 metals, nickel, arsenic, and cadmium, known to affect DNA methylation in a complex way (hypo- and hypermethylation observed), including depletion of methyl donors; 3 estrogenic compounds ethynylestradiol (EE2), diethylstilbestrol (DES), bisphenol-A (BPA), known to affect DNA methylation through activation of estrogen receptors; and PFOA, a non-estrogenic endocrine active compound. After exposure to these compounds, starting within 2h after fertilization, epigenetic effects were evaluated after 72 hours of exposure in whole embryo DNA extracts. This was done through analysis of 1) global DNA methylation after DNA digestion and single nucleotide HPLC chromatography, and 2) methylation at three specific loci with shown (*vasa*, *vtgI*) or suggested (*cyp19a2*) DNA methylation plasticity, through pyrosequencing of defined DNA fragments.

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The second model was the primary human umbilical cord derived mesenchymal stem cells, which can be differentiated into adipocytes. Epigenetic changes play an important role in cell differentiation. Therefore, this model could perhaps serve to causally relate epigenetic changes in differentiation-related genes to morphological effects on cell differentiation. A disadvantage of primary cell cultures is that the cell yield is low, which we compensated through generation of cultures from multiple umbilical cords. We tested a set of 5 compounds that

have known effects on adipocyte differentiation. Although we observed patterns suggesting compound specific effects on adipocyte differentiation, there was considerable variation of responses between and within the explored cell lines. Overall, we conclude that this model is not sufficiently robust to serve a phenotype controlled evaluation of epigenetic effects.

1 Introduction

There is a global rise in the incidence of chronic diseases, such as conditions associated with the metabolic syndrome (obesity, diabetes, cardiovascular disease), cancer, and autoimmune disease. This cannot be fully explained by genetic variation or by life style factors, such as decreased physical activity and diet. Some 15 years ago, an alternative hypothesis was put forward, stating that during early life stages the homeostatic capacity of an individual is fine tuned to the organism's future environment¹. This Developmental Origins of Health and Disease (DOHaD) concept originated from epidemiological observations showing that undernutrition during early life stages was associated with a higher incidence of chronic disease at later ages. The reasoning was that the body of the fetus was programmed to deal with poor nutrition conditions and, consequently, not with excessive food intake.

In this line, later epidemiological observations indicated associations between maternal intake of food contaminants during pregnancy and affected health condition of the child at later ages, such as impaired carbohydrate and lipid metabolism, decreased vaccination responses or decreased learning performance. These observations suggested yet unidentified risks of exposure to food ingredients and contaminants during early life stages for health and disease later in life. A potential mechanism which could mediate such effects is epigenetic programming, because the epigenome, which is largely defined early in life, is the molecular layer which filters and shapes the functions of each cell and thus of the body as a whole².

The relevance of epigenetic modifications as a causative factor for disease has already been implicated in the onset and progression of cancer, resulting in identification of diagnostic epigenetic markers and, more recently, in new therapeutic modalities^{3,4}. However, the observed associations of epigenetic modifications with other diseases have not yet been established as a causative factor.

The potential of food ingredients and contaminants to affect the homeostatic program of the body was investigated in various experimental animal models. Such studies indeed showed effects on metabolic parameters in adult animals after in utero and lactational exposure to selected food contaminants. Specific epigenetic modifications have been suggested as an underlying mechanism in some of such studies, but there is considerable variation of conclusions among study models, and no consistent, comprehensive explanation has been put forward until now.

To bring the insight on the epigenetic programming potential of chemical substances further, several position papers, supported by opinions of international bodies such as OECD and EFSA, emphasized the need to systematically characterize the epigenetic modifying effects of substances in a

¹ Desai M, Hales CN. Role of fetal and infant growth in programming metabolism in later life. *Biol Rev Camb Philos Soc.* 1997;72:329-48.

² Gluckman PD, Hanson MA. Developmental origins of disease paradigm: a mechanistic and evolutionary perspective. *Pediatr Res.* 2004;56:311-7.

³ Huang Y, Rao A. Connections between TET proteins and aberrant DNA modification in cancer. *Trends Genet.* 2014;30:464-74.

⁴ Katz TA, Huang Y, Davidson NE, Jankowitz RC. Epigenetic reprogramming in breast cancer: from new targets to new therapies. *Ann Med.* 2014;46:397-408.

screening model^{5,6}. These papers list several potentially suitable models, ranging from a rodent reporter model to a variety of in vitro models, which all have their specific advantages and disadvantages. In this project, we set out to study two of such suggested models, that is, zebrafish embryos and human umbilical cord mesenchymal stem cells (huMSC). Both models have the potential for high throughput screening, while huMSC obviously has the advantage of human relevance, whereas the zebrafish embryo measures the effects in a whole organism. In the huMSC, the epigenetic effects can potentially be related to phenotypical effects on cell differentiation. In the zebrafish embryo, epigenetic changes can either be linked to morphological effects (teratology and delayed development), or, preferred, to affected functions later in life, such as effects on neurobehaviour, oxidative stress resistance, immunity.

As a first step in our approach we defined the phenotype which can be used to relate DNA methylation effects to, that is morphological embryotoxic effects (teratology, delayed development) in the zebrafish embryo, and adipogenic differentiation in the mesenchymal stem cells.

Only in the zebrafish embryo, we could proceed with the next step, that is to check the effect of control compounds on DNA methylation, at the level of global methylation and at the level of locus specific methylation (with a defined marker selection).

The following steps were also partly addressed in zebrafish embryos only: to verify DNA methylation effects in expanded test compound set; and to test predictivity of DNA methylation effects for future health effects. These steps need further exploration, together with optimization of the marker set through analysis of genome wide locus specific methylation.

⁵ Rasoulpour RJ, LeBaron MJ, Ellis-Hutchings RG, Klapacz J, Gollapudi BB. Epigenetic screening in product safety assessment: are we there yet? *Toxicol Mech Methods*. 2011;21:298-311.

⁶ Greally JM, Jacobs MN. In vitro and in vivo testing methods of epigenomic endpoints for evaluating endocrine disruptors. *ALTEX*. 2013;30:445-71.

2 part 1 – Zebrafish embryos

2.1 methods

zebrafish embryos

The zebrafish embryo toxicity test (OECD test guideline 236) is a subacute toxicity test using freshly fertilized zebrafish eggs. According to European legislation, zebrafish embryos are considered as an alternative non-animal test model until yolk sac independent feeding, which is at 120 hours post fertilization (hpf). Zebrafish embryos are easily generated in large quantities and easily maintained, and allowing for medium or high throughput testing. Principle of the test is that eggs/embryos are exposed to a test compound dissolved in water (solvents like ethanol or DMSO added as required) starting immediately after fertilization and until 72h of age. Embryos are then scored for morphological effects (embryotoxicity) and DNA is extracted for further analysis.

test compounds

5-Aza-cytidine and the metals arsenic, nickel and cadmium were used as control compounds because of their known effects on DNA methylation. 5-Azacytidine (5AC) is a DNA methyltransferase (DNMT) inhibitor, known to produce DNA hypomethylation. The three used metals arsenite, nickel, and cadmium have been reported to interact with DNMTs, although with variable effects. This variation may depend on the experimental model (and associated specific DNMT interaction), or on exposure duration (e.g. nickel and cadmium can initially inhibit DNMT with resulting DNA hypomethylation, but after prolonged exposure increase DNMT expression and produce DNA hypermethylation). Arsenic has been suggested to affect DNA methylation either through interaction with methyltransferases, leading to hypermethylation, or through depletion of the methyl donor S-adenosylmethionine during its metabolisation, leading to DNA hypomethylation. Nickel is a methyltransferase inhibitor.

As test compounds we used 17 α -ethinylestradiol (EE2), diethylstilbestrol (DES), bisphenol-A (BPA) and perfluorooctanoic acid (PFOA). BPA is a food contaminant, and EE2, DES were used as a reference for the estrogenic activity of BPA. PFOA is a non-estrogenic contaminant, acting mainly via PPAR α . DES is known to induce programmed health effects after in utero exposure in humans and experimental animals, and programmed health effects were also suggested for BPA and PFOA .

The exposures were conducted in a dose-response at subtoxic concentrations, to find epigenetic markers which potentially are predictive of future, programmed health effects, rather than markers of developmental toxicity.

DNA analysis – global methylation

Analysis of global methylation of DNA is widely used as a non-specific screen for epigenetic effects at all. To this aim, DNA was digested with DNase I, Nuclease P1, and alkaline phosphatase, to produce single nucleotides, which were quantified by HPLC. In this way, the ratio of 5-methyl-2'-deoxycytidine (mdC) and unmethylated 2'-deoxycytidine can be derived.

Global methylation is a measure of methylation in all cytidines in CpG combinations in the entire genome in all cells. Although small differences may indicate deranged activity of methylation and demethylation processes, the absence of any specific information, e.g. regarding overall or locus specific effects, or magnitude of effects at specific loci, excludes any functional interpretation. Global methylation is therefore not more than an exploratory marker.

DNA analysis – locus specific methylation

Next, we performed targeted analysis in three specific loci, vitellogenin I (*vtg1*), *vasa* (*vasa*, *ddx4*), and aromatase B (*cyp19a2*, also known as *cyp19a1b*, *cyp19b*, and *aroB*), all three derived from literature and described as markers of toxic effects of chemical compound exposures, with known or suggested effects in their DNA methylation profile. The target sequence in the promoter region of *vtgI* analyzed in this study is based on Strömqvist et al⁷, who observed EE2 induced effects at this locus at concentrations that affect sex differentiation. It is 174 basepairs and contains three CpG-sites. The *vasa* promoter showed reduced methylation after exposure to embryotoxic concentrations of benzo[a]pyrene in zebrafish larvae⁸. The target sequence in the promoter region of *vasa* used in this study is a 198 basepair fragment and contains 5 CpG sites⁹. Selection of *cyp19a2* was based on compound induced expression by BPA and EE2. The in-house designed target sequence of 247 basepairs of *cyp19a2* is in its promoter region and contains 7 CpG-sites.

For the targeted analysis, DNA was treated with bisulfite prior to CpG-analysis to distinguish between methylated and non-methylated CpG sites, because the bisulfite reaction converts non-methylated cytosine to uracil. After this treatment the specific region of each of the three targets was amplified using Polymerase Chain Reaction (PCR). The amplicon was then analyzed by pyrosequencing. In the case of BPA, the effect in *vasa* was verified by a method which measures the integrated methylation status over the 5 CpG sites in the complete PCR fragment (high resolution melting, HRM).

Measurement of locus specific methylation in the embryo indicates the average of that specific locus in all cells in the body of the embryo. Overall small effects may therefore mark a simultaneous small change in all tissues, or a relatively large change one or few tissues. Biological significance can be anticipated particularly in the latter case, although this needs further exploration.

functional effects

Finally, as a proof of principle, the case of one of the test compounds, BPA, was used to analyze for programmed functional effects later in life after embryonal exposure. To this end, a batch of embryonally exposed fish were maintained until 8 weeks of age, and then analyzed for body length and weight, fin regeneration, and neurobehavioural functions. Neurobehaviour was analyzed through automated recording of swimming patterns (Viewpoint system).

statistical analysis

All exposures were designed to allow for dose-response analysis (PROAST software). Dose-responses are given as the function that deviates significantly from the no effect function $y=a$ and provides the most optimal description of the entire dataset. The critical effect dose (CED) derived from this function is the concentration that induces a critical effect size (CES), arbitrarily defined at 5% (5% deviation of the background value a). As indicated above, a small methylation difference may be good marker in the case of global methylation, and have biological significance in the case of locus specific methylation, and a CES of 5% is therefore justified.

Illustrations of such dose-responses are given for the case of BPA, in Fig. 2-1, and further explained in the Figure legend. CEDs are a practical way to compare effect sensitivity of various parameters (see Table 2-1).

⁷ Strömqvist M, Tooke N, Brunström B. DNA methylation levels in the 5' flanking region of the vitellogenin I gene in liver and brain of adult zebrafish (*Danio rerio*)--sex and tissue differences and effects of 17 α -ethinylestradiol exposure. *Aquat Toxicol.* 2010;98:275-81.

⁸ Fang X, Thornton C, Scheffler BE, Willett KL. Benzo[a]pyrene decreases global and gene specific DNA methylation during zebrafish development. *Environ Toxicol Pharmacol.* 2013;36:40-50.

⁹ Lindeman LC, Winata CL, Aanes H, Mathavan S, Alestrom P, Collas P. Chromatin states of developmentally-regulated genes revealed by DNA and histone methylation patterns in zebrafish embryos. *Int J Dev Biol.* 2010;54:803-13.

2.2 Results

defining a compound-induced phenotype

All compounds induced embryotoxic effects in a range finding test, indicating that they were absorbed by the embryo. Based on that, the test concentration in the final tests, summarized in Table 2-1, were aimed to produce embryotoxicity in only the highest concentration.

effect of control compounds on DNA methylation

The control compound 5-azacytidine (5AC) produced the expected DNA hypomethylation at the level of global methylation, and also in the three analyzed targets (Table 2-1). However, effects in these three targets were observed at different concentrations relative to embryotoxicity, *vasa* being the most sensitive target.

With two of the three metals, there was also a response of *vasa*, but this

Table 2-1: Summary of dose-response effects (global and locus specific methylation)

Compounds	test range μM	Embryo- toxicity (ET) CED μM	Global methylation CED /ET	Locus specific methylation CED /ET			
				<i>vtg1</i>	<i>vasa</i>	<i>vasa</i> CpG 1-5 ^c	<i>cyp19a2</i>
5AC	10-320	307	↓ 0.3	↓ >1.0 ³	↓ 0.05 ³ 0.2 ⁴ 0.1 ⁵		↓ >1.0 ^{6,7}
Arsenite	100-3200	2900	-	-	-		-
Nickel	7.7-250	183	-	-	↑ >1.4 ⁵		↓ >1.4 ⁶
Cadmium	3.2-100	13.9	-	-	↑ 4.4 ³ 10.4 ⁵		-
EE2	0.1 – 3.2	3.2 ^a	-	-	↓ 0.9 ³ 0.4 ^{5*}		-
DES	0.1 – 3.2	2.4	-	-	↓ 0.8 ³		↓ 1.6 ¹
BPA	4.9 – 22.7	20	-	-	↓ 0.2 ³	↓ 0.6	↑ () ^{3,b}
PFOA	10 – 320	320 ^a	-	↑ 0.02 ¹	↓ 0.7 ³		-

Values of effects in global and locus specific methylation are Critical Effect Doses (CEDs), calculated at Critical Effect Size (CES) = 0.05 (explained under *statistical analysis*, above) and presented relative to the CED of embryo toxicity. In this way, the CEDs of methylation can be valued as the relative sensitivity of the methylation effect, with values >1, <1 representing methylation effects at higher, respectively lower concentrations than embryotoxic effects. Dose responses are visually explained in dose response graphs for BPA in Fig. 2.1. Values are derived from single experiments.

¹⁻³⁻⁴⁻⁵⁻⁶⁻⁷ Superscripts indicate the respective CpG in the analyzed fragment. ↑, ↓ hypermethylation, hypomethylation. ^a Observed in a preceding range-finding; ^b Effect was <5%, therefore a CED is not available at CES = 5%; ^c Additional analysis with high resolution melting, a procedure to measure integrated DNA methylation over all CpGs in the fragment (see Fig.2-1C). -, no observed dose response; empty cells, not tested.

occurred at a higher concentration than embryotoxic effects. Nickel also produced a DNA methylation effect in *cyp19a2*, also at a higher concentration compared to embryotoxicity. Arsenite didn't show an effect in any of the assayed targets.

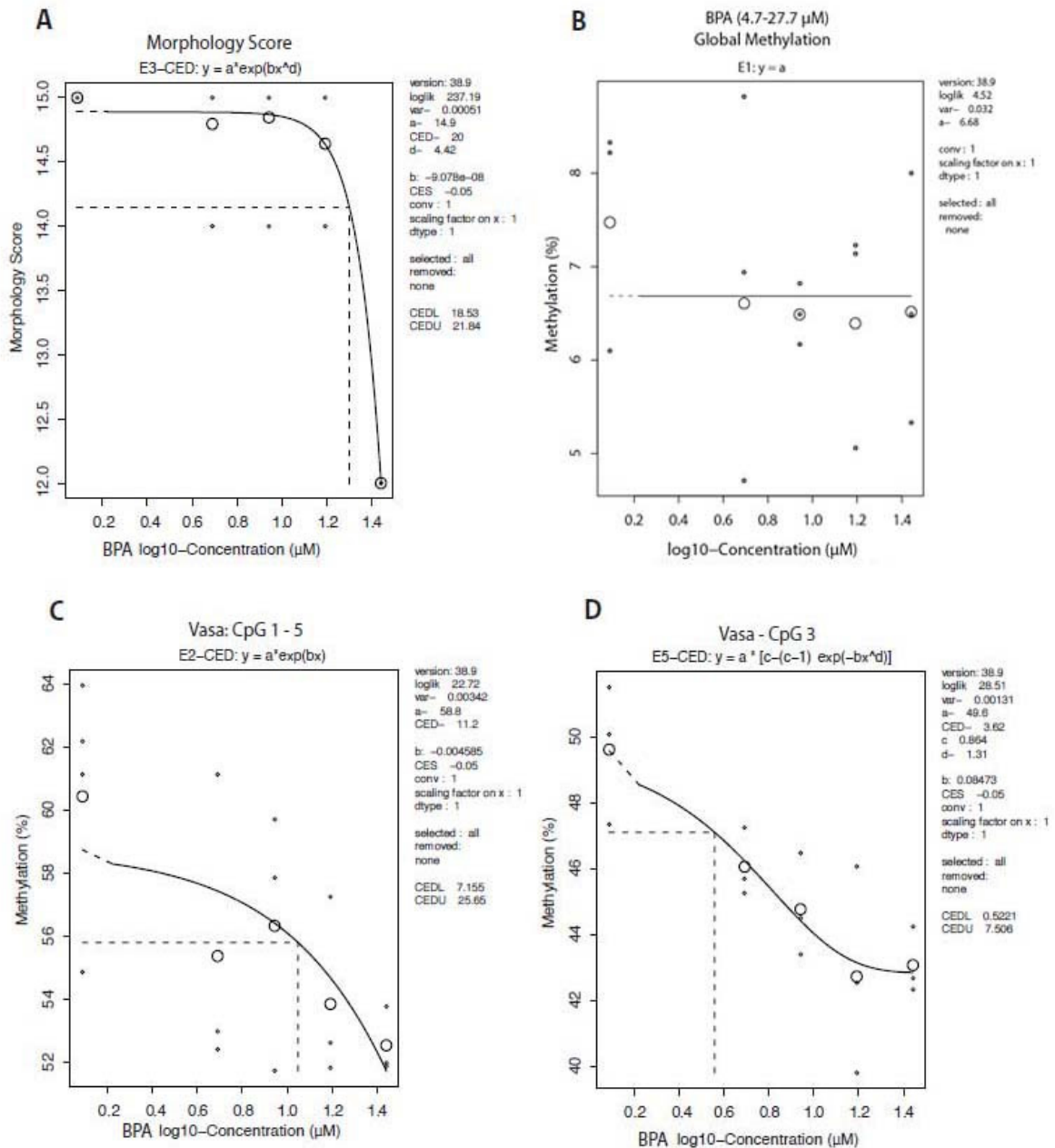


Figure 2-1: Dose responses for BPA. A – Morphology score for development toxicity, with a critical effect dose (CED) near the highest tested concentration, B – global methylation (no effect), C – Integrated analysis over all 5 CpGs in the *vasa* fragment (CED at an intermediate concentration), D – *vasa*, effect in CpG 3 only (CED at a low concentration). Horizontal dotted line represents the effect level (critical effect size, CES), which is defined at 5%. The vertical dotted line indicates the corresponding critical effect dose (CED), which can be read as the sensitivity of the effect.

verifying DNA methylation in an expanded compound set

All four endocrine active compounds induced a DNA methylation effect in *vasa* at a lower concentration compared to embryotoxic effects. The ratio of CEDs of *vasa* / embryotoxicity was lowest for BPA (hypomethylation in *vasa* CpG3; Table 2-1), and this sensitive effect in *vasa* was confirmed by HRM. DES also affected methylation of *cyp19a2*. The non-estrogenic PFOA showed an effect in *vtg1* at a very low concentration compared to embryotoxicity.

Table 2-1 further shows that overall, *vasa* is the most responsive target, showing effects with 7 out of 8 tested compounds. The effect of the two metals in *vasa* separates by producing hypermethylation, in contrast to the observed hypomethylation with all other effective compounds.

Apart from effects with 5AC, *vtg1* showed only effect with PFOA, and the three compounds inducing methylation effects in *cyp19a2* did not do so with a particular pattern.

Further methylation analyses with additional compounds are ongoing to complement the current dataset, and to reproduce preliminary results.

predictivity of DNA methylation effects on functions later in life

The DNA methylation effects of the endocrine active compounds in *vtg1* and *vasa* occurred at lower concentrations than developmental effects. Although these targets were selected to study DNA methylation effects at all, without a specific mechanistic hypothesis, it should be considered that the observed DNA modifying effects indeed operate independently of direct embryotoxicity and predict health effects later in life. To test this, as a proof of principle, functional effects were evaluated in juvenile fish which had embryonal exposure to BPA. In this pilot evaluation there were no effects on body metrics and fin regeneration. However, minor effects were detected in the neurobehaviour. Juvenile fish that were embryonally exposed to BPA showed a statistically significant but slight increase of time spent in inactivity, and also a deviating response to an artificial predator: where control fish responded with a statistically significant increase in high activity (both in terms of duration and distance), BPA fish didn't (Fig. 2-2 for duration). However, the equipment needs further adjustments which may improve the accuracy of the measurements. This is presently discussed with the supplier.

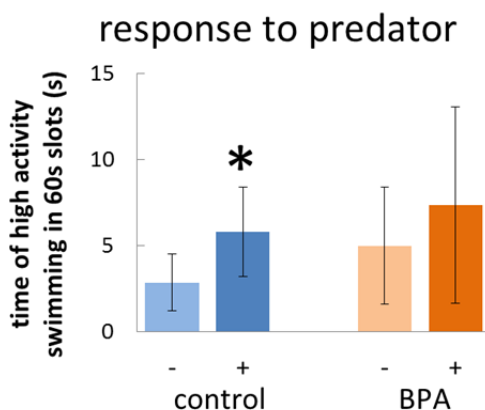


Figure 2-2 Response to predator. After 60s blank measurement, an image of a predator was moved along the tank during the next 60s. The system measurements were inactivity, normal and high activity swimming. $n=10$ fish per group.

optimizing the marker set

From the observations so far, it can be concluded that compound exposure in early stage zebrafish embryos affects DNA methylation in a compound and target specific way. *Vasa* is the most informative target, but probably does not fully represent DNA methylation effects in the whole genome. The representative value of the present selected set of targets can be verified by a genome-wide analysis in BPA exposed samples. Similarly, the absence of DNA methylation effects with arsenite on the selected targets also needs verification by means of a genome-wide analysis. These tests are ongoing, and may also produce additional informative targets.

2.3 Conclusions and Recommendations

- With the zebrafish embryo, effects on DNA methylation can be detected after exposure to environmental contaminants.
- Of the three selected loci, DNA methylation of *vasa* was responsive after exposure to 7 of the 8 tested compounds. *Vtgl* and *cyp19a2* both only responded 1 or 4 cases, respectively.
- Effects in *vasa* occurred at lower concentrations than embryotoxic effects with all 4 tested endocrine active compounds (and with the control compound 5AC), and at higher concentrations than embryotoxic effects with 2 of the 3 tested metals.
- Global methylation is not an informative endpoint in this model, since there was only an observed response with the control compound 5AC.
- Long term effects of embryonal exposure to BPA may be detected in neurobehaviour, but the present results are preliminary and need to be confirmed.

The primary aim of the present study was to explore whether the zebrafish embryo model is suitable to screen for DNA methylation modifying effects. The preliminary results confirm that this can be done, and compound and class specific effects were suggested. However, further substantiation of such effects and their impact is required. Testing more substances to produce a robust database may better underpin compound and class-specific effects. The presently used set of locus-specific markers may need revision to include new informative markers. This requires genome wide analysis of selected cases. Finally, the potential of epigenetic effects in the early embryo to predict an adverse phenotype at later age needs further exploration.

In the OECD Fourth Meeting of the Advisory Group on Endocrine Disrupters Testing and Assessment (EDTA) of the Test Guidelines Programme, this October, it was suggested to update TG236 (fish embryo toxicity test) to include epigenetic endpoints. The explorative studies described here are thus timely, and we have lined up with an initiative responding to this suggestion from EDTA, which will be worked out in a Horizon 2020 proposal. The importance of such a test is that it may classify factors that increase the risk of non-communicable chronic diseases through disrupted epigenetic programming, and thus support regulation of consumer goods, including food additives and contaminants, cosmetics and drugs.

3 part 2 - human umbilical cord mesenchymal stem cells (huMSC)

3.1 methods

human umbilical cord mesenchymal stem cells

Development of huMSC as a screening model for epigenetic modifications had a step-wise design. The first step was to replicate the described generation of primary cultures and the differentiation protocol of established primary cultures^{10,11}. Secondly, in an operational differentiation model, DNA was to be harvested to evaluate effects of compound exposure on DNA methylation, and link this with effects on differentiation.

For the first step, umbilical cord segments (approx. 15 cm length) were obtained during Caesarian sections at Wilhelmina Children's Hospital, Utrecht. These tissues were kept in PBS at 4°C, until further processing, usually within 16h after delivery. Cord segments were rinsed, vessels removed, and the tissue minced with scalpel knives before digestion with collagenase-B (Sigma). Various incubation times (1h-overnight) were tested to optimize the yield of stem cells, however without noticeable differences. After washing of digests with PBS, cells were allowed to attach in tissue culture flasks during 3 days. Then, erythrocytes and tissue debris were removed, and adherent cells further cultured for 3 passages, whereafter the culture was harvested, subdivided in at least 10 vials, frozen and banked until use in exposure experiments. The entire procedure takes several weeks, and therefore, to advance the exposure experiments, a culture of commercially prepared huMSC was obtained (Promocell).

According to literature, characterization of huMSC can be done operationally, through testing of the differentiation potential. MSC are multipotent cells, which can be differentiated in cell types of the mesenchymals lineage, including osteocytes, chondrocytes, adipocytes. Here, adipogenic differentiation was applied, in view of comparability with results in the 3T3-L1 cells, which is a pre-adipocyte model assayed by a partner institute (VU-IVM). Adipogenic differentiation was tested after seeding 3×10^4 cells per well in a 24-wells plate in DMEM-F12 culture medium with 10% fetal calf serum, and addition of an adipogenic cocktail, consisting of insulin (5 µg/ml), dexamethasone (1 µM), 3-isobutyl-1-methylxanthin (500 µM), and indomethacin (60 µM)⁸. This cocktail allows further stimulation as well as inhibition of adipogenic differentiation with simultaneously added test compounds. The test duration was 7 days, with culture medium renewal at day 3 or 4. After 7 days, adipogenesis was evaluated through staining of intracellular lipid with the fluorescent dye Adipored, which was expressed relative to fluorescent Hoechst as a measure of the final cell number in the well. This relative value is the adipogenic index.

compounds

After setting up the exposure system, tests were initiated with control compounds tributyltin chloride (TBTC 0.01-5µM, an inducer of adipogenesis in

¹⁰ Karahuseyinoglu S et al. 2007. Biology of stem cells in human umbilical cord stroma: in situ and in vitro surveys. *Stem Cells* 25:319-331.

¹¹ Biemann R et al. 2012. Endocrine disrupting chemicals affect the adipogenic differentiation of mesenchymal stem cells in distinct ontogenetic windows. *Biochem Biophys Res Commun* 417, 747-752.

3T3-L1 cells¹²) and sodium arsenite (NaAs 0.01-10 μM , a known modifier of DNA methylation), diethylstilbestrol (DES 0.1-100 μM , potent synthetic estrogen, known for its programming effects), and a selection of food contaminants, bisphenol-A (BPA 1-100 μM , weak estrogen), and bis(2-ethylhexyl)phthalate (DEHP 0.01-100 μM , anti-androgen).

3.2 Results and Discussion

defining a compound-induced phenotype

Twelve umbilical cords were processed. Stem cells were hard to identify in initial digests because of tissue debris and erythrocytes. Adherent cells were first observed after 3 days of incubation after removal of the collagenase, but workable populations were only generated after at more than three weeks of incubation. Not all processed cords produced sufficient cells, and some cultures were lost due to contamination, mostly fungus. In the end, only four cords produced sufficient cells for exposure experiments, which were supplemented with a fifth, commercially obtained culture.

Even successfully produced cultures showed slow cell replication and tended towards growth in clusters (bodies), complexing controlled seeding and evaluation during experiments. In the end, these factors limited the number of experiments that could be performed per cell line, and not enough cells could be generated to extract sufficient DNA for analysis of effects on methylation.

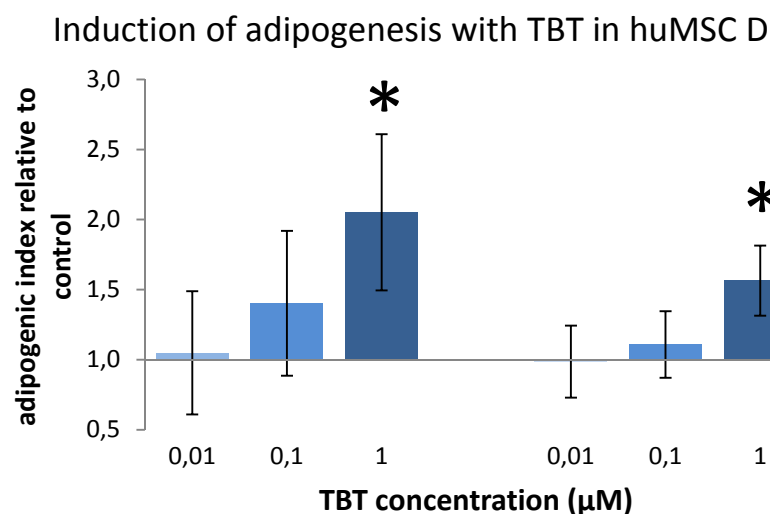


Figure 3-1 - Illustration of stimulated adipogenic differentiation in a huMSC line. Shown is cell line D (see also Table 3-1) in a replicate experiment (left and right panels). Adipogenesis was measured as an adipogenic index (Adipred fluorescence per Hoechst fluorescence), and shown relative to average of control medium; $n=6$ wells per condition. In both experiments, 1 μM of TBT produced a significant stimulation of adipogenic differentiation (*).

¹² Grun F et al. 2006. Endocrine disrupting organotin compounds are potent inducers of adipogenesis in vertebrates. Mol Endocrinol 20, 2141-2155.

Table 3-1 – Effects on adipogenic differentiation of human umbilical cord mesenchymal stem cells

	range of test concentrations (µM)	cell line					
		A	B		C	D	E
TBT	0.01-5	1	0.0	0.5-1	1-10	1	1
			5			1	1
			0.0	1			
arsenite	0.01-10	10	10	10	1-10	10	
				10	1	10	
BPA	1-100			100	100	100	
DES	0.1-100			100	10	100	
DEHP	0.01-100	100	100	1(-100)	1	100	
			5-100		1		
			100				

Green, induction of adipogenesis; **red**, inhibition of adipogenesis; **blue**, no effect observed. Data in the green and red cells represent the concentrations at which the effect was observed (T-test of adipogenic index of exposed compared to control). Data in blue cells indicate the highest tested concentration in that experiment (which was possibly sub-optimal in some cases).

In the exposure experiments, TBT was used as a positive control for adipogenic differentiation, because it is known to have this effect in rodent-based in vitro and in vivo models. TBT induced adipogenic effects in 4 of the 5 tested mesenchymal stem cell lines (illustrated for line D in Fig. 3-1; see Table 3-1 for overview of experiments). This induction however, was not consistent nor reproducible in cell line B (Table 3-1), which also responded with inhibition of adipogenic differentiation. The TBT test concentration in the non-responsive cell line E was comparable to that in the other lines, which can therefore not explain the absence of an effect.

Arsenite is a known modifier of DNA methylation and because DNA methylation is a basis for cell differentiation, this compound could also be expected to affect differentiation in the present model. Indeed, arsenite inhibited adipogenic differentiation in 3 out of 5 tested cell lines (B,C,D), but this effect was not reproduced in the 2 other tested cell lines (A,E).

Of the other 3 compounds, BPA inhibited adipogenic differentiation at the highest test concentration in 2 out of 3 tested cell lines (repeated in one of the responsive cell lines). Due to its (weak) estrogenicity, the effect of BPA could be consistent with the inhibitory effect of the synthetic estrogen DES. The effect of DES was observed in 2 out of 3 tested cell lines, where the absence of an effect in the third cell line could be explained by the use of a suboptimal test concentration. DEHP induced adipogenic differentiation in 2 out of 5 tested cell lines, however this effect was not reproduced in repeated testing in one of the

two responsive cell lines. The absence of an effect in one of the three non-responsive cell lines could be due to the use of a suboptimal test concentration.

Overall, cell lines B-C-D appeared to be more responsive than cell lines A and E, particularly when considering that the test concentrations in non-responsive experiments of cell line D might be sub-optimal. Although not systematically explored, the passage number used in the exposure experiments appeared to affect the response in these primary cultures, which may explain within cell line variation. Furthermore, since the available resources didn't allow phenotyping the cultures, contamination by non-stem cells cannot be excluded.

In conclusion, all five tested compounds were able to induce effects in two or more cell lines, although not reproducible in each case. Overall, when effects were induced, these were mostly consistent regarding the direction of the effect (stimulation or inhibition of adipogenesis) across cell lines, with exception of TBT effects in cell line B. Arsenite and the estrogenic compounds BPA and DES appear to be potential inhibitors of adipogenic differentiation in primary human umbilical mesenchymal stem cells, whereas TBT and DEHP are a potential stimulator of adipogenic differentiation in this model (TBT not consistent in cell line B). Critical, non-defined factors determine observed responses in these cell lines.

effect of control compounds on DNA methylation

The analysis of DNA methylation in this model was not performed because not enough cells could be generated to extract sufficient DNA. Furthermore, in view of the variable effects on adipogenic differentiation, the model was not deemed sufficiently robust to proceed with further efforts in epigenetic analysis.

3.3 Conclusions and Recommendations

- Although the primary human umbilical mesenchymal stem cell is a highly relevant model to detect disruption of adipogenesis after contaminant exposure in humans, there are drawbacks purity of the stem cell population, passage number, and differences between umbilical cord samples, which limit robust testing. These factor may also explain the observed variability of responses within and between cell lines.
- The limited number of cells that can be generated in this model of primary cells doesn't allow a high-throughput screening application for evaluation of effects on adipogenic differentiation, nor on methylation of DNA.
- The disadvantages of this model in view of screening for epigenetic modifying potential of food contaminants are predominating, and it is recommended to discontinue further exploration of this model. This does not exclude exploration of other stem cell models, particularly established stem cell lines.

