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An Alkylphenol Mix Promotes Seminoma Derived Cell Proliferation through an ER α 36-Mediated Mechanism

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Abstract

Long chain alkylphenols are man-made compounds still present in industrial and agricultural processes. Their main use is domestic and they are widespread in household products, cleansers and cosmetics, leading to a global environmental and human contamination. These molecules are known to exert estrogen-like activities through binding to classical estrogen receptors. *In vitro*, they can also interact with the G-protein coupled estrogen receptor. Testicular germ cell tumor etiology and progression are proposed to be stimulated by lifelong estrogeno-mimetic exposure. We studied the transduction signaling pathways through which an alkylphenol mixture triggers testicular cancer cell proliferation *in vitro* and *in vivo*. Proliferation assays were monitored after exposure to a realistic mixture of 4-tert-octylphenol and 4-nonylphenol of either TCam-2 seminoma derived cells, NT2/D1 embryonal carcinoma cells or testis tumor in xenografted nude mice. Specific pharmacological inhibitors and gene-silencing strategies were used in TCam-2 cells in order to demonstrate that the alkylphenol mix triggers CREB-phosphorylation through a rapid, ER α 36-PI3kinase non genomic pathway. Microarray analysis of the mixture target genes revealed that this pathway can modulate the expression of the DNA-methyltransferase-3 (Dnmt3) gene family which is involved in DNA methylation control. Our results highlight a key role for ER α 36 in alkylphenol non genomic signaling in testicular germ cell tumors. Hence, ER α 36-dependent control of the epigenetic status opens the way for the understanding of the link between endocrine disruptor exposure and the burden of hormone sensitive cancers.

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Introduction

Over the last 50 years, the incidence of male reproductive disorders such as hypospadias, cryptorchidism, hypofertility and testis cancer has dramatically risen. For instance, testicular germ cell tumor (TGCT) has become the leading cause of cancer in men aged 15 to 45 years from industrialized countries. Among malignant tumors of the testis, 95% are testicular germ cell tumors, which are classified into two main categories based upon histologic, molecular and epigenetic traits: seminoma and non-seminoma [1]. Both derive from a common precursor cell type called carcinoma in situ (CIS) [2] which is believed to originate from misdifferentiated primordial germ cells or gonocytes in response to altered hormone signaling [3]. CIS cells appear during fetal life and then enter a period of dormancy in infancy until after puberty when TGCT emerge [4]. This prepubertal dormancy suggests a hormone sensitive mechanism for TGCT development and tumor progression at puberty. A wide range of published data dealing with TGCT geographic incidence variation, epidemiological studies performed on migrant men, and exposure model analyses *in vivo* and *in vitro* strongly suggest the participation of endocrine disrupting compounds (EDCs) in both initiation and progression of testis cancer. In occidentalized countries, this hypothesis also emerge to explain the burden of testis cancer associated pathologies such as cryptorchidism, hypospadias,

hypofertility, as well as increased incidence of other hormone sensitive cancer (breast, prostate, ovary, endometrium) [5–8].

Among the great diversity of compounds potentially able to alter hormone signaling, plasticizers are of great concern because of (i) the ubiquitous and persistent environmental and human contamination, (ii) their presence in everyday life used cosmetics, food, drinking water, home cleansers and (iii) their ability to trigger estrogenic signaling [9].

These molecules such as bisphenol A (BPA), 4-nonylphenol (NP) or 4-tert-octylphenol (OP) belong to the alkylphenol family and are still used in various industrialized processes. Once released in the environment, they become persistent pollutants that are poorly eliminated by liver detoxification enzymes in mammals and can enter cells, especially in body fat due to their lipophilic properties [10,11]. BPA and NP are also able to cross the seminiferous tubules basal lamina, alter the testis-blood barrier and trigger differentiating germ cell sloughing and apoptosis by disrupting Sertoli/germ cell attachment and communication [12]. Moreover, this class of EDCs appears to promote the development and progression of estrogen-dependent cancers [13]. BPA was also reported to promote mitogenic effect in JKT-1 seminoma derived cells [14,15].

Binding experiments indicate that alkylphenols could mimic estrogen mitogenic signaling since BPA, NP and OP display

a relative binding affinity to 17 β -estradiol (E₂) in the range of 0.1% for the nuclear receptor ER α 66 and 50% for the transmembrane g-protein coupled estrogen receptor (GPER) [9,16]. Besides, nothing is known about alkylphenol binding to ER α 36, a novel 36 kDa NH₂-term truncated form of the canonical human estrogen receptor ER α 66, retaining the DNA-binding, partial dimerisation and an altered ligand-binding domains [17]. ER α 36 was previously shown by us and others to mediate estrogen non conventional mitogenic signaling in TCam-2 seminoma cells, triple-negative breast cancers cells and endometrial cancer cells [18–20]. Depending on the cell lines tested, ER α 36 acts as a membrane located ER, sometimes collaborates with either GPER or EGFR, and triggers several kinase-dependent pathways, such as MAPK, STAT5 or PKA [20–23].

In an attempt to understand the mechanisms underlying the deleterious effects of EDCs on neoplastic germ cells, we aimed to decipher the alkylphenol-dependent transduction pathways in TCam-2 cells, a unique seminoma cell line [24]. The test compounds, 4-tert-octylphenol (4-t-OP) and 4-nonylphenol (4-NP) were mixed based on their realistic concentration ratio (1:30) in food from Raecker and colleagues [25]. The resulting mix was called M4 and used at concentrations that mimic human environmental exposure. First, we show that M4 increases the TCam-2 seminoma cell proliferation rate *in vitro* by triggering the stimulation of ER α 36 dependent mitogenic pathways. Second, we confirm the M4 stimulates NT2/D1 embryonal carcinoma cell proliferation *in vitro* as well as tumor growth in NT2/D1 xenografted nude mice. Finally, we demonstrate that alkylphenol signaling pathway ends on target genes involved in epigenetic modifications.

Materials and Methods

Reagents

The test compounds, 4-nonylphenol (CAS number: 84852-15-3), 4-tert-octylphenol (CAS no: 140-66-9), and 17 β -estradiol (E₂, CAS no: 50-28-2) were purchased from Sigma Aldrich (France). 4-tert-octylphenol (4-t-OP) and 4-nonylphenol (4-NP) were mixed based on their realistic concentration ratio (1:30) in infant food [25], thus forming the working mix called M4. Stock solutions of 10 mM for M4 (the concentration refers to 4-NP therein) or E₂ were prepared in dimethylsulfoxide (DMSO) and further diluted in RPMI medium without phenol red. All working solutions were freshly made just before the cell treatment assays.

Wortmanin (PI3K inhibitor), G1 (GPER agonist) and G15 (GPER antagonist) were purchased from Sigma-Aldrich (France). Control cells were treated with DMSO, diluted with the same factor as M4 and indicated as “vehicle” in the figures and ranging from 0.01% to 10^{−12}%.

Ethic Statement

Animals used in the present research have been treated humanely according to institutional guidelines (Directive EU/63/2010), with due consideration to the alleviation of distress and discomfort. Protocol for animal handling and experiments was approved by the “Lorraine Regional Committee for Animal Experiments” and carried out by competent and authorized persons (personal authorization number 54–89 issued by the Department of Veterinary Services) in a registered establishment (establishment number C-54-547-03 issued by the Department of Veterinary Services).

Animal experiment was planned in respect to the 3R rule: minimal number of mice necessary and sufficient to reach statistical significance was calculated *a priori*. For each animal,

several organs (testis, liver, tumors, blood.) were harvested at the end of treatment for further analysis. Animals were housed in cages sized and filled with appropriate litter respectfully to European ethic guidelines, with free access to tap water and food *ad libitum*, in filtered atmosphere to avoid pathogen contamination.

Males were reared in individual cages to avoid fight stress and aggressiveness. Animals were housed for 3 weeks before the beginning of any experiment. Tumor grafts were performed rapidly under sodium pentobarbital anesthesia in a warm separate room. Tumor grafts, radiotherapy and resection were performed under general anesthesia. All s.c. injections and animal handling were performed by the same technician in a separate room and all efforts were made to minimize suffering. At the end of treatment, mice were anesthetized with 8 mg/kg xylazine and 90 mg/kg ketamine injection, blood was collected by cardiac puncture and animals sacrificed by cervical dislocation.

Nude Mouse Xenograft Model

Pathogen-free, 5–7 week-old male athymic NMRI-nu (*nu/nu*) mice were purchased from Janvier Laboratories (Le-Genest-St-Isle, France). Animals were housed in solid-bottomed plastic individual cages with free access to tap water and standard food *ad libitum*.

Primary tumors were obtained after intra-testicular injection of 1×10⁷ NT2/D1 cells. Six weeks later, tumors reached the ethic volume (0.5 cm³). Primary tumors were harvested and cut into 1 mm³ pieces in order to be grafted sub-cutaneously in Nude male mice.

Six males bearing bilateral NT2/D1 grafts were daily inoculated s.c. with either vehicle, or M4 (1.0 μ g/kg bw or 10 μ g/kg bw), 5 days per week. Nude mice were pre-treated for 2 weeks before graft, tumor pieces were grafted and treatment was applied for 4 additional weeks (Figure S1). The low dose was relevant to daily children intake (0.8 μ g/kg bw/day) [25]. The tumor-take rate ranged from 95–100%. Mice weight (Figure S1) and tumor volume were monitored twice a week by caliper measurement of the length, width, and height and were calculated using the formula $V = D \cdot d^2 / 2$. At the end of treatment, tumors were removed, weighed, and fixed in 4% formalin for histological and immunohistochemical characterization (Figure S1).

Cell Culture

TCam-2 and NT2/D1 cells were respectively maintained in RPMI1640 (GIBCO) and DMEM/F12 (1:1, GIBCO) supplemented with 10% fetal calf serum (FCS, Invitrogen) and 2 mM L-glutamine and cultured in a 5% CO₂ containing atmosphere at 37°C. Briefly, cells were plated in 10% FCS containing medium for 24 h and then starved for 24 h in 0.5% charcoal-stripped FCS-containing medium without phenol red. Treatments were performed on 0.5% charcoal-stripped FCS cultured cells, plated at a density of 8×10⁴ cells per well in 6-well plates. In case of inhibitor use, the corresponding compound was added to the medium 30 minutes before M4 or E₂ treatment.

Cell Proliferation Assay

Cells were seeded in 96-well plates at a density of 1×10³ cells/well, in 0.2 ml medium supplemented with 10% FCS and 2 mM L-glutamine. They were washed with PBS (GIBCO) once they had attached and then incubated in phenol red-free medium containing 0.5% charcoal-stripped FCS for 24 h. Cells were then submitted to the indicated treatments for 48 h. At the end of the treatment, cells were counted by using an inverted microscope. Each treatment was replicated six times. For proliferation assays,

the DMSO dilution retained was similar to the one of M4 having the most important effect (1 nM M4 corresponding to $10^{-5}\%$ DMSO). However, none of the DMSO doses tested displayed proliferation stimulation effect compared to non treated cells.

Real-time PCR Analysis

Reverse transcription and real-time PCR analyses were performed as previously described [19]. Large ribosomal protein (RPLPO) encoding gene was used as a control to obtain normalized values. Primers are listed in Table S1. Assays were performed at least in triplicate, and the mean values were used to calculate expression levels, using the $\Delta\Delta C(t)$ method referring to RPLPO housekeeping gene expression. When treatments were performed, the variation of expression was measured as treated/DMSO treated cells (control).

RNA Interference

The small-interfering RNA (siRNA) duplexes for targeting GPER (Table S1) and scrambled control (SR-CL000-005) were purchased from Eurogentec (Angers, France). TCam-2 cells (8×10^4) were plated into 6-well plates, in 2 ml of medium supplemented with 10% FCS and 2 mM L-glutamine the day before transfection. Cells were transiently transfected with either GPER (200 nM of the duplex) or scrambled siRNAs by using the OligofectamineTM Reagent (Invitrogen) according to the manufacturer's instructions. After 24 h, cells were washed with PBS and the medium was replaced with phenol red-free RPMI supplemented with 0.5% charcoal-stripped FCS and 2 mM L-glutamine. 24 h later, cells were treated in phenol red-free and 0.5% stripped FCS RPMI and harvested for further analyses. Efficacy of RNA interference is presented in Figure S2A.

Western Immunoblotting

Western blot were performed as described previously [19]. The following primary antibodies were used: anti-ER α 36 (diluted 1:5000, Cell Applications, San Diego, USA), anti-DNMT3A (diluted 1:500, Active Motif n°39897, La Hulpe, Belgium), anti-DNMT3B (diluted 1:1000, Active Motif n°39899), anti-DNMT3L (diluted 1:5000, Active Motif n°39907) and anti-phospho CREB (1:1000; Epitomics #1113-5, Montrouge, France). The anti-alpha tubulin antibody (1:5000, sc2004, Santa Cruz Biotechnology Inc., Santa Cruz, USA) was used as a control. The membranes were developed with ECL detection reagent using chemiluminescence (Amersham, Orsay, France). Protein quantification was performed using the Quantity One Geldoc software (Biorad, Marnes-la-Coquette, France).

Transient Transfection and Establishment of Stable Cell Line

TCam-2 cells were transfected with the empty expression vector or the ER α 36-specific shRNA expression vector kindly provided by Dr Wang ZY (Creighton University medical school, Omaha, USA) using the ExGen500 *in vitro* transfection reagent (Euro-medex, France) as described previously [19]. Efficacy of shRNA knock-down is shown in Figure S2B.

Statistical Analysis

Data were summarized as the mean $\pm$ s.e.m. Proliferation analysis data from each dose group were compared by analysis of variance (one-way ANOVA) followed by the Bonferroni multiple procedure with SPSS software (SPSS Inc., Chicago, USA). Real-time PCR and western blot experiments results were analyzed as follows: variance analysis of treated *versus* control cells was

performed using Dunnett's test for multiple comparisons. Differences in which *P* was less than 0.05 were considered as statistically significant.

Results

The M4 Alkylphenol Mix Stimulates Testicular Germ Cell Tumor Growth *in vitro* and *in vivo*

To test if an alkylphenol mix such as M4 could act as a proliferation inducer in seminoma-derived (TCam-2) and embryonal carcinoma (NT2/D1) cell lines, the growth rate of TCam-2 and NT2/D1 cells was determined by counting the number of cells exposed to different concentrations of M4. After a 24 h serum deprivation, cells were treated for 48 h with M4 decimal dilutions starting from 1.0 μ M to 0.01 pM and counted by using an inverted microscope. M4 stimulated TCam-2 and NT2/D1 proliferation whatever the dose tested (Figure 1A). The dose-response curves of these cells to M4 exhibited non-monotonic or biphasic pattern. When compared to vehicle exposure, a maximum proliferation increase was observed for cells treated in the nanomolar range, which corresponds to environmental doses. Therefore, the dose of 1.0 nM was retained for further analyses.

To assess the effect of *in vivo* M4 exposure in male (androgenic; low estrogenic) hormonal context, a NT2/D1 derived germ cell tumor xenograft model was established (see material and methods section for details). Figure 1B shows that, at the end of treatment, tumor weight was significantly higher in M4 (1 μ g/kg bw) *versus* vehicle injected mice. These data confirmed that an exposure to a low dose of M4, which corresponds to human daily intake [25] stimulates embryonal carcinoma growth *in vivo*. Notably, It is noteworthy that NT2/D1 cells knocked down for ER α 36 are not viable. They can be selected and isolated after shRNA transfection but do not divide and therefore cannot be amplified for *in vivo* injection or used *in vitro* for proliferation assays. We also tried twice to obtain sh36-TCam-2 derived tumors after intra testicular injection of 1×10^6 , 5×10^6 , 1×10^7 or 2×10^7 cells but we never observed any tumor take, even 10 weeks after injection.

M4 Triggers CREB Phosphorylation Through an ER α 36 Dependent Pathway

Since we previously demonstrated that E₂ and E₂BSA both trigger CREB phosphorylation and in TCam-2 cells through GPER-ER α 36 dependent mechanisms [19], we tested the potential estrogenicity of M4 by assessing phosphorylated CREB level. Western blot analysis clearly indicated an increase of CREB phosphorylation level (Figure 2A) after a 20 minute exposure to 1.0 nM M4. Several membrane receptors such as EGFR or GPER have been previously described to trigger estrogen-like signaling in various cancer cell lines [19,26]. However, CREB phosphorylation induction was still observed in scrambled siRNA, EGFR-targeted transfected cells, suggesting that EGFR is not required for M4 signaling in TCam-2 seminoma-derived cells (data not shown). In order to check GPER involvement in M4 signaling, we used GPER agonists or antagonists in several contexts: scrambled siRNA transfected cells and their GPER-targeted counterparts were exposed to vehicle, 1.0 nM M4, 1.0 nM E₂, 100 nM G1 (a GPER agonist) or 100 nM G15 (a GPER antagonist) for 20 minutes. M4, G1 and G15 appeared to be powerful inducers of CREB phosphorylation whereas E₂ displayed lower efficiency in control cells (Figure 2B). In GPER-knocked down cells, M4, G1 and G15 could still stimulate CREB phosphorylation, even if this effect was milder, demonstrating that GPER activity was not fully required. As previously demonstrated, GPER knockdown pre-

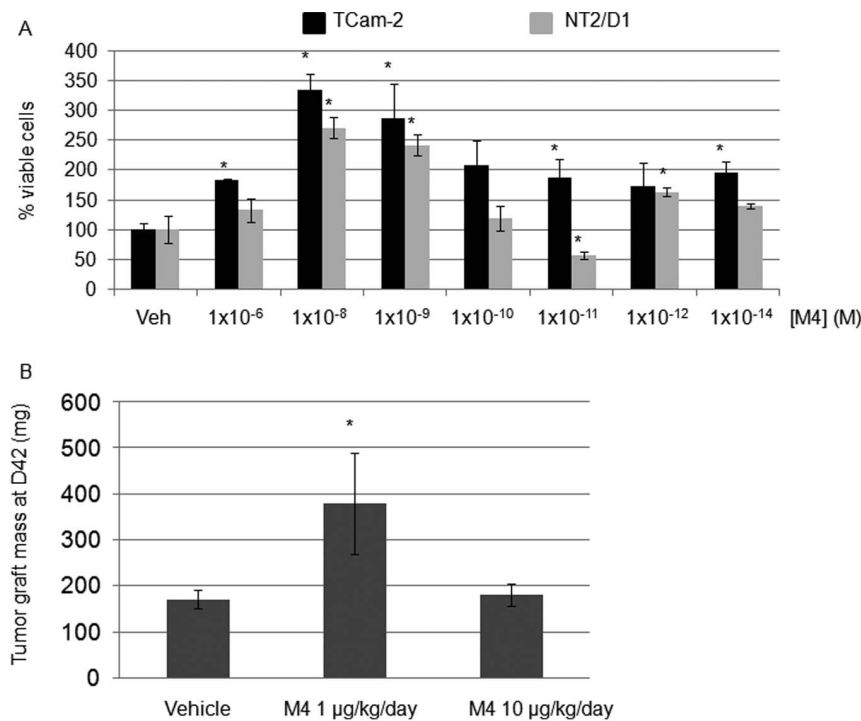


Figure 1. Alkylphenols stimulates TCam-2 and NT2/D1 cell proliferation *in vitro* and *in vivo*. (A) Cells were treated by M4 concentrations ranging from 1×10^{-6} to 1×10^{-14} M or vehicle for 48 h and counted under inverted microscope (see text for details). The DMSO dilution retained and indicated as “vehicle” was similar to the one of M4 having the most important effect: 1 nM M4 corresponding to 0.00001% DMSO. Notably, none of the DMSO doses tested (ranging from 0.01% to $10^{-12}\%$) displayed proliferation modulating effect compared to non treated cells. Values indicated are the mean of 6 counts $\pm$ SEM. * $P < 0.05$ vs vehicle treated. (B) Exposure to M4 alkylphenol mix stimulates tumor growth in nude male mice, injected 5 times a week with a M4 dose corresponding to human exposure or with a ten-fold higher dose. Tumors were weighed after a 6-week long treatment duration. Indicated values are the mean of at least 10 tumor weights $\pm$ SEM. doi:10.1371/journal.pone.0061758.g001

vented E₂-dependent CREB phosphorylation, which suggests that the mechanisms involved in M4 signaling do not fully mimic those of estrogens [19]. Since G1 and G15 can act as ER α 36 agonists and stimulate non genomic signaling pathways [21,27] the results presented in Figure 2B also suggest that ER α 36 in addition to GPER could trigger M4 dependent CREB phosphorylation.

ER α 36 Mediates M4 Induced Cell Proliferation and CREB Phosphorylation in TCam-2 Cells

Normal human germ cells from the testis, malignant germ cells and their derived cell lines TCam-2 or NT2/D1 do not express the long form of ER α , ER α 66. Nevertheless, they express the ER α 36 isoform, which is necessary for mitogenic response to estrogens [19]. To address the hypothesis of ER α 36 involvement in M4 proliferative effects, we performed dose-response experiments in the neo-TCam-2 and sh36-TCam-2 stable cell lines, which contain respectively, an empty plasmid or the corresponding vector expressing ER α 36 targeted shRNA [19]. Figure 3A shows that a 48 h exposure to 1.0 nM M4 could stimulate neo-TCam-2 cell proliferation whereas this mitogenic effect was not observed in sh36-TCam-2 cells. Besides, CREB phosphorylation level did not increase in M4-treated ER α 36-knocked down TCam-2 cells (Figure 3B). Both results strongly suggest that ER α 36 is required for M4 signaling in seminoma-derived cells.

Neo-TCam-2 and sh36-TCam-2 cells were pre-treated with antagonists for several signaling pathways for 30 minutes before M4 exposure: a PKC inhibitor (BIM), the PKA inhibitor H89 and the PI3K inhibitor wortmanin. BIM did not prevent M4 induced CREB phosphorylation whereas H89 totally blocked it as well as

its basal level, even in the non-M4 treated cells (data not shown). Wortmanin seemed to be the only antagonist able to prevent an M4 specific effect in neo-TCam-2, suggesting that M4 triggers ER α 36-PI3kinase-dependent CREB phosphorylation in TCam-2 cells (Figure 3B).

The key role of PI3K-dependent signaling was further confirmed in both TCam-2 and NT2/D1 cells since wortmanin pre-treatment also impaired M4-enhanced cell proliferation (Figure 3C). Noteworthy, wortmanin, by itself, seemed to trigger a mild stimulation of NT2/D1 proliferation at high doses (1 μ M to 10 μ M; data not shown). Such an effect was not observed in TCam-2 cell line.

M4 Represses DNA-methyltransferase Expression Through ER α 36 Dependent Mechanisms in TCam-2 Cells

In order to determine the transcriptional profile of TCam-2 cells exposed to M4, we performed a microarray analysis of gene expression after a 60 minutes or a 24 h treatment. TCam-2 cells were cultured for 24 h in 0.5% FCS containing medium in the presence of vehicle or 1.0 nM M4. Total RNA was extracted for global analysis of gene expression on Nimblegen microarray. Venn diagram presented in Figure 4 indicates that 1124 transcripts and 633 transcripts were up or down regulated (absolute variation factor ≥ 2 in duplicate RNA samples, $P < 0.05$) after 1 hour or 24 hours M4 exposure, respectively. Among them, 264 genes were similarly regulated in both conditions and the corresponding list was analyzed for functional classes and networks with the Ingenuity software (Tables S2, S3, S4, [28]). As expected, main networks and biological functions associated to the list of M4

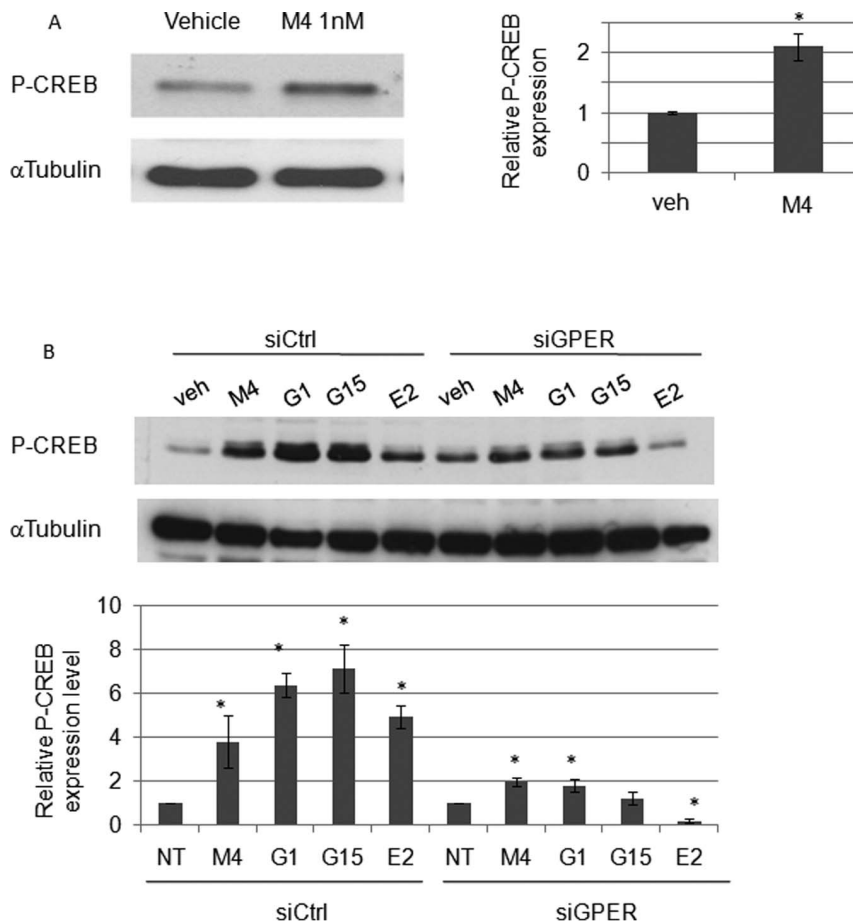


Figure 2. M4 induces CREB phosphorylation in TCam-2 cells through a GPER-independent pathway. (A) Cells were treated for 20 minutes with 1.0 nM of M4 and the level of phosphorylated CREB (P-CREB) was assessed by western-blot analyses compared to vehicle treated cells. (B) Western blot analyses of CREB phosphorylation upon exposure to 1.0 nM M4, 10 nM G1, 10 nM G15, or 1.0 nM E₂ for 20 minutes in TCam-2 cells transfected by scrambled siRNA (siCtrl), or GPER-targeted siRNA (siGPER). Alpha tubulin stands for a loading control. Quantification means $\pm$ SEM and corresponding statistical analyses from at least three similar experiments are indicated. * $P < 0.05$ vs vehicle treated. doi:10.1371/journal.pone.0061758.g002

regulated genes referred to cancer, developmental disorder, cell growth and proliferation (Tables S2 and S3). Moreover, the predicted upstream regulators were all related to estrogens (Table S4).

Among the functional classes of genes whose expression is significantly up- or down-regulated (top list provided in Table S5), we focused on those involved in epigenetic modifications which seemed related to PI3K/CREB and estrogen receptor signaling in Ingenuity sorting (Figure S3). Indeed, two of the three Dnmt3 genes display predicted CREB response elements half-sites (TGACG/CGTCA) in their promoter region (Dnmt3A: -684; -783; -1468; -2644; -3605; Dnmt3L: -2297; -2884 from the transcription start site) and therefore are good candidates for CREB-dependent expression control. Namely, the Dnmt3 gene family displayed a mild but reproducible down regulation after both 60 minutes and 24 h M4 exposures (Table 1). The results from microarray analysis were confirmed by quantitative RT-PCR (Figure 5A) and western-blot (Figure 5B) analyses. Indeed, M4 triggered a downregulation of DNMT3 expression in TCam-2 and neo-TCam-2 cells whereas such a repression was not observed in sh36-TCam-2 cells or after a 30 minute wortmanin pre-treatment (Figure 5C). Similar downregulation of Dnmt3A and Dnmt3L gene expression was observed in NT2/D1 cells (Figure 6A). Hence, M4- and PI3K-dependent repression of DNMT3B and

DNMT3L was further observed at the protein level (Figure 6B). This suggests that the ER α 36-dependent M4 signaling ending at target genes involved in DNA-methylation status could be a common feature of both seminoma and embryonal carcinoma cells.

Discussion

Although numerous chemicals are now known or suspected to have endocrine disruption effects, a relevant classification based on comprehensive understanding of their mode of action and targets is still failing. More confusing is the wide variety of cocktails detected in the environment, when trying to decipher dose-response consequences for lifelong human exposure. In the present study, we chose to focus on a well defined mix of alkylphenols - M4 -composed of 4-tert-octylphenol and 4-nonylphenol (1:30 ratio). Despite the burden of recent research on BPA which belongs to the same chemical family and exert various estrogenic effects, 4-tert-octylphenol and 4-nonylphenol are still neglected. High doses of tert-octylphenol or nonylphenol ranging from 25 to 200 mg/kg bw were previously shown to significantly decreased sperm count and quality in male mice, and affect uterine weight, vaginal opening and reproductive ability in female rats [29,30]. However, both molecules have never been associated in a realistic mixture

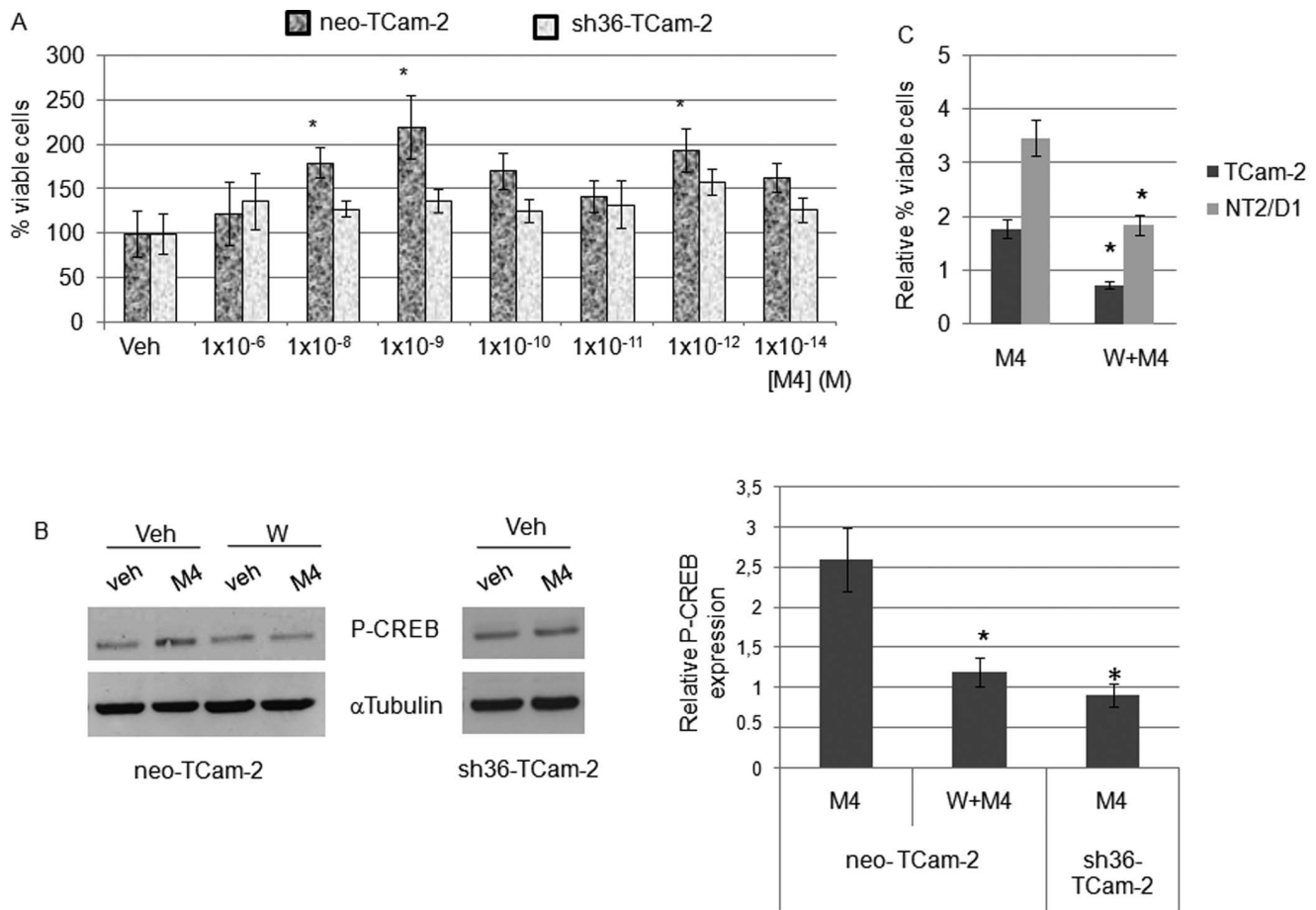


Figure 3. ERα36 mediates M4-induced PI3K-dependent proliferation and CREB-phosphorylation. (A) neo-TCam-2 or sh36-TCam-2 were previously described [-]: briefly, the neo-TCam-2 cell line was transfected with a control vector whereas sh36-TCam-2 stably expresses ERα36 targeted shRNA. Both cell lines were treated by M4 concentrations ranging from 1×10⁻⁶ to 1×10⁻¹⁴ M or vehicle for 48 h and cells were counted by using an inverted microscope. Values indicated are the mean of 6 counts ± SEM. *P<0.05 vs vehicle treated. (B) neo-TCam-2 or sh36-TCam-2 cell lines were treated for 20 minutes with 1.0 nM of M4 alone or after a 30 minute pre-treatment with 0.2 μM wortmanin. The level of phosphorylated CREB was then assessed by western-blot analysis and compared to vehicle treated cells. Quantification means ± SEM and corresponding statistical analyses from at least three similar experiments are indicated. *P<0.05 vs vehicle treated. (C) TCam-2 and NT2/D1 cells were pre-treated with 0.2 μM wortmanin for 1 hour before a 48 hour exposure to 1 nM M4. Cells were then counted by using an inverted microscope. Quantification means ± SEM. *P<0.05 vs M4 treated. doi:10.1371/journal.pone.0061758.g003

mimicking daily human contamination from household products, cosmetics and food. Here, estrogen-like mechanisms of action were addressed in a model of TGCT lacking the long form of ERα receptor (ERα66). As in the case of 17β-estradiol or its BSA-coupled counterpart, M4 doses ranging from 10 nM to 0.1 nM stimulated both seminoma and embryonal carcinoma cell pro-

liferation in a non monotonic dose-response manner. Moreover, we observed a positive impact of M4 exposure on tumor growth in a TGCT xenograft nude mouse model after a treatment corresponding to human intake (1 μg/kg bw) [25]. No stimulating effect was detected after exposure to the higher dose (10 μg/kg bw), suggesting (i) that tumor growth in xenografted mice could



Figure 4. Venn diagram of M4 regulated genes as revealed by microarray analysis in TCam-2 cells. 860 genes and 369 genes were regulated by at least two-fold (p<0.05) after 1 hour or 24 hour 1 nM M4 treatment, respectively. 264 genes were commonly regulated at both exposure times. doi:10.1371/journal.pone.0061758.g004

Table 1. DNMT3 gene expression level variation after either 1 hour or 24 h M4 exposure as observed in microarray analysis in TCam-2 cells.

Sequence ID	Gene name	1 h M4 exposure		24 h M4 exposure	
		Fold	p value	Fold	p value
NM_175630	DNMT3A	-2.25	0.03	-2.16	0.01
NM_006892	DNMT3B	-2.65	0.01	-3.19	0.01
NM_013369	DNMT3L	-2.70	0.001	-2.96	0.001

doi:10.1371/journal.pone.0061758.t001

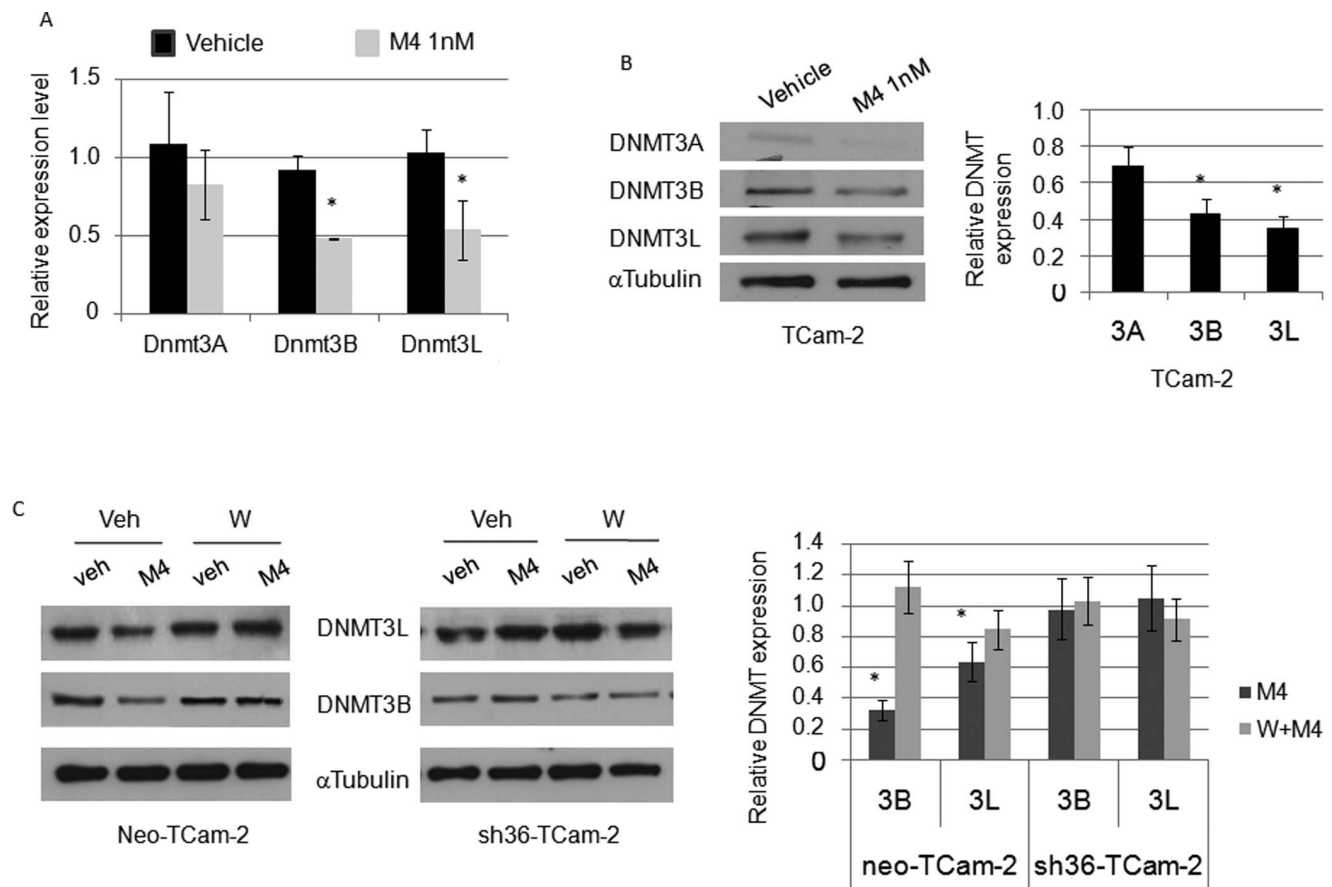


Figure 5. M4 represses the expression of Dnmt3A, Dnmt3B and Dnmt3L. (A–B). TCam-2 cells were treated for 24 hours with 1.0 nM of M4 and Dnmt3A, 3B or 3L expression was assessed by real-time PCR (A) or western-blot analysis (B). (C) M4-dependent Dnmt3B and Dnmt3L down-regulation was also observed in neo-TCam-2 cells but was impaired after a 30 minute pre-treatment with 0.2 μ M wortmannin or in sh36-TCam-2 cells. Quantification means $\pm$ SEM and corresponding statistical analyses from at least three similar experiments are indicated. * P <0.05 vs vehicle treated. doi:10.1371/journal.pone.0061758.g005

respond to M4 in a non-monotonic way, as observed for in vitro cell proliferation or (ii) that a mild toxicity could appear after exposure to high doses of the mix. Taken together, these results suggest that alkylphenol exposure may on the one hand, alter normal germ cell multiplication and differentiation during development [12,31] through mutagenic or clastogenic mechanisms at high doses [32,33] as described by others and on the other hand, elicit neoplastic germ cell proliferation at low doses, as shown in this study.

Therefore we investigated the rapid non-genomic transduction pathways potentially involved. Whereas estradiol and BPA were previously shown to bind and exert such mitogenic effects through GPER in both SKBR3 breast cancer cells and JKT-1 seminoma derived cells [15,34], we demonstrated that M4 acts mainly via an ER α 36-dependent pathway. Indeed, we evidence here that M4 triggers PI3K activity and CREB phosphorylation. Nevertheless, preliminary data indicate that both GPER and ER α 36 may activate downstream signaling such as src phosphorylation and thus modulate the expression of M4 target gene subclasses as well as cell proliferation (A. Chesnel, personal communication). Since GPER was shown to partially govern ER α 36 expression in our TCam-2 model [19] and may collaborate with ER α 36 for estrogenic activities in other cancer cell lines [26,35], it would be relevant to test the participation of ER α 36 in alkylphenol

response in hormone-sensitive cancers such breast or prostate cancers.

The microarray analysis performed in order to describe the gene expression pattern of M4-treated TCam-2 cells, indicated that several epigenetic modification enzymes encoding genes are affected. We focused on M4-dependent down-regulation of DNMT3 expression because (i) other estrogeno-mimetic such as genistein and resveratrol or anti-androgenic compounds such as vinclozolin that are present in food have been previously demonstrated to modulate tumor suppressor gene expression through epigenetic mechanisms [36,37], (ii) DNMT3 proteins have been shown to be involved in germ cell proliferation and differentiation control during a developmental window when neoplastic germ cells (CIS) are believed to emerge [38], (iii) polymorphism of these genes is clearly associated with gastric and breast cancer, as well as ovarian endometriosis susceptibility [39–41]. Indeed, “Ingenuity sorting” clearly classifies DNMT3 down-regulation into functional networks involved in cancer progression and cell proliferation downstream estrogen receptors and estrogens (Tables S2, S3, S4). Moreover, several studies point out the key role of DNA methylation in testicular tumor initiation, progression and resistance to chemotherapy [42–46], highlighting the importance for examining carefully which upstream compounds or regulation factors are able to modulate DNMT expression and activity.

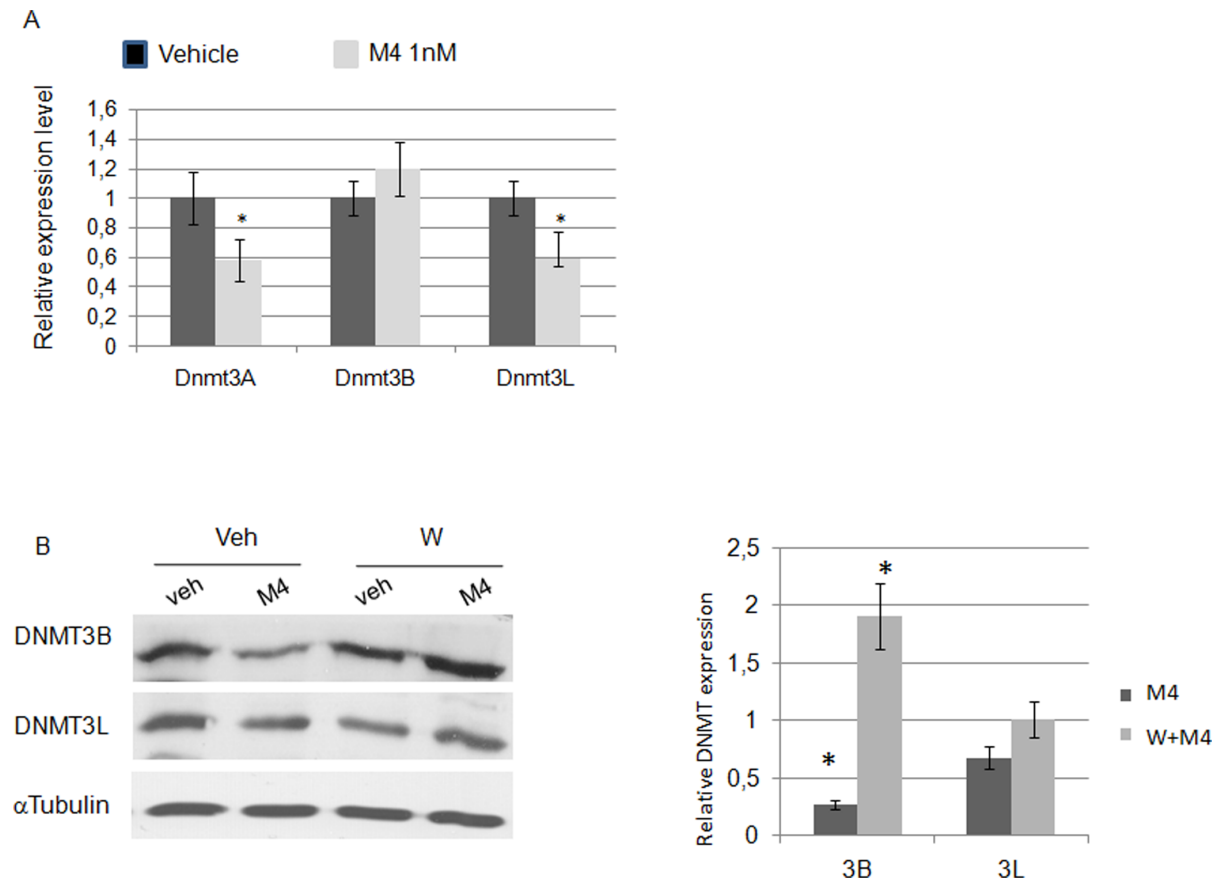


Figure 6. M4 modulates the expression of Dnmt3A, Dnmt3B and Dnmt3L through PI3K-dependent signaling. (A–B). NT2/D1 cells were treated with 1.0 nM of M4 and Dnmt3A, 3B or 3L expression was assessed by real-time PCR (A) or western-blot analysis without or after a 30 minute pre-treatment with 0.2 μ M wortmanin (B). Quantification means $\pm$ SEM and corresponding statistical analyses from at least three similar experiments are indicated. * $P < 0.05$ vs vehicle treated. doi:10.1371/journal.pone.0061758.g006

Hence, our results indicate that either wortmanin treatment or ERalpha36 knockdown can impair M4-dependent Dnmt3 repression while ERalpha36 expression appears to be necessary for M4-dependent enhanced proliferation.

CREB target gene database detects CREB response elements half-sites in Dnmt3A and Dnmt3L promoters and further suggest that both gene could be a target for the PI3K/CREB dependent pathway [47]. Moreover, Hervouet and coworkers [48] demonstrated that DNMT3B and 3A can physically interact with several transcription factors involved in proliferation control, such as CREB, FOSB, KLF12, EGR1 or JUN, which were proposed to direct methylation on specific gene promoter sequences. DNMT3 can also regulate each other expression through promoter methylation [38].

Finally, since ERalpha36 promoter is located into the first intron of ESR1 gene, balanced expression of either ERalpha36 or ERalpha36 could be regulated by differential methylation. This point was already addressed by others who demonstrated that downregulation of DNMT3A and DNMT3B led to regulation of ESR1 or ESR2 via promoter DNA aberrant methylation in acute myeloid leukemia, endometriosis, prostate and ovarian cancer [49–52].

Endocrine disruptors such as alkylphenols are also suspected to alter germ cell epigenetic reprogramming during fetal and perinatal development, thus triggering long-term disruption of gene expression which, in turn, could be a main risk factor for

hormone-dependent cancers. Anway and colleagues [37] also found DNMT3A and DNMT3L isoforms to be repressed in the testis after embryonic exposure to the endocrine disruptor vinclozolin. This commonly used fungicide suspected to have antiandrogenic effects triggered transgenerational epigenetic reprogramming associated with increased adult onset diseases, namely prostate disease, testis abnormalities, and tumor development [53]. Therefore, it could be relevant to address the effects of delayed consequences of a M4 exposure.

Surprisingly, DNMT3L expression was clearly detected at both mRNA and protein level in TCam-2 seminoma-derived cells by using commercially available antibody contrary to previous work on human biopsies using homemade polyclonal antibody and indicating that DNMT3L expression was restricted to embryonal carcinoma [54]. In the germ cell lineage, DNMT3L is involved in *de novo* retrotransposon methylation and appears to be a signature of spermatogonia stage [55]. Therefore, the expression of DNMT3L could be a hallmark of undifferentiated stage.

DNMT3A and DNMT3B are usually described as enzymes responsible for the establishment of specific CpG dinucleotides methylation essential for embryonic development and gene repression at the time of implantation [56]. However, a growing number of evidences support the hypothesis for their contribution in the maintenance of DNA methylation [57]. DNMT3L also participates in a complex coupling H3K4 methylation and DNMT3A-dependent DNA methylation, thus modifying chroma-

tin accessibility [58]. Therefore, M4-dependent downregulation of these enzymes could trigger DNA hypomethylation and chromatin opening, thus leading to aberrant gene expression pattern, which can be maintained transgenerationally. These observations are of particular interest in the field of testicular germ cell carcinogenesis since CIS are proposed to originate in non-differentiated primordial germ cells displaying a gene expression pattern of pluripotency. This could be also relevant in the context of testis tumor growth since lifelong M4 exposure may maintain a population of non-differentiated/highly proliferative cancer cells in the tumor.

Taken together, these data suggest that M4 exposure may elicit a positive feedback loop beginning at ER α 36 activation, triggering PI3K-dependent CREB phosphorylation, ending on Dnmt3 repression which, in turn, could stimulate and maintain a high level of ER α 36 expression and rapid proliferation. Epigenetic regulation of ESR1 locus remains to be carefully examined in various cell contexts in order to address such a hypothesis.

Supporting Information

Figure S1 Characterization of testis tumor xenograft model in nude mice. Germ cell tumor xenograft models were first established after intra testicular injection of 1×10^7 TCam-2 or NT2/D1 cells in 0.9% NaCl in nude mice. Tumors developed to approximately 0.5 cm^3 in 6 weeks. MRI imaging was used (Spectro-imageur Bruker Biospec Avance 24/40; 2.4 teslas magnetic field) to confirm the presence of tumors into the scrotum. Tumors were harvested and seminoma or embryonal carcinoma identity was attested by histological and immunohistochemical analyses. The slices presented are hematoxylin/eosin/safran colorations of TCam-2 derived or NT2/D1 derived tumors. Tumor tissue was harvested and subcutaneously (s.c.) grafted in the inguinal pit of male nude mice. Because NT2/D1, but not TCam-2 derived tumors developed, we focused on the NT2/D1 model to examine the effects of M4 on tumor growth. Nude mice were s.c. implanted with 1–2 mm^3 tumor pieces harvested from previously grown (0.5 cm^3) NT2/D1 tumor (third passage). For alkylphenol assay, M4 or vehicle treatment was injected five days per week subcutaneously in male nude mice 2 weeks before, and 4

weeks after tumor graft in order to mimic everyday life contamination (see text for details). Bars are $100 \mu\text{m}$ long. (TIF)

Figure S2 Real time PCR analysis (A) and western blot (B) showing the efficacy of GPER- or ER α 36 -silencing in TCam-2 cells, respectively. (TIF)

Figure S3 General scheme of Ingenuity software analysis showing the regulation network which involves DNMT3 gene family. (TIF)

Table S1 Primer and siRNA sequences. (DOCX)

Table S2 Main results from Ingenuity analysis: top five networks in which M4 regulated genes are involved. (DOCX)

Table S3 Main results from Ingenuity analysis: biological functions in which M4 regulated genes are involved. (DOCX)

Table S4 Main results from Ingenuity analysis: Top five predicted upstream regulators of M4 regulated genes. (DOCX)

Table S5 Top list of the 20 genes deregulated by M4 after 1 hour or 24 hour exposure. (DOCX)

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Author Contributions

Corrected the manuscript: SP SF. Conceived and designed the experiments: HD AC SP. Performed the experiments: HA AC SP FP HD. Analyzed the data: AC SP FP HD. Wrote the paper: AC HD.

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