Fetal and Neonatal Exposure to the Endocrine Disruptor Methoxychlor Causes Epigenetic Alterations in Adult Ovarian Genes

Aparna Mahakali Zama and Mehmet Uzumcu

Department of Animal Sciences, School of Environmental and Biological Sciences, Rutgers, The State University of New Jersey, New Brunswick, New Jersey 08901-8525

Exposure to endocrine-disrupting chemicals during development could alter the epigenetic programming of the genome and result in adult-onset disease. Methoxychlor (MXC) and its metabolites possess estrogenic, antiestrogenic, and antiandrogenic activities. Previous studies showed that fetal/neonatal exposure to MXC caused adult ovarian dysfunction due to altered expression of key ovarian genes including estrogen receptor (ER)-β, which was down-regulated, whereas ERα was unaffected. The objective of the current study was to evaluate changes in global and gene-specific methylation patterns in adult ovaries associated with the observed defects. Rats were exposed to MXC (20 mg/kg d or 100 mg/kg d) between embryonic d 19 and postnatal d 7. We performed DNA methylation analysis of the known promoters of ERα and ERβ genes in postnatal d 50–60 ovaries using bisulfite sequencing and methylation-specific PCRs. Developmental exposure to MXC led to significant hypermethylation in the ERβ promoter regions (P < 0.05), whereas the ERα promoter was unaffected. We assessed global DNA methylation changes using methylation-sensitive arbitrarily primed PCR and identified 10 genes that were hypermethylated in ovaries from exposed rats. To determine whether the MXC-induced methylation changes were associated with increased DNA methyltransferase (DNMT) levels, we measured the expression levels of Dnmt3a, Dnmt3b, and Dnmt3l using semiquantitative RT-PCR. Whereas Dnmt3a and Dnmt3l were unchanged, Dnmt3b expression was stimulated in ovaries of the 100 mg/kg MXC group (P < 0.05), suggesting that increased DNMT3B may cause DNA hypermethylation in the ovary. Overall, these data suggest that transient exposure to MXC during fetal and neonatal development affects adult ovarian function via altered methylation patterns. (Endocrinology 150: 4681–4691, 2009)

Recent reports describing the role of epigenetic mechanisms in the fetal and neonatal basis of adult disease have highlighted the concerns of the scientific community. The emerging threat of adverse environmental conditions such as improper nutrition, stressors, and endocrine-disrupting chemicals (EDCs) and the consequent damage to the epigenome resulting from such exposures has serious implications on human health. EDCs are of specific concern among the detrimental environmental factors because they are widespread in the environment (1–6). A sex- and stage-specific exposure to EDCs during early development could alter the epigenetic programming of the genome and result in adult-onset disease (4, 7). Importantly, these effects can be epigenetically transmitted to the next generation (1, 5, 7). Furthermore, the observations that endocrine disruptors alter reproductive behaviors in generations that are not directly exposed to these compounds raise the possibility that EDCs have evolutionary and trans-population health implications (8).

Epigenetic mechanisms such as DNA methylation, histone modifications, and noncoding RNAs are heritable elements of DNA that influence gene expression without changing the gene sequence (9). DNA methylation is one of the most commonly studied epigenetic mechanisms in-
involved in the regulation of gene expression and chromosomal stability (10, 11). Alterations in DNA methylation patterns are implicated in several complex diseases including cancer (12–14). However, the full impact of epigenetic mechanisms in disease etiology is not completely understood.

Among the functions of the adult ovary are the interdependent processes of folliculogenesis and steroidogenesis, which are required for ovulation and corpus luteum formation. These processes occur through a dynamic bidirectional communication between the germ line and the supporting somatic cells (granulosa cells and theca cells). Many critical events in early follicular development such as oocyte nest breakdown, primordial follicle assembly, and the initial primordial to primary follicle transition occur between embryonic day (E) 19 and postnatal day (PND) 7 in female rats. When exposed to EDCs during their development, other organ systems including the mammary gland, prostate, uterus, and testis develop adult dysfunction associated with alterations in DNA methylation patterns (1, 4, 7, 15). It is likely that similar defects could occur in the ovary (16). Importantly, female germ cell epigenetic reprogramming (i.e. remethylation of DNA) occurs during early ovarian development and could be perturbed by exposure to EDCs. Therefore, it is imperative to explore the possibility that EDCs lead to such epigenetic alterations in the ovary.

Two stages of early development, embryogenesis and gametogenesis, have heightened DNA methylation activity wherein DNA methylation patterns are actively erased and reestablished (epigenetic reprogramming) (17). Normal mammalian development requires the action of DNA methyltransferases (DNMTs) for the de novo establishment (DNMT3A and B) and maintenance (DNMT1) of DNA methylation within the genome. In addition, DNMT3L (DNMT3-like) that has similar sequence as DNMT3A and DNMT3B but lacking in enzymatic activity functions as a regulator of DNMT3A and DNMT3B (18, 19). The expression level of these enzymes is highly regulated and peaks during specific stages of postnatal ovary development (20). Therefore, measurement of the levels of DNMTs can be used to assess the epigenetic status of the ovary.

Methoxychlor (MXC) was used in the United States as a replacement for dichlorodiphenyltrichloroethane for more than 50 yr and is a well-studied EDC (21). Whereas the use of MXC is currently restricted within the United States, its use in the rest of the world is unknown. The levels of MXC in the environment range from 160 mg/liter, or 160 ppm (in waters downstream from MXC sprayed areas), to 0.1 ng/kg-d, the latter being the Food and Drug Administration’s calculated average daily intake of MXC in adults (22). Studies of MXC’s effects in the adult ovary have shown that antral follicles are particular targets for its adverse actions (23, 24), which are likely to be through an estrogen receptor (ER)-mediated pathway (25). MXC along with its metabolites possesses estrogenic, antiestrogenic, and antiandrogenic activities. EDCs with similar activities are common in the environment; therefore, MXC represents a model EDC (21). The MXC metabolite 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) possesses the ability to bind to both ER subtypes and acts as an estrogen agonist when bound to ERα or an antagonist when bound to ERβ (26–28).

ERα and ERβ are nuclear receptors as well as steroid receptors that act as ligand-regulated transcription factors. Models of cooperation as well as competition between the two ERs have been proposed (29–32). The N-terminal A/B domain is the most variable region of ERα and ERβ sharing less than 20% amino acid identity in this region, indicating that this domain may contribute to ER subtype-specific actions on target genes. Both nuclear ER subtypes are expressed in the ovary during development as well as adulthood. Gene deletion studies in mice show that ERβ, expressed in the granulosa cells, plays a more significant role locally in the ovary. ERβ is known to be essential for FSH-directed granulosa cell differentiation as well as for LH responsiveness (33, 34). ERα, expressed in the theca cells, is necessary for steroidogenesis and estrogen-mediated feedback in the hypothalamus and pituitary (35).

A recent study investigated the effects of transient exposure to environmentally relevant low and high doses of MXC during the periods of fetal and neonatal ovarian development (36). This report investigated several reproductive parameters including morphology and levels of key regulatory markers in the ovary. The high dose of MXC caused accelerated entry into puberty and the first estrus, increased irregular cyclicity, reduced litter sizes, and caused premature reproductive aging. In addition, the high dose of MXC collectively reduced the superovulatory response and serum progesterone and increased serum LH levels. We also found altered follicular composition, specifically, an increase in the number of preantral and early antral follicles and a reduced number of corpora lutea. In addition, this ovarian dysfunction was associated with a reduction in levels of ovarian ERβ expression along with reduced LH receptor and cytochrome P450 side-chain cleavage (36).

The objective of the current study is to evaluate changes in global and gene-specific methylation patterns that are associated with dysfunction in adult ovaries after a transient fetal and neonatal exposure to MXC.

Materials and Methods

Animals

Fischer (CDF) inbred rats were obtained from Charles River Laboratories (Wilmington, MA) to generate timed-pregnant fe-
mals. The inbred strain was used because it has minimal polymorphisms, which facilitates the detection of treatment effects. The animals were maintained in a room with controlled illumination (lights on 0700–2100 h), temperature (26–28 °C), and humidity (30–70%). Rats were given a soy-free diet rat chow (SV01, Lab Diet; PMI Nutrition International LLC, Brentwood, MO) and tap water ad libitum. The soy-free diet was given to reduce the quantity of phytoestrogens in the feed and to minimize background-level exposure to estrogenic compounds (37). All procedures were carried out in accordance with the guidelines of the Rutgers University Animal Care and Facilities Committee.

Treatments

Timed-pregnant females received two different treatment dosages of MXC (Sigma, St. Louis, MO): 20 μg/kg/d (low dose MXC) and 100 mg/kg/d (high dose MXC) in 1 ml/kg vehicle. Control animals received only vehicle (dimethyl sulfoxide-sesame oil; 1:2; control). The rats were treated for 12 d between E19 and PND7. On E19, three to five dams were randomly assigned to each treatment group. To precisely control the dosage, we used parenteral routes. The treatment was administered to the dams via an ip injection. The day of birth was designated as PND0. The litter size was culled to eight to ten offspring/dam. The female offspring were treated via sc injection daily from PND0 to PND7. The first injection was within the first 24 h after birth. No significant differences were observed in the weight of the animals at collection, suggesting no overt toxicity.

Tissue collection

On the proestrus day of the third regular cycle, PND50–60 animals were killed by decapitation and ovaries were collected. One ovary from each animal was snap frozen and stored at −80 °C until further use, whereas the other ovary was fixed in Bouin’s solution. The frozen ovary was bisected and one half used for DNA and RNA extractions, respectively. Ovaries from five to six animals per treatment group were collected. At least three female offspring belonging to different litters were used in the following experiments/assays, except in control and 20 μg/kg/d MXC groups (see Fig. 4), in which two ovaries for each were used.

Histology and immunohistochemistry

Histology and immunohistochemistry was performed as previously described (36). Briefly, sections were dewaxed in Citrisolv (d-limonene; Fisher catalog no. 04-355-121) and rehydrated in PBS for 10 min. After the antigen retrieval and blocking procedures, primary antibodies, anti-ERα, or anti-ERβ (rabbit polyclonal antibodies, catalog no. sc-542 or PA1 310B; Santa Cruz Biotechnology, Santa Cruz, CA, or Affinity Bioreagents, Golden, CO, respectively) were diluted to 1:100 or 1:50 in 1 ml/kg vehicle. Negative control sections were treated identically, except that the primary antibody was replaced with PBS (data not shown). Sections were observed under an Eclipse E800 microscope with epifluorescence attachments (Nikon, Tokyo, Japan). Images were acquired with a Nikon DXM1200F camera with ACTI software (version 2), and minimal and equal adjustments for brightness on all images were made with Photoshop CS (Adobe, San Jose, CA). As previously described by Armenti et al. (36), mean staining intensity per unit area of selected structures for each marker was determined using Image J software (National Institutes of Health, http://rsb.info.nih.gov/ij/). The polygonal selection tool of the software was used to select the specific structures. The mean staining intensity was determined for granulosa cells (ERβ) or theca cells (ERα) of each follicle, excluding oocyte and antral space, in randomly selected preantral/early antral stage follicles for ERβ and large antral follicles for ERα. At least five follicles per animal (n = 3, total of 15 follicles per treatment group) were quantified in this manner.

Methylation-sensitive arbitrarily primed PCR

(AP-PCR)

Global changes in the DNA methylation pattern of the genome of MXC-treated rats were identified by AP-PCR as previously described (1). Briefly, 4 μg of genomic DNA were digested with 10 U/μl each of Rsal alone (R), Rsal and methylation-sensitive HpaII (H), or Rsal and methylation-insensitive MspI (M) overnight at 37 °C. This was followed by PCR using 10 primer sets designed to amplify methylation sites, as previously described (1, 38). PCR products were separated using PAGE and visualized by SYBR green staining (Invitrogen). Hypo- or hypermethylation was determined by the relative band intensity using Kodak 1D Image Analysis software (Eastman Kodak Digital Sciences, New Haven, CT). The H-digested PCR products that were differentially amplified between control and MXC groups were isolated, reamplified, cloned (pGEM-T Easy Vector system; Promega, Madison, WI), and sequenced. The identity and chromosomal location was determined by using the basic local alignment sequence tool (BLAST; National Center for Biotechnology Information, United States National Library of Medicine, Bethesda, MD) in the rat Ensembl database.

Bisulfite treatments

Genomic DNA (5 μg) was digested with Rsal (10 U/μl) at 37 °C overnight and then treated with bisulfite solution using a commercially available kit (EZ-DNA methylation kit; Zymo Research, Orange, CA) according to the manufacturer’s instructions. After purification, the bisulfite-converted DNA was used as a template for bisulfite-sequencing PCR (BSPCR) and methylation-specific PCR (MSPCR).

BSPCR

Sequence-specific primers to amplify the CpG-rich regions of interest were designed using the MethPrimer program (www.urogene.org/methprimer). PCR products were amplified and cloned (pGEM-T Easy; Promega). Clones were selected through blue-white screening. Five to eight clones were sequenced per animal per treatment (Genewiz, South Plainfield, NJ) to characterize the methylation status of the CpG sites in ERα and pregnancy-associated plasma protein-A (PAPP-A). Direct sequencing was conducted for ERβ with the same primers as used for PCR amplification. The peak heights were used to calculate the levels of methylation (C/C+T×100) at each predicted CpG.
been previously described, and all primer sets were adapted to rat
malized to the
levels of
Chr, Chromosome.

### MSCPFR

Due to lack of robust bisulfite sequencing primers in the region of interest of rat ERβ exon 0N, methylation levels were measured using MSCPFR. Primers were designed to amplify unmethylated alleles (UM) and methylated alleles (M) from both ERβ and Hprt (housekeeping gene) promoters. Levels of methylation were calculated based on the ratio of intensity of amplimers from PCRs amplifying methylated to unmethylated alleles as normalized to the Hprt amplicons. Primer sequences and conditions are presented in supplemental Table 1, published as supplemental data on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org.

### Semiquantitative RT-PCR for analysis of mRNA levels of Dnmt3a, Dnmt3b, and Dnmt3l

Two micrograms of RNA (extracted by the Trizol method; Invitrogen) were reverse transcribed using the ABI high-capacity-cDNA synthesis system (Applied Biosystems Inc., Foster City, CA). Samples were diluted to 20 ng and used for PCR. Primers to amplify Dnmt3a, Dnmt3b, Dnmt3l, and Hprt have been previously described, and all primer sets were adapted to rat Dnmts (details are included in Table 1). NIH Image J was used to quantitate the gel band intensities, and all values were normalized to the Hprt values. Samples were collected from three to four animals per treatment group.

### Data analysis

The data were analyzed using one-way ANOVA for all experiments except for percent methylation of ERβ promoter region –625 to –392, in which two-way ANOVA was used. Statistically significant differences were determined using the Dunnett’s test for comparing to the vehicle-treated control or the Bonferroni test for multiple comparisons. GraphPad Prism graphing and analysis software (version 4a; GraphPad Software, Inc., San Diego, CA) was used for all statistical analyses. A statistically significant difference was confirmed at $P < 0.05$.

### Results

**ERα expression is unaffected by MXC exposure, whereas ERβ expression is down-regulated**

Previous studies from our laboratory demonstrated that MXC exposure during fetal and neonatal periods inhibits the levels of ERβ but has no effect on ERα in adult females. To confirm our previous observations (36) and also assess the status of estrogen receptors in adult ovarian follicles, we conducted immunohistochemical evaluation of ERα and ERβ expression. ERα was found predominantly in theca and interstitial cells surrounding all stages of follicles with intense expression in the corpora lutea of control ovaries (shown in large antral follicles, Fig. 1A). Mean staining intensity in theca cells was quantified using Image J software. Control ovaries were found to have $24.13 \pm 13.34$ (arbitrary intensity value; mean $\pm$ s.d.), and MXC treatment did not significantly alter the expression of ERα, as shown in Fig. 1, B and C ($23.87 \pm 7.39$ in 20 $\mu$g/kg treated ovaries and $19.33 \pm 6.96$ in 100 mg/kg treated ovaries). On the other hand, ERβ expression was predominantly in the granulosa cells of multiple stages of follicles in the control ovaries, with a more intense staining in the preantral and early antral follicles (Fig. 1D, $36.71 \pm 5.47$). The 100 mg/kg MXC treatment caused a significant reduction in the levels of ERβ staining intensity in these follicles (Fig. 1F, $19.9 \pm 1.06$, $P < 0.05$), whereas the staining intensity in preantral and early antral follicles of 20 $\mu$g/kg-treated ovaries (Fig. 1E, $31.78 \pm 5.22$) had a trend to be reduced.

**ERα and ERβ promoter regions are differentially methylated by transient MXC exposure**

To determine the role of epigenetic regulation in ER expression in MXC-treated ovaries, we performed methylation profiling of the known promoters of ERα and ERβ genes.

We performed BSPCR and cloned a 140-bp PCR product from the ERα promoter spanning –186 to –45 and found no significant changes in the methylation patterns of the six CpG dinucleotides in this region (supplemental Fig. 1). This finding correlates with the lack of significant effects on the levels of ovarian ERα protein (Fig. 1, 36).

As shown in Fig. 2A, the promoter region of rat ERβ has two CpG islands that include important regulatory elements (39). Whereas one of these regions spans the canonical proximal promoter of ERβ (–625 to –392), CpG
Global DNA methylation is altered by developmental MXC exposure in adult ovaries

To further explore the role of epigenetic alterations on ovarian dysfunction, we assessed global DNA methylation changes using methylation-sensitive AP-PCR. To identify the maximum number of potential methylation events, we conducted AP-PCR with ovarian DNA obtained from control and MXC-treated (100 mg/kg) rats. Preliminary screening of H-digested PCR products revealed more than 25 alterations in methylation patterns. These PCR products were cut out, re-amplified, and cloned. At least five to six clones were sequenced, and the sequences were matched to genes with 95–100% homology. After elimination of repeats and nonhomology matches, 10 genes were identified to be hypermethylated in ovaries from MXC-exposed rats (Fig. 3 and Table 1). Most of these proteins serve critical functions in the ovary (40–45). Among the candidate genes, PAPP-A is particularly relevant to the MXC-induced ovarian phenotype, i.e. a reduction in both follicular maturation and ovulation. Therefore, we analyzed PAPP-A using bisulfite sequencing (Fig. 4). We examined both the promoter and exon/intron regions for changes in methylation patterns. Our results demonstrated that whereas the promoter region (−566 to −329) was unchanged, the exon region (+8 to +300) of the PAPP-A gene was hypermethylated in ovaries from rats exposed to not only 100 mg/kg MXC but also 20 μg/kg MXC.

Developmental MXC exposure up-regulates expression of DNMTs in the adult ovary

*De novo* DNMTs are responsible for the addition of new methyl groups on DNA. To determine whether the MXC-induced gene-specific and global DNA hypermethylation is associated with increased DNMT levels, we measured the mRNA expression levels of *Dnmt3a*, *Dnmt3b*, and *Dnmt3l* in ovaries from control and MXC-treated (20 μg/kg and 100 mg/kg) rats between E19 and PND7 (Fig. 5A). Whereas *Dnmt3a* was unchanged, *Dnmt3b* expression was stimulated, nearly 2-fold, in ovaries from MXC-treated (20 μg/kg and 100 mg/kg) rats compared to control and MXC-exposed (100 mg/kg) rats. Interestingly, the levels of *Dnmt3l* were reduced in the 20 μg/kg-treated ovaries and unchanged in 100 mg/kg dose ovaries.

Discussion

We previously demonstrated that a transient exposure to MXC during early development causes reduced fertility...
due to dysregulation of numerous ovarian genes, including ERβ (36). In addition, superovulation studies using exogenous gonadotropins in prepubertal females showed that 100 mg/kg MXC treatment reduced the number of eggs ovulated, suggesting a direct effect on the ovary. In the current study, we have provided evidence that there is a direct correlation between such an exposure and global and gene-specific hypermethylation in the adult ovary. This study is the first to show that DNA hypermethylation events, possibly via increased Dnmt expression, are associated with the changes in gene expression and dysfunction in the adult ovary.

Human epidemiological studies have shown that women exposed to EDCs such as agricultural pesticides (organochlorines) have prolonged/irregular estrous cycles and difficulty in achieving pregnancy, failed assisted reproductive technology attempts, and loss of pregnancies (46, 47). Studies of female reproductive function after exposure to EDCs have documented defects such as aneuploidy in oocytes, formation of multioocyte follicles, and disruption of estrus cyclicity in rodent and other species, which collectively support the data obtained from the human epidemiological studies (16, 48–53).

Pathologies of several organs (e.g. uterus, prostate, mammary gland, and cardiovascular tissues) are associated with alterations in DNA methylation patterns and the resulting changes in gene expression (13, 54–56). For example, increased ERβ gene expression and decreased DNA methylation were observed in endometriotic tissues compared with normal endometrial tissues (57). Hypermethylation and reduced gene expression of ERβ were also observed in atherosclerosis and during vascular aging (58). Similar observations were made in breast and prostate cancers (59–63). The current study has confirmed that exposure to high dose MXC during fetal and neonatal periods dramatically down-regulates ERβ expression in the adult ovary due to pronounced hypermethylation in the promoter regions of ERβ. Our results indicating that ERα was unaffected suggest that MXC primarily acts via ERβ.

ERs are expressed in the neonatal stages during which MXC exposure occurred in the current study. Our unpublished observations have suggested that ERα is primarily expressed in the germinal epithelium, stromal cells, and theca cells in the rat ovary starting at PND 1. This pattern was disrupted by MXC treatment, and granulosa cells from MXC-treated ovaries had strong expression of ERα. In addition, intense immunoreactivity was observed in the oocytes in MXC-treated ovaries. These data are similar to previous studies using prepuber-
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Digested PCR products that were differentially amplified between control and MXC set). C, Control; MXC, 100 mg/kg.

Heterodimerize with ER cell types (66, 67). These isoforms have been found to result from the misexpression of ERs, heterodimerization of hitherto noncoexpressing isoforms of ER can occur (72), resulting in the transactivation of noncanonical targets.

Another interesting possibility is that alterations in ER methylation patterns due to stress, such as the early exposure to MXC, potentiate the ovarian epigenome as a whole to further epigenetic changes (73, 74) that are then either erased or fixed in the epigenome through later hormonal interplay (75–77). The 100 mg/kg MXC-treated ovaries had high levels of Dnmt3b, whereas Dnmt3l was unchanged. On the other hand, the 20 μg/kg-treated ovaries had high levels of Dnmt3b with low levels of Dnmt3l. Because DNMT3L modulates DNMT3B activity, these data potentially correlate with an overall increase in DNMT activity in the 100 mg/kg MXC-treated ovaries. It has been documented in different types of cancers that differential expression of the Dnmt genes, as seen in the current study, might contribute to the CpG island methylator phenotype wherein hypermethylation patterns are observed in clusters of genes related to the particular cancer (60, 78–82). Detailed analysis of follicle stage-specific DNMT protein expression patterns, using immunohistochemistry, is necessary to further examine possible effects of MXC on DNMTs.

We have now identified candidate genes using AP-PCR. The majority of these genes were either transcription factors or ribosomal proteins; some of these have documented roles in ovarian function. One of the most promising results was that MXC caused hypermethylation of exon regions of the PAPP-A gene. PAPP-A is an IGF binding protein (IGFBP) endo-

FIG. 3. Effects of MXC on the global DNA methylation in ovaries at PND 50–60.

Methylation-sensitive AP-PCR was used. Four micrograms of ovarian genomic DNA were digested with RsaI (R) or Rsal with methylation-sensitive HpaII (H) or -insensitive MspI (M) restriction enzymes, followed by PCR with 10 degenerate primer sets designed to amplify methylation sites (five representative PCR product sets are shown, 1–5; each panel shows one set). C, Control; MXC, 100 mg/kg MXC. See Materials and Methods for details. The H-digested PCR products that were differentially amplified between control and MXC (arrowheads; enlarged inset, and arrow) were identified and listed as potential candidates for further investigation (Table 1); (n = 3 animals per treatment). All experiments were repeated three times.

FIG. 4. Effect of MXC on CpG sites in PAPP-A gene in adult ovary.

Bisulfite sequencing was conducted to examine the regions from 566 to −329 and +8 to +300 upstream and downstream, respectively, from the transcription start site of the PAPP-A gene (shown above are data from the +8 to +300 group). Eight separate clones were analyzed from two or three animals from each treatment group. All predicted CpGs that were converted to thymine are shown as upper circles and methylated cytosines that remain as cytosines are shown as filled circles. Although no changes were observed in the upstream region of the start site, numerous alterations were observed in the 16 CpGs in the +8 to +300 region. Only one predicted CpG site was methylated in control ovaries. However, numerous CpGs were methylated in ovaries exposed to 20 μg/kg and 100 mg/kg MXC.

FIG. 5. Enlarged arrowheads pointing to the DNA methylation site in representative HpaII-sensitive (H) and -insensitive (M) regions.
peptidase that maintains IGFBP/IGF balance and is thus necessary for normal folliculogenesis (45). IGFs stimulate processes of FSH-directed follicle selection and final follicular maturation (41, 83). Reduced PAPP-A expression due to increased methylation could limit its availability in follicles and thus increase IGFBP content and sequester IGF-I. This could lead to the observed defect in follicle selection and maturation (36). PAPP-A expression pattern is well documented in late stage antral follicles and mid to late corpora lutea in both humans and rodents (84, 85). Our studies detected reduced luteal expression of PAPP-A in MXC-treated ovaries (not shown); however, studies to confirm the expression patterns after hormonal stimulation are pending. Another interesting candidate is the enzyme involved in dopamine biosynthesis, dopamine β-hydroxylase (Dbh). Catecholamines have been found in the oocyte (44) and norepinephrine has been found in the ovarian sympathetic nerve fibers. The function of catecholamines is essential for follicular maturation in the ovary (86, 87). Potential alteration in the Dbh methylation profile could cause reduced dopamine production in the ovary, which also could lead to reduced follicular maturation. Whereas the candidate search using AP-PCR is not exhaustive, it provides a good starting point for revealing the effects of MXC on DNA methylation patterns. In the future, oocyte- vs. somatic cell-specific DNA methylation arrays can be conducted.

The physiological levels/ratio of its metabolites could define the overall activity of MXC as being estrogenic vs. antiestrogenic (88). Although the low dose (20 μg/kg·d) is higher than the estimated average daily intake (0.1 ng/kg·d) for adults (22), it is considered an environmentally relevant dose. We observed that the low dose leads to significant alterations in some ovarian parameters (36) and DNA methylation changes in the PAPP-A gene and a hypermethylation trend in the ERα promoter (current study). Developmental exposure to this low level of MXC can lead to alterations in reproductive behavior and increase uterine growth (88, 89). These described effects were observed after a short-term exposure to MXC done under controlled experimental conditions. Most humans are exposed to multiple EDCs during their lifetime. Therefore, the results of the low-dose exposure studies are worth pursuing with longer-term exposure protocols in conjunction with exposures involving combinations of EDCs.

Additional work is needed to fully understand the developmental epigenetic role of EDCs on adult ovarian dysfunction. Other studies should also investigate the possibility that these effects are transmitted to subsequent generations via epigenetic alterations in female germ cells because the exposure to MXC is occurring during the period of female germ cell epigenetic reprogramming. This understanding is likely to provide us with better prevention and treatment options against the abnormalities induced by EDCs, thus improving human health and reducing future health costs.

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Address all correspondence and requests for reprints to: Mehmet Uzumcu, Department of Animal Sciences, School of Environmental and Biological Sciences, Rutgers, The State University of New Jersey, 84 Lipman Drive, New Brunswick, New Jersey 08901–8525. E-mail: uzumcu@aesop.rutgers.edu.

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