Perinatal Programming of Adult Rat Germ Cell Death After Exposure to Xenoestrogens: Role of microRNA miR-29 Family in the Down-Regulation of DNA Methyltransferases and Mcl-1

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Different studies have pointed out that developmental exposure to environmental endocrine disruptors can induce long-term testicular germ cell death probably through epigenetic mechanisms. By using a model of early neonatal post-natal day (PND) 1 to 5 exposure of male rats to a xenoestrogen, estradiol benzoate (EB), we investigated the role of microRNA and DNA methyltransferases (DNMT) on the developmental effects of EB on the adult germ cell death process. Neonatal exposure to EB induced adult germ cell apoptosis together with a dose-dependent increase in miR-29a, miR-29b, and miR-29c expression. Increased miR-29 expression resulted in a decrease in DNMT1, DNMT3a, and DNMT3b and antiapoptotic myeloid cell leukemia sequence 1 (Mcl-1) protein levels as shown in 1) germ cells of adult rats exposed neonatally to EB and 2) in spermatogonial GC-1 transfected with miR-29. The DNMT decrease was associated with a concomitant increase in transcript levels of DNA methylation target genes, such as *L1td1–1 ORF1* and *ORF2*, *Cdkn2a*, and *Gstp1*, in correlation with their pattern of methylation. Finally, GC-1 cell lines transfection with miR-29a, miR-29b, or miR-29c undergo apoptosis evidenced by Annexin-V expression. Together, the increased miR-29 with a subsequent reduction in DNMT and Mcl-1 protein levels may represent a basis of explanation for the adult expression of the germ cell apoptosis phenotype. These observations suggest that the increased expression of the "apoptomir" miR-29 family represents the upstream mechanism identified until now that is involved in adult germ cell apoptosis induced by a neonatal hormonal disruption. **(*Endocrinology* 153: 1936–1947, 2012)**

Human epidemiological studies have indicated that the risk of developing diseases in later life might be related to disorders occurring during the developmental period. Fetal/neonatal life are vulnerable periods of the lifespan, during which exposure to adverse environmental factors could result in late-onset diseases, such as hypertension, type 2 diabetes, and cancer, which have become major medical and public health concerns (1–3). Such a concept could be also applied to the reproductive pathology, such as male infertility, reproductive tract malforma-

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Abbreviations: Bcl-2, B-cell CLL/lymphoma 2; CASP3, caspase-3; Ct, threshold cycle; DAPI, 4′,6′-diaminido-2-phenylindole; DNMT, DNA methyltransferase; EB, estradiol benzoate; ED, endocrine disruptor; EED, environmental ED; FITC, fluorescein isothiocyanate; GC-1, GC-1 spg; GD, gestational day; Mcl-1, myeloid cell leukemia sequence 1; MeDIP, methylated DNA immunoprecipitation; miRNA, microRNA; PCNA, proliferating cell nuclear antigen; PND, post-natal day; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyl transferase 2′-deoxyuridine, 5′-triphosphate nick end labeling.

tions, and testicular germ cell tumors (4). In recent years, a potential causal link between *in utero* and/or neonatal exposure to compounds termed endocrine disruptors (ED) or xenoestrogens that alter endocrine functions and the development of genital tract abnormalities, such as impaired spermatogenesis with adult testicular germ cell apoptosis, has emerged from studies in rodents. Indeed, *in utero* or neonatal exposure to xenoestrogens induces a wide range of abnormalities of the male genital tract, including small testes, cryptorchidism, hypospadia evidenced at birth, and male infertility in adulthood (5–11). Even though the exact molecular basis of the long-term testicular germ cell apoptosis at the origin of the infertility, induced by neonatal exposure to estrogenic compounds, remains unknown, some studies have suggested that epigenetic mechanisms and specifically DNA methylation might be at play (for review see, Refs. 12, 13). Furthermore, the recent identification of several families of microRNA (miRNA) involved in the different cellular processes, especially apoptosis via the "apoptomir" family (14), led us to hypothesize as to whether these short noncoding RNA might represent the upstream step involved in the neonatally programmed adult germ cell death process after early developmental exposure to xenoestrogens.

This study aims to identify the apoptomir expression alteration, specifically miR29 and their targets, the DNA methyltransferases (DNMT)1, DNMT3A, and DNMT3B and the antiapoptotic factor Mcl-1, potentially involved in the adult programmed germ cell death process, induced by neonatal exposure to xenoestrogens (15, 16).

Materials and Methods

In vivo experimental studies

Pregnant Sprague Dawley rats at gestational day (GD)15 (Janvier, Le Genest Saint Isle, France) were individually housed in temperature-controlled rooms with 12-h light, 12-h dark cycles and given free access to water and feed. At birth, each pup was sexed, weighed, and identified. Pups were administered vehicle (corn oil; MP Biomedicals, Illkirch, France) or estradiol benzoate (EB) (Sigma-Aldrich, L'Isle D'Abeau, France) by daily sc injections from post-natal day (PND) 1 to PND5 at doses of 0, 0.75, 1.25, 2.5, or 25 μg/d. From PND6, rats were left without treatment and killed at PND6, PND30, or PND90 by CO_2 inhalation. During necropsy, the position of each testis was carefully noticed, and testes were removed and weighed. Only bilateral descended testes were studied. One of the testes was snap frozen for quantitative molecular approaches, whereas the other was fixed for morphological studies. At least seven different animals from four different litters were used for each treatment group. This study was conducted in accordance with current regulations and standards approved by Institut National de la Santé et de la Recherche Médicale Animal Care Committee (protocol no. 2008-43).

Histology

Testes were immediately fixed for 48 h in Bouin's fluid, dehydrated stepwise in graded ethanol baths, and embedded in paraffin. Bouin-fixed testes were sectioned into 5 μm thick and prepared and stained with hematoxylin, eosin, and safran (n = 5 animals per group).

Terminal deoxynucleotidyl transferase 2′-deoxyuridine, 5′-triphosphate nick end labeling (TUNEL) and immunofluorescence

TUNEL experiments were performed as previously described (6), using terminal deoxynucleotidyl transferase (Euromedex, Mundolsheim, France), biotin-11-2′-deoxyuridine, 5′-triphosphate (Roche Diagnostics, Meylan, France), streptavidin-fluorescein conjugate (Merck KGaA, Darmstadt, Germany). At the end of the experiment, testis sections were counterstained with 4′,6′-diaminido-2-phenylindole (DAPI), mounted with coverslips using mounting medium. The results were expressed as the number of TUNEL-positive cells per 100 random round seminiferous tubules. For immunofluorescence experiments, after unmasking treatment, sections were incubated 10 min in PBS 0.1% Triton X-100 at room temperature, then in PBS-5% FCS-1% protease inhibitor cocktail (Sigma, St. Louis, MO) for 30 min. The section were incubated overnight at +4 C with anti proliferating cell nuclear antigen (PCNA) antibody (C-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), antiphospho-histone H2A.X (Ser139) (Merk Millipore, Bedford, MA), or anti heat shock protein 70 kDa (Cell Signaling, Beverly, MA) diluted at 1:50, or antiactive caspase-3 (CASP3) (Abcam, Cambridge, MA) diluted at 1:100. Sections were then washed three times in PBS 0.1% Triton X-100 and incubated 1 h at room temperature with a fluorescein isothiocyanate (FITC) or Texas Red-conjugated secondary antibody either donkey antirabbit (Amersham, Piscataway, NJ), rabbit antimouse, or swine antirabbit (Dako, Glostrup, Denmark) diluted at 1:30. The sections were washed in 1× PBS and counterstained as described for TUNEL.

Western blot analysis

Frozen testicular tissues were ground in liquid nitrogen to obtain tissue powders. Aliquots of powder were homogenized in ice-cold hypotonic buffer (25 mM Tris-HCl, 0.1% sodium dodecyl sulfate, and 1% protease inhibitor cocktail) (Sigma-Aldrich). Tissue homogenates were further sonicated (10 sec at 80 W). Protein concentration was determined using the bicinchoninic acid assay. The experimental procedures were carried out as previously described (5). The antibodies used in this study were Dnmt3A (1:1000, no. 2160; Cell Signaling), Dnmt3B (1:1000, no. 2161; Cell Signaling), Dnmt1 (1:1000, KAM-TF040; Stressgen, Farmingdale, NY), cleaved CASP3 (1:500, no. 9661; Cell Signaling), Mcl-1 (1:5000; Rockland, Gilbertsville, PA), and actin (1:20,000; The Jackson Laboratory, Bar Harbor, ME). Membranes were scanned using a Luminescent Image Analyzer 3000 CCD Camera (Fujifilm, Dusseldorf, Germany) and quantified using MultiGauge logiciel (Fujifilm).

Real-time quantitative PCR

Total RNA were isolated from frozen testicular powders using TRIzol reagent (Invitrogen, Cergy Pontoise, France) coupled to an on-column purification and deoxyribonuclease treatment

with an RNeasy kit (QIAGEN, Courtaboeuf, France). cDNA was synthesized from total RNA (1 μg) with M-MLV RT (Moloney Murine Leukemia Virus Reverse Transcriptase) (10 U/μl) (Invitrogen) and random hexamer primers (5 μM) (Invitrogen) in a final volume of 20 μl according to the manufacturer's instructions. A 1:20 dilution of each RT product was used for the real-time RT-PCR analyses. The real-time RT-PCR measurement of individual cDNA (2 μl of 1:20 dilution) was performed using Quantitect SYBR Master Mix (4 μl) (QIAGEN), PCR primers (2 μl of 10 μM solution), and ultrapure water (2 μl) to measure the duplex DNA formation with the Roche Lightcycler system. The primer sequences that used are listed in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org, or for *L1td1* (previously known as *Line1*) *ORF1* and *ORF2*, *Sine B2* (17), and *Gstp1* (18), have been previously described. Gene amplification was carried out as follows: initial activation of HotStarTaq DNA polymerase at 95 C for 10 min; 45 cycles in three steps: 95 C for 15 sec, 60 C for 15 sec, and 72 C for 15 sec. Melting temperature analysis was carried out by a slow increase in temperature (0.1 C/sec) up to 95 C. Standard curves were generated with testicular cDNA pools from animals with different treatments. The data were normalized to β-actin levels using the threshold cycle (Ct) method.

Real-time RT-PCR analysis of miRNA expression

RT-PCR reactions were performed using the stem-loop RT-PCR method as previously described (19), which is specific for mature miRNA (TaqMan miRNA assays; Applied Biosystems, Foster City, CA). Ten nanograms of total RNA were reverse transcribed in a 7.5-μl reaction using Multiscribe Reverse Transcriptase and a TaqMan miRNA (29a, 29b, 29c) RT primer (Applied Biosystems). The reaction mixture was incubated at 16 C for 30 min, 42 C for 30 min, 85 C for 5 min, and finally held at 4 C until subsequent analysis or stored at −20 C. Five microliters of the reverse transcribed product (5-fold dilution from RT-PCR) were assayed using TaqMan Universal PCR Master Mix, no AmpErase, and 1 μl of TaqMan miRNA (29a, 29b, 29c; Applied Biosystems) PCR primers/probe mix in a 15-μl reaction mix. Real-time RT-PCR was performed on a ABI7900 system (Applied Biosystems) using the following conditions: after 10 min at 95 C, 40 cycles were performed at 95 C for 15 sec, and 60 C for 1 min. The data were normalized to small nucleolar RNA using the Ct method.

Detection of methylation by methylated DNA immunoprecipitation (MeDIP)-qPCR

DNA was isolated from frozen testicular powder by using DNeasy Blood & Tissue kit (QIAGEN), and 6 μg were digested with 24 U of *Mse*I enzyme (New England Biolabs, Ipswich, MA) supplemented with 100 ng/μl BSA overnight at 37 C. The reaction was stopped by heating the samples for 20 min at 65 C. Digested DNA was purified using the QIAquick PCR Purification kit (QIAGEN). *Mse*I-digested DNA was run on a 2% agarose gel to verify a fragment size of 200-1000 bp. *Mse*I-digested DNA corresponded to input DNA. One microgram of *Mse*I-digested DNA was incubated with magnetic beads coupled with methyl-CpG-binding domain protein 2 protein (EpiXploreTM Methylated DNA Enrichment kit; CLONTECH, Mountain View, CA). Beads were washed to remove non- and hypomethylated DNA. The enriched methylated DNA fraction was eluted with high salt buffer and purified by precipitation (MeDIP DNA). Five nanograms of the input and the MeDIP DNA were used for real-time quantitative PCR. The levels of methylated DNA were calculated according to the following formula: 2^[(Ct (Input) − Ct (MeDNA-IP))]. The primer sequences used are described in Supplemental Table 1.

Cell cultures and transfection

GC-1 spg (GC-1) mouse spermatogonia type B-spermatocyte cell lines, provided by Pierre Chambon (Institut de la Génétique et de la Biologie Moléculaire et Cellulaire, Strasbourg, France), were maintained in DMEM/Glutamax medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) at 37 C in a humidified, CO_2-controlled (5%) incubator. GC-1 cells grown on 12-well plates were transfected with 50 nM miR-29a, miR-29b, or miR-29c analogs (Applied Biosystems) using Hiperfect Transfection reagent (QIAGEN) according to the manufacturer's protocol. To knockdown DNMT expression, cells were transfected with 25 nM small interfering RNA (siRNA) targeting DNMT1 or DNMT3b (Applied Biosystems) or negative control siRNA (Eurogentec, Fremont, CA); 48 h after transfection, cells were harvested for protein and RNA extraction. For the visualization of apoptotic cells by immunofluroscence, we performed an Annexin-V-FITC and PI staining on GC-1 cells grown on glass coverslips (Miltenyi Biotec, Auburn, CA).

Data analysis

The data from the different experiments were analyzed with GraphPad software version 4.0 (GraphPad Software, Inc., San Diego, CA). The values were expressed as the mean ± SEM to account for sample and animal variation within a dataset. The Student's *t* test for single comparison analysis or one-way ANOVA for multiple comparisons was performed to determine whether there were differences between all groups ($P < 0.05$). For ANOVA, this was followed by the Bonferroni *post hoc* test if $P < 0.05$ to determine the significance ($P < 0.05$) of differences between the pairs of groups.

Results

Adult testicular apoptotic phenotype induced by neonatal exposure to EB

The adult germ cell apoptosis induced by neonatal exposure to the estrogenic analog EB was investigated by histological and molecular approaches. At the highest EB dose (25 μg/d) used, adult (PND90) testes showed severe atrophy (Fig. 1A) with alterations in the seminiferous epithelium ranging from partial (spermatocytes and spermatids) to massive germ cell loss (Fig. 1B). At 2.5 μg/d EB, minor histological alterations in the seminiferous tubules were observed (Fig. 1B) and none at lower doses (0.75, 1.25 μg/d) (data not shown). These data were correlated with the absence of modification in body and testicular weight at 2.5 μg/d and lower doses of EB (Supplemental Table 2). To minimize or avoid massive germ cell loss that

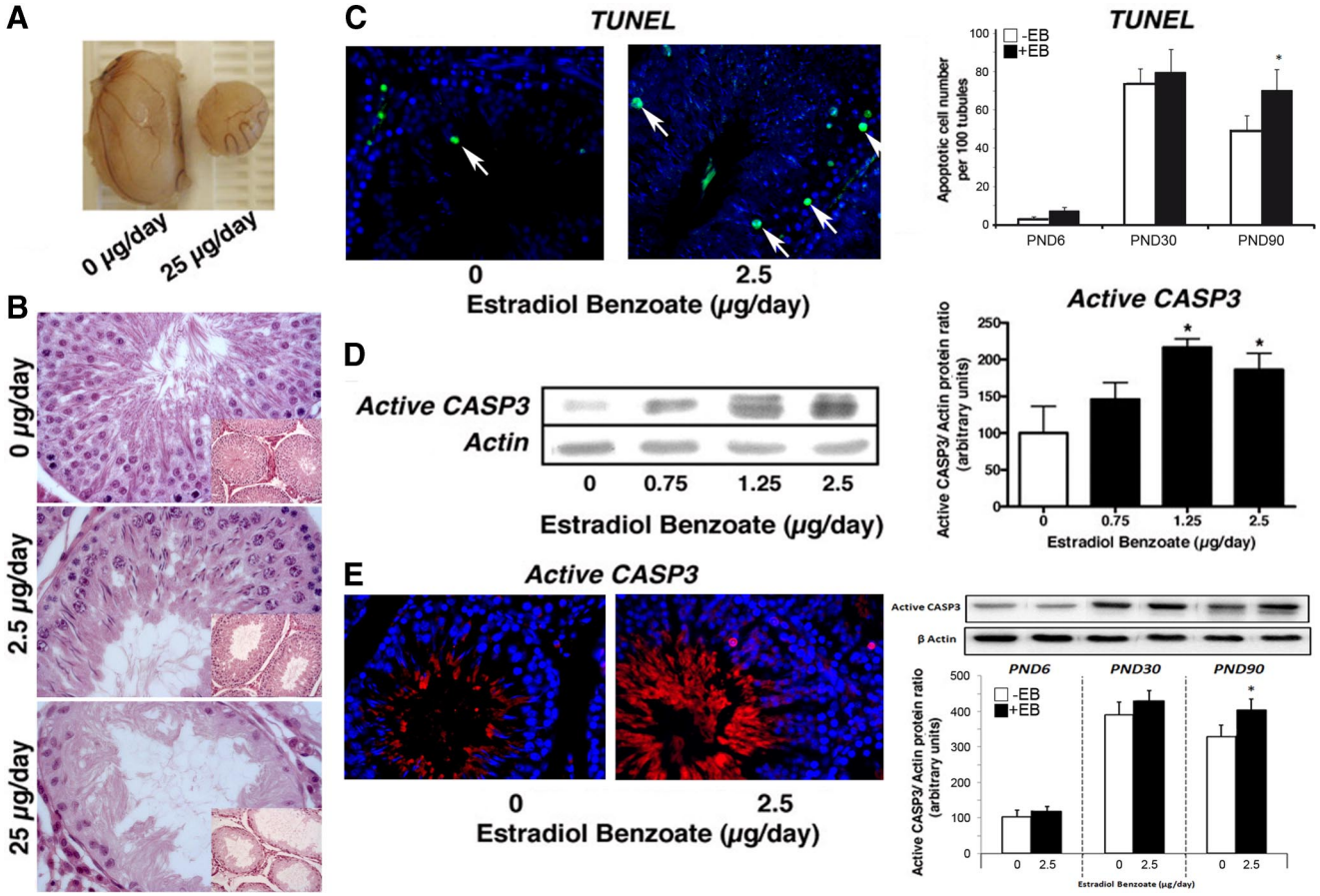

FIG. 1. Characterization of the adult testicular phenotype induced by neonatal exposure to EB. A, Macroscopic examination of adult testes showed a severe atrophy induced by neonatal EB (25 μg/d) exposure compared with untreated rats (0 μg/d). B, Testicular morphology in adult rat testes from untreated (0 μg/d), 2.5 μg/d-treated, and 25 μg/d-treated animals (magnification, ×400; *inset*, ×200). C, Adult rat testes were subjected to TUNEL analysis to visualize and count apoptotic cells in untreated (0) and EB-treated males (2.5 μg/d). The *green* fluorescent signal corresponds to TUNEL-positive (apoptotic) cells (*white arrows*). Nuclei were counterstained with DAPI (*blue signal*). The histograms represent the number of TUNEL-positive (apoptotic) germ cells per 100 seminiferous tubules from immature (PND6), juvenile (PND30), and adult (PND90) rats exposed neonatally to EB. The results are expressed as the mean ± SEM determined from at least four different animals per condition (*, $P < 0.05$). D, Cleaved CASP3 levels were determined by Western blot analysis in adult rat testes from unexposed (0) or 0.75, 1.25, and 2.5 μg/d EB-exposed animals. Representative autoradiograms were shown, whereas histograms represent cleaved CASP3 protein levels expressed as a percentage of the ratio (target protein/actin protein) detected in the untreated group. The results are expressed as the mean ± SEM determined from seven different animals (*, $P < 0.05$). E, Cleaved CASP3 levels were determined by Western blot analysis in immature (PND6), juvenile (PND30), and adult (PND90) rats exposed neonatally to EB (2.5 μg/d). The results are expressed as the mean ± SEM determined from five to seven different animals per condition (*, $P < 0.05$). CASP3 was localized in adult (PND90) rat testes from untreated (0) and EB-treated (2.5 μg/d) rats. The *red* fluorescent signal corresponds to cleaved CASP3-positive cells; nuclei were counterstained with DAPI (*blue* signal).

might confound the interpretation of EB effects on testicular germ cell gene expression and protein levels, we decided to analyze the testicular phenotype at a molecular level at the EB doses of 0.75, 1.25, and 2.5 μg/d.

Neonatal exposure to EB induced a cell death process in adult (PND90) rat testes but not in immature (PND6) or juvenile (PND30) ones as monitored through TUNEL (Fig. 1C) and active CASP3 (Fig. 1, D and E) approaches. At PND90, a significant increase was observed (30%, $P < 0.05$) in the TUNEL-positive cell number (Fig. 1C) and active CASP3 (Fig. 1E) levels. Active CASP3 protein levels were significantly increased in testes from 1.25 μg/d-treated (2-fold, $P < 0.05$) and 2.5 μg/d-treated animals (1.8-fold, $P < 0.05$) (Fig. 1D). The TUNEL-positive cells corresponded to germ cells (spermatocytes and/or spermatids) (Fig. 1, C and E, and Supplemental Fig. 1). With regards to the rate of proliferation, although adult spermatogonia and preleptotene spermatocytes specifically displayed PCNA staining, no significant change in the accumulation of PCNA was observed between untreated and EB-exposed male rats (Supplemental Fig. 1).

Neonatal exposure to EB induces long-term alteration of miR-29 in adult rat testes

Early postnatal exposure to EB induced a dose-dependent increase in miR-29 (a, b, and c) levels in adult

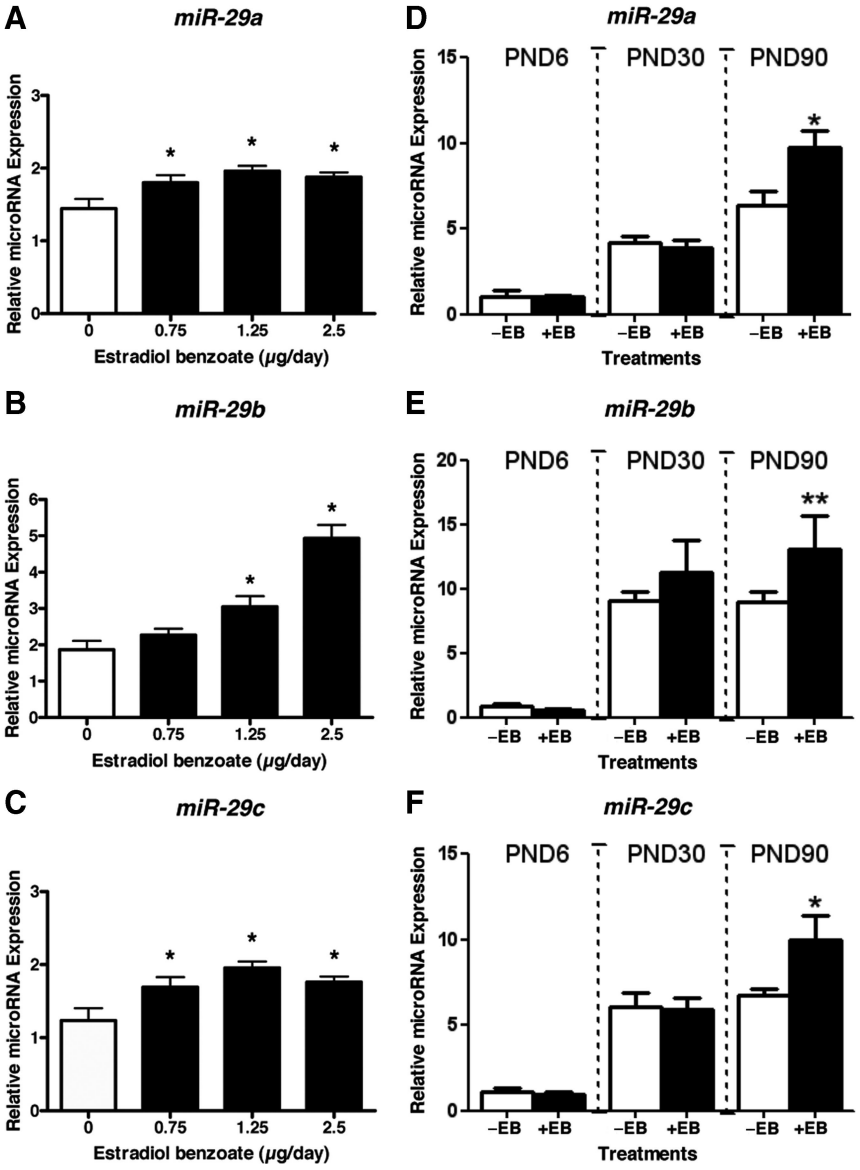

FIG. 2. Neonatal exposure to EB induces a long-term alteration in miR-29 levels. Mature miR-29a (A), miR-29b (B), and miR-29c (C) levels were determined by real-time RT-PCR in adult rat testes from unexposed (0) or 0.75, 1.25, and 2.5 μg/d EB-exposed animals. Mature miR-29a (D), miR-29b (E), and miR-29c (F) levels were determined by real-time RT-PCR in immature (PND6), juvenile (PND30), or adult (PND90) rat testes from unexposed (−EB) or exposed (2.5 μg/d, +EB) animals. Histograms represent relative miRNA levels normalized to small nucleolar RNA levels. The results are expressed as the mean ± SEM determined from seven to eight different animals (*, $P < 0.05$).

(PND90) rat testicular tissue. Indeed, miR-29a displayed a significant increase at 1.25 μg/d (1.4-fold, $P < 0.01$) and 2.5 μg/d (1.3-fold, $P < 0.05$) of EB (Fig. 2A). A highly significant increase in the miR-29b accumulation was observed at 1.25 μg/d (1.6-fold, $P < 0.05$) and 2.5 μg/d (2.6-fold, $P < 0.01$) of EB (Fig. 2B). Again, miR-29c levels were significantly increased at 0.75 μg/d (1.35-fold, $P < 0.05$), 1.25 μg/d (1.6-fold, $P < 0.01$), and 2.5 μg/d (1.4-fold, $P < 0.05$) of EB (Fig. 2C). miR-29a (Fig. 2D), miR-29b (Fig. 2E), and miR-29c (Fig. 2F) miRNA levels were affected at PND90 but not at PND6 (immature testes) or PND30 (juvenile testes).

Among the proteins targeted by the miR-29 members are, at least, two potential candidates that might be involved directly and/or indirectly in the germ cell apoptotic process, because their alterations induce germ cell death (20): Mcl-1 and DNMT. Mcl-1 is a B-cell CLL/lymphoma 2 (Bcl-2) (CASP3) family protein that promotes cell survival by interfering at an early stage in a cascade of events leading to the release of cytochrome c from mitochondria (21). In adult testes from rats exposed during early postnatal life to EB, Mcl-1 protein levels (but not mRNA) (data not shown) were significantly reduced at a dose of 2.5 μg/d (41% decrease, $P < 0.05$) of EB (Fig. 3G). Mcl-1 protein levels were affected at PND90 but not at PND6 (data not shown). Similarly, in adult testes from rats exposed to EB during early postnatal life, DNMT3A protein levels were significantly reduced at doses of 0.75 μg/d (40% decrease, $P = 0.036$), 1.25 μg/d (65% decrease, $P < 0.0001$), and 2.5 μg/d (70% decrease, $P < 0.0001$) of EB (Fig. 3A). DNMT3B (3B2 isoform) protein levels were decreased at doses of 0.75 μg/d (60% decrease, $P = 0.0075$), 1.25 μg/d (85% decrease, $P =$ (CASP3) 0.0005), and 2.5 μg/d (70% decrease, $P = 0.0029$) of EB (Fig. 3B). Again, DNMT1 protein levels were decreased by 60% at 1.25 μg/d ($P = 0.0004$) and 2.5 μg/d ($P = 0.0006$) in adult rat testes (Fig. 3C). Concerning transcript levels, although DNMT3A (Fig. 3D) and DNMT3B (Fig. 3E) mRNA levels remained unchanged, the DNMT1 mRNA level showed a modest but significant decrease (1.2-fold, $P = 0.0222$) in the adult testes from 2.5 μg/d-treated animals (Fig. 3F). DNMT3A2 mRNA levels were unchanged in adult rat testes (data not shown). Moreover, because antibodies raised against DNMT3A2 protein lack specificity, we were unable to quantify the potential changes in these protein levels. We were also unable to detect and quantify the *Dnmt3L* levels in the adult testes, confirming previous reports that *Dnmt3L* expression was extremely

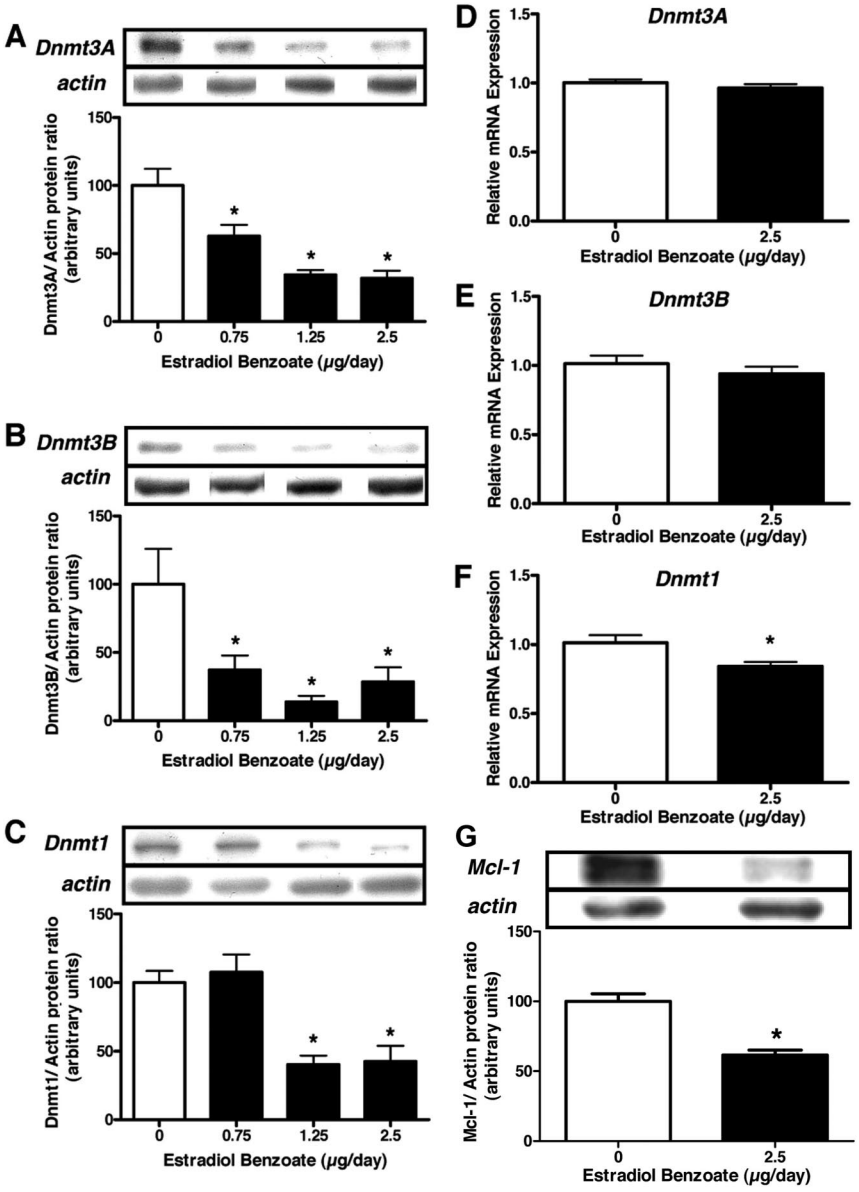

FIG. 3. Neonatal exposure to EB induces a long-term alteration in testicular DNMT protein levels in adult rats without mRNA modification. Protein levels for DNMT3A (A), DNMT3B (B), and DNMT1 (C) were determined by Western blot analysis in adult rat testes from unexposed or 0.75, 1.25, and 2.5 μg/d EB-exposed animals. Representative autoradiograms were shown, whereas histograms represent protein levels expressed as a percentage of the ratio (target protein/actin protein) detected in the untreated group. The results are expressed as the mean ± SEM determined from seven different animals (*, $P < 0.05$). Transcripts levels of *Dnmt3A* (D), *Dnmt3B* (E), and *Dnmt1* (F) were determined by real-time RT-PCR analysis in adult testes from unexposed (0) and 2.5 μg/d EB-exposed rats. Histograms represent relative mRNA levels normalized to β-actin levels. The results are expressed as the mean ± SEM determined from eight different animals (*, $P < 0.05$). G, Protein levels for Mcl-1 were determined by Western blot analysis in adult (PND90) testes from untreated (0) or 2.5 μg/d EB-exposed animals. Representative autoradiograms were shown, whereas histograms represent protein levels expressed as a percentage of the ratio (target protein/actin protein) detected in the untreated group. The results are expressed as the mean ± SEM from seven different animals (*, $P < 0.05$).

low or even undetectable in adult testes (22). Although DNMT3A (Fig. 4A), DNMT3B (Fig. 4B), and DNMT1 (Fig. 4C) protein levels were significantly ($P < 0.05$) decreased in adult (PND90) testes from rats exposed during early postnatal life to EB, DNMT protein levels were unchanged in immature (PND6) rat and juvenile (PND30) testes (Fig. 4, A–C) after exposure to EB.

To demonstrate the inhibitory effects of miR-29 on DNMT protein levels, we used an *in vitro* model of transient transfection with synthetic miRNA in a rodent testicular germ (GC-1) cell line, which harbors features of type B spermatogonia and primary spermatocytes (23). Compared with scrambled oligonucleotide, miR-29a transfection had a clear repressive effect on DNMT3A (50% decrease, $P = 0.027$) (Fig. 5A) and DNMT1 (50% decrease, $P = 0.0191$) (Fig. 5C) protein levels, whereas miR-29a transfection had no significant effect in down-regulating DNMT3B protein levels (Fig. 5B). The most marked inhibitory effect of miR-29 overexpression on DNMT protein levels was observed for miR-29b, which highly reduced DNMT3A (75% decrease, $P = 0.0042$) (Fig. 5A) and DNMT3B (75% decrease, $P = 0.0052$) (Fig. 5B) protein levels as well as, to a lesser extent, DNMT1 protein levels (50% decrease, $P = 0.007$) (Fig. 5B). Transfection of miR-29c reduced only DNMT3A protein levels (75% decrease, $P = 0.0017$) (see figure 7 below), because no modification was observed in DNMT3B (Fig. 5B) and DNMT1 (Fig. 5C) protein levels. Interestingly, on the other hand, miR29a expression was not affected by DNMT knockdown (Supplemental Fig. 2).

To identify the functional consequences of DNMT protein level alterations in adult testes, we further investigated the expression of genes (*Cdkn2a*, previously known as *p16* and *Gstp1*) or sequences (*L1td1*, previously known as *Line-1*) known to be controlled by a DNA methylation mechanism. The transcripts originating from transposable elements *L1td1* and *SINE B2* were chosen because they normally remain silent through DNA methylation. Interestingly, at PND90, a significant increase of

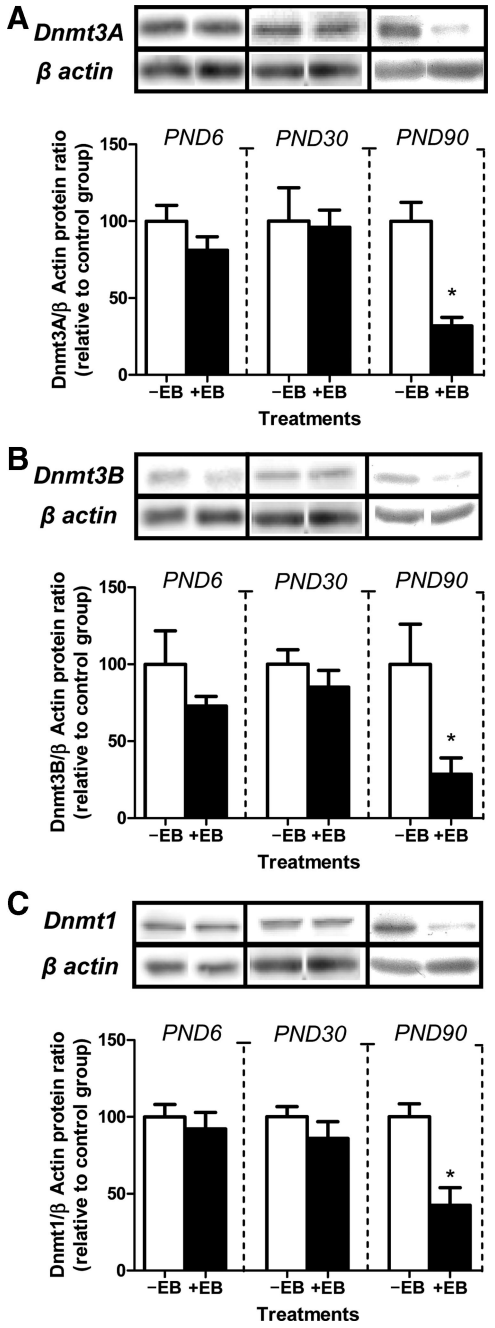

FIG. 4. Neonatal exposure to EB induces a long-term alteration in testicular DNMT protein levels in adult rats but not in immature or juvenile rats. Protein levels for DNMT3A (A), DNMT3B (B), and DNMT1 (C) were determined by Western blot analysis from immature testes (PND6), juvenile (PND30), and adult testes (PND90) from untreated (−EB) or EB-exposed (2.5 μg/d, +EB) animals. Representative autoradiograms were shown, whereas histograms represent protein levels expressed as a percentage of the ratio (target protein/actin protein) detected in the untreated group at the same age. The results are expressed as the mean ± SEM from five (PND6) or seven (PND30 and PND90) different animals (*, $P < 0.05$).

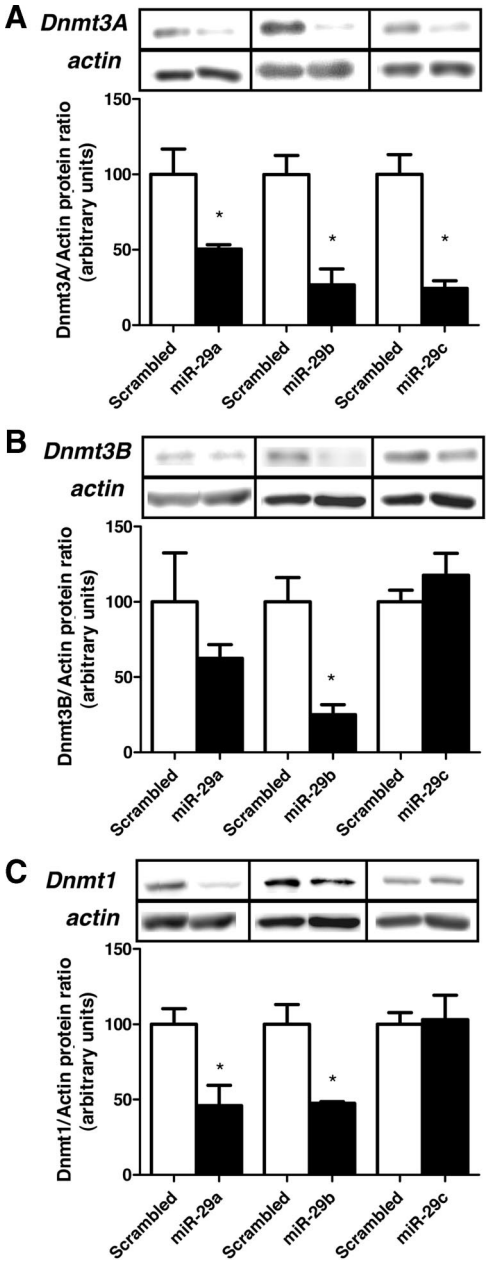

FIG. 5. Effects of transfection of miR-29 on DNMT protein levels in germ cells. Protein levels for DNMT3A (A), DNMT3B (B), and DNMT1 (C) were determined by Western blot analysis in the GC-1 germ cell line transiently transfected (48 h) by miR-29a, miR-29b, and miR-29c. Representative autoradiograms were shown, whereas histograms represent protein levels expressed as the percentage of the ratio (target protein/actin protein) detected in the scramble-treated control cells. The results are expressed as the mean ± SEM from at least four independent experiments (*, $P < 0.05$).

L1td1 ORF1 (1.5-fold, $P = 0.01$) (Fig. 6A) and *L1td1 ORF2* (1.2-fold, $P = 0.04$) (Fig. 6C) transcripts was observed, whereas the *SINE* (*B2_Rn*) transcript levels were unchanged (data not shown). The analysis of DNA methylation status of *L1td1* showed a significant decrease (30%, $P < 0.03$) in EB-exposed animals (Fig. 6A). Moreover, *Cdkn2a* (1.5-fold, $P < 0.01$) (Fig. 6B) and *Gstp1* (1.3-fold, $P < 0.04$) (Fig. 6C) mRNA levels were increased in the testis from adult EB-exposed rats. These data are correlated with a significant decrease in their DNA methylation status: *Cdkn2a* (80%, $P < 0.02$) (Fig. 6B) and

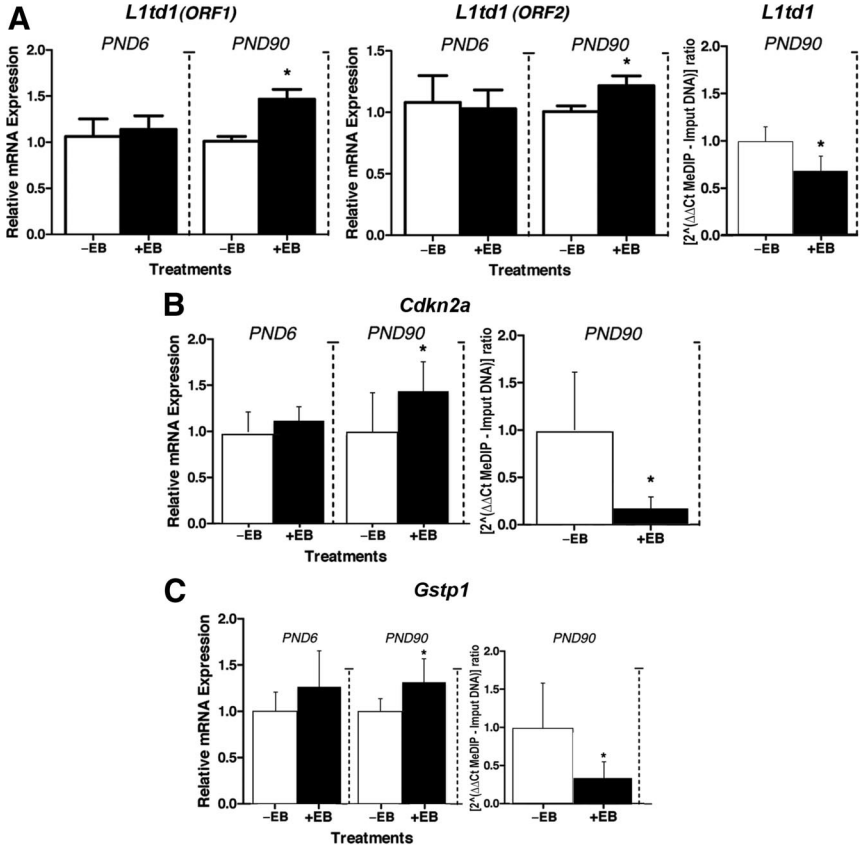

FIG. 6. Long-term effects of neonatal exposure to EB on the transcript levels of gene expression controlled by DNA methylation and pattern of methylation. Transcript levels of *L1td1-ORF1* and *L1td1-ORF2* (A), *Cdkn2a* (B), and *Gstp1* (C) were determined by real-time RT-PCR analysis in immature and adult rat testes untreated (−EB) or exposed to EB (2.5 μg/d, +EB). Histograms represent transcripts levels normalized to actin levels compared with the untreated group at the same age. The results are expressed as the mean ± SEM determined from eight different animals (*, $P < 0.05$). The methylation status of *L1td1* (A), *Cdkn2a* (B), and *Gstp1* (C) was performed by comparing expression between MeDIP DNA and input DNA by real-time RT-PCR analysis in adult rat testes untreated or exposed to EB (2.5 μg/d). The results are expressed as the mean ± SEM determined from at least three different animals (*, $P < 0.05$).

Gstp1 (65%, $P < 0.03$) (Fig. 6C). Consistently with the absence of changes in the protein levels in DNMT in immature (PND6) testes, the transcript levels corresponding to methylated-target sequences (*L1td1 ORF1*, *L1td1 ORF2*, *Cdkn2a*, and *Gstp1*) remained unchanged in immature (PND6) rats (Fig. 6, A–C).

Transfection of GC-1 germ cell line with synthetic miR29a, miR29b, or miR29c induced an apoptotic process. Indeed, compared with scrambled oligonucleotide, miR-29a, miR-29b, and miR-29c transfection had a clear repressive effect on the protein level of antiapoptotic MCL-1 (Fig. 7A), whereas it enhanced that of proapoptotic cleaved CASP3 (Fig. 7A) and the number of Annexin-V and PI positive cells (Fig. 7C). Interestingly, DNMT knockdown induced also apoptosis as monitored by active CASP3 expression after transfection of the siRNA of DNMT3A (Fig. 7B). Such observations suggest that the induction of active CASP3 by miRNA overexpression might be mediated through the inhibition of DNMT levels.

Discussion

In this study, we demonstrated that early (PND1–PND5) postnatal exposure to a xenoestrogen compound could result in late-onset testicular diseases, such as adult germ cell apoptosis, and lead to male infertility. Indeed, testicular germ cell apoptosis was observed in adult (PND90) animals but not immature (PND6) or juvenile (PND30) rats. High EB doses (25 μg/d) induce severe impairment of spermatogenesis with a decreased relative weight of the testes associated with a massive germ cell loss. These data are in accordance with the results of Putz *et al.* (24). Exposure to high doses of EB, and more generally to environmental ED (EED), is relevant to assess a clear infertile testicular phenotype but induce massive germ cell loss, which leads to important modifications in the relative cell-type proportions. Such changes could lead to false interpretations, because the observed differences in gene expression are the results of changes in testicular cell content rather than the regulation of gene expression. Through a dose-effect experiment of EB, we first determined the concentration that did not dramatically affect testicular weights but maintained the cellular testicular phenotype. Indeed, lower EB doses (0.75–2.5 μg/d) did not affect testicular weights but induced discrete germ cell alterations into the seminiferous tubules (2.5 μg/d) and increased germ cell apoptosis (1.25 and 2.5 μg/d) without modifying the proliferation rate. This allowed us to assume that at the doses used (0.75–2.5 μg/d) for the molecular analyses, the modifications of gene expression were significant, not the consequences of altered cell content. Designing the exposure conditions (age, doses, time, developmental periods) of individuals to EED presents by definition limitations. Indeed, individuals are generally exposed to several compounds. Even if exposure occurs at low doses for each compound, additive, synergistic, or even inhibitory effects are possible, leading therefore to

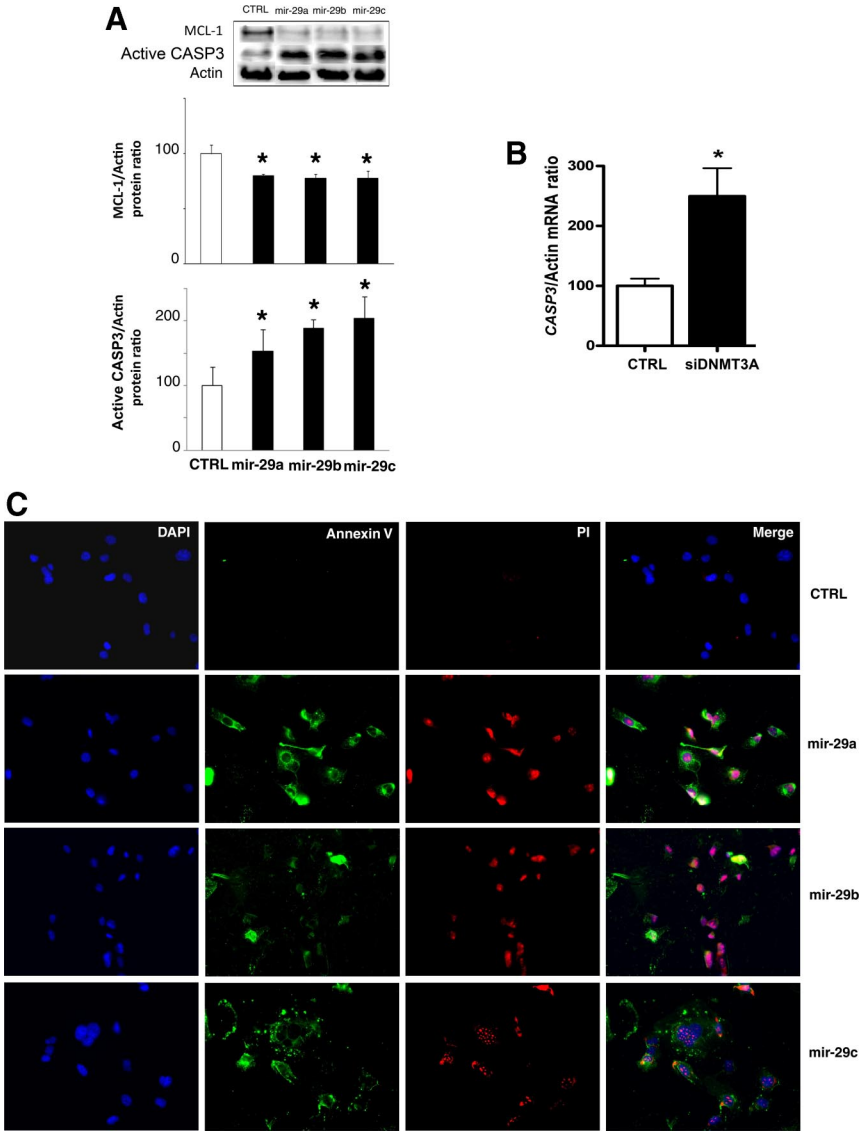

FIG. 7. Effects of miR-29 transfection on germ cell apoptosis. Protein levels for (A) CASP3 and MCL-1 were determined by Western blot analysis in the GC-1 germ cell line transiently transfected (48 h) by miR-29a, miR-29b, and miR-29c. Protein levels for (B) CASP3 were determined by Western blot analysis in the GC-1 germ cell line transiently transfected by DNMT3A. Representative autoradiograms were shown, whereas histograms represent protein levels expressed as the percentage of the ratio (target protein/actin protein) detected in the scramble-treated control cells. The results are expressed as the mean ± SEM determined from at least three independent experiments (*, $P < 0.05$). C, Apoptosis in GC-1 cells was visualized by Annexin-V-FITC and PI staining after transfection with control miR (CTRL), miR-29a, miR-29b, or miR-29c.

observable pathological effects. It is difficult if not impossible to determine, at the present time, the number of hormonally active chemicals to which individuals are exposed to, the duration of exposure, and the developmental periods at which exposure occurs. By using an experimental model based on the use of one compound with exposure at the lowest effective dose during a critical vulnerable period, we aimed, in the present study, at dissecting the upstream molecular mechanisms involved in the testicular adult germ cells triggered by exposure to a xenoestrogen.

The exposure of pups to EB from d 1 to 5 corresponds to the *in utero* second and third trimester development of their human counterparts, because this window of exposure corresponds to the period of testicular development that begins in fetal life and continues after birth by the proliferation of germ cells and somatic Sertoli cells (25).

Here, we report that adult germ cell apoptosis induced by early postnatal exposure to EB might be related to the increased expression of the three members of the miR-29 family (miR-29a, miR-29b, and miR-29c). miRNA are a class of short noncoding RNA (~22 nucleotides) that are involved in the post-transcriptional regulation of gene expression in most eukaryotic organisms. miRNA sometimes silence their targets through the induction of direct cleavage, but in most cases, they do so through either a cleavage-independent degradation mechanism or translational repression (26). miRNA play important roles in cellular processes, such as cell differentiation, growth/proliferation, migration, apoptosis/death, metabolism, and defense (26). With regards to miR-29, they are termed apoptomir, because it has been shown that miR-29a, miR-29b, and miR-29c up-regulate p53 by targeting p85 and cell division cycle 42 and induce apoptosis in a p53-dependent manner in breast and colorectal cancer cell lines (27). miR-29b has also been shown to target Mcl-1 and sensitize cells to TNF-related apoptosis-inducing ligand (16) and might promote apoptosis through a mitochondrial pathway that involves Mcl-1 and Bcl-2 (28). In this context, we have shown that the increased expression of miR-29 is associated with decreased levels of the antiapoptotic Mcl-1 in adult rat testis perinatally exposed to EB.

The second target genes of miR-29 that might drive germ cell apoptosis are DNMT. Indeed, miR-29 (particularly miR-29b) control DNMT levels not only in human normal and cancer cells (15, 29) but also in murine germ cells (30). Although paternal irradiation has been shown to lead to up-regulation of the miR-29 family in the exposed male germ line and to cause decreased expression of

de novo methyltransferase DNMT3a (31), to our knowledge, this is the first report indicating that exposure during an early neonatal period to an ED activity triggers, in an irreversible manner, the increased expression of a miR family, which modulates key epigenetic factors such as DNMT in the adult germ cells. Indeed, early postnatal exposure to EB induces a dramatic dose-dependent decrease (reduction higher than 50%) in DNMT protein levels observed at the lowest doses tested (0.75 μg/d) for both DNMT3A and DNMT3B and 1.25 μg/d for DNMT1. Our findings also indicate that DNMT3A and DNMT3B might be directly targeted by miR-29, whereas DNMT1 might be indirectly targeted by miR-29 up-regulation. Increased miR-29 levels might induce the deregulation of transcription factors involved in *Dnmt1* transcriptional regulation, consistent with the fact that it is the only one altered at the transcript level by the neonatal exposure to EB. Moreover, the up-regulation of other miRNA, such as miR-148b-3p and miR-152 (as predicted by the Target scan database), can target DNMT1 to destabilize its mRNA or inhibit its translation.

Therefore, a miR-29 increase induced changes in DNA methylation status in the testes through decreased levels in the adult (PND90) testes of *de novo* (DNMT3A and DNMT3B) and maintenance (DNMT1) DNMT. These decreased DNMT levels might be involved in the adult germ cell death process induced upon early neonatal EB exposure, given that the conditional knockout of *Dnmt3A* induced a dramatic spermatogenesis failure characterized by decreased testicular weight and azoospermia (22, 32). Moreover, the transient knockdown of *Dnmt1* in the germ stem cells (33) or of *Dnmt3a* in GC-1 cell line (this study) induced a dramatic apoptotic phenotype. The administration of DNMT inhibitors (5′-azacytidine or 5-aza-2′-deoxycytidine) to adult rats or mice induced a disruption of DNA methylation activity in spermatogenesis associated to reduced fertility decreased testicular weight, severe alterations in the seminiferous epithelium, and increased germ cell apoptosis (20). Although the precise mechanisms through which reduced DNMT can induce apoptosis in germ cells remain to be identified, one possible explanation could be that the increased expression of *L1td1* elements induces chromosomal alteration and elicits the apoptotic process in germ cell meiosis. Indeed, decreased DNMT levels induced functional consequences, such as a decrease in methylation status of some genes (*Cdkn2a*, *Gstp1*, and *L1td1*) regulated through promoter methylation (34–36) associated with an increase in their mRNA expression in adult (PND90) rats (this study). Testicular DNMT expression changes have previously been reported by at least two other laboratories in rats exposed *in utero* to the antiandrogenic fungicide vinclozolin. Firstly, a decrease in DNMT1, DNMT3a mRNA levels was observed in fetal (GD18) testes from rats exposed *in utero* to vinclozolin (100 mg/kg·d from GD8 to GD14) (12). However, DNMT protein levels were not evaluated in this study (12). Secondly, a decrease in DNMT1 mRNA and protein levels, but an increase in DNMT3b mRNA and protein levels, was reported in adult rats after *in utero* exposure to vinclozolin (100 mg/kg·d from GD14 to GD18) (37). Compared with these two studies, in our study, no change in DNMT levels at the transcriptional level (except for DNMT1 with a slight decrease in RNA levels) was observed. In the present study, the alterations are mainly related to a dramatic decline in DNMT protein levels (60–70% decrease) and in an increase in miR29, which target DNMT protein but also other factors, such as the antiapoptotic Mcl-1. Such a decrease in DNMT levels provides a basis of explanation for the adult germ cell death process as we previously mentioned (20, 22, 32). The discrepancies observed in DNMT expression between the three different studies (Refs. 12, 37 and the present study) after perinatal endocrine disruption could be attributed to 1) the use of different types of endocrine disrupters (antiandrogenic *vs.* estrogenic compounds) and 2) the period of exposure (*in utero vs.* postnatal). Although postnatal exposure to EB appears not to affect adult testicular androgen receptor mRNA and plasma testosterone levels in adult animals (our unpublished data), it will be of interest in the future to determine as to whether testosterone administration to the adult may revert the adult germ cell death process and DNMT protein levels (37).

The increased expression of the miR-29 family members, reported here, represents the highest upstream mechanisms identified to date that provide a basis of explanation for the adult germ cell death process in neonatal rats exposed to xenoestrogens. That the GC-1 germ cell line transfected with the miR-29 family members undergoes the apoptosis process strengthens such an observation. However, the remaining questions are about the upstream control of miR-29 expression, including for example the nature and role of transcription factors involved as well as the epigenetic control mechanisms (for a review see, Ref. 26) potentially altered in adult germ cells after neonatal estrogen activity disruption. Potential putative transcription factors controlling miRNA 29 expression could be identified with the use of *in silico* analysis (see www.microrna.gr/microT). Moreover, the epigenetic control of miR-29 expression alterations at the level of DNA methylation and/or chromatin remodeling processes might be at play. It is unlikely that DNA methylation changes occurring after neonatal exposure to EB deregulate miR-29 expression, because 1) *in silico* analysis (miRGen database) has shown that miR-29 members do not harbor CpG

islands in their genomic content and 2) miR-29 levels are not affected by DNMT in the GC1 cell line (as reported here). Finally, among the potential mechanisms involved, chromatin remodeling has been suggested to be at the origin of the long-term deregulation of specific miRNA after exposure to estrogenic compounds. Indeed, the *in vitro* exposure of mammary epithelial cells to diethylstilbestrol can modulate histone H3 methylation marks (H3K27me3 and H3K9me2) at the miR-9-3 gene, leading to epigenetic inactivation (38). Specifically for the miR-29 family, studies have demonstrated that during muscle differentiation, miR-29b and miR-29c expression could be regulated by the YY1 transcription factor involved in the recruitment of different histone-modifying enzymes such as histone deacetylases and Polycomb group proteins (39). It is thus tempting to speculate that in our model, neonatal exposure to EB induced histone modifications at specific miRNA clusters, leading to permanent chromatin remodeling and the transcriptional activation of specific miRNA genes. Therefore, it would be of interest to investigate the status histone marks (*e.g.* H3K27me3 and H3K9me2) and their related histone methyltransferase enzymes. As a first clue that argues for such a regulation of miRNA genes, a work previously published by our laboratory demonstrated that the neonatal exposure of mice to estrogenic compounds (EB or diethylstilbestrol) induced the downregulation of the histone methyltransferase G9a at the mRNA and protein levels and decreased H3K9me1 and H3K9me2 marks as well as the transcript accumulation of several G9a target genes (40). Another interesting point is that miR-29 is a hallmark of sexual differentiation in female but not male gonads (30). Such exposure to estrogenic compounds that reinduce the expression of miR-29 is reminiscent to the demasculinizing effects of the EED reported at hormonal levels (41, 42).

In this article, the delayed apoptotic effects on adult germ cells after neonatal exposure to xenoestrogens is an observation which is reminiscent of the Barker hypothesis (2) related to the concept of the developmental origins of health and disease (1). It is of interest to note that the changes in miR-29a, miR-29b, and miR-29c levels are not observed immediately (PND6) after the endocrine disruption between d 1 and 5 after birth but later in adulthood (PND90) concomitant to the appearance of the adult germ cell death phenotype. As expected, these delayed changes in miRNA profile were accompanied by delayed changes in their target DNMT, and Mcl-1 levels, factors actively involved in the adult germ cell, as previously indicated. Whether these delayed changes in DNMT and Mcl-1 driven by the miR-29 in the adult germ cells provide some initial bases for the explanation of the developmental origins of health and disease would be of major interest to investigate. In this context, it is noteworthy that altered miRNA patterns might be linked to disturbed developmental processes during fetal or neonatal life. For example, the high-fat treatment of gestating mice induces the long-term deregulation of several miRNA in the liver of the progeny (43). The exposure of mice to ethanol during fetal life has also been shown to induce a long-term increase of miRNA in the brains of exposed animals (44).

In summary, our data showed that early postnatal hormonal disruption induces an adult testicular germ cell apoptosis linked to the increased expression of apoptomirs (miR-29 family). The consequences of these increased levels of miR-29 are, at least, the decreased expression of DNMT and the antiapoptotic factor Mcl-1, two types of targets that elicit cellular apoptosis. The involvement of miR-29 in the adult germ cell death also makes of the miRNA a potential therapeutic target for male infertility.

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