Gestational bisphenol-A exposure lowers the threshold for autoimmunity in a model of multiple sclerosis

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Environmental and hormonal factors are implicated in dysimmunity in multiple sclerosis. We investigated whether bisphenol-A, a prominent contaminant with endocrine-disrupting capabilities, altered susceptibility in an inflammatory model of multiple sclerosis. We found that gestational, but not adult, exposure to bisphenol-A increased the development of experimental autoimmune encephalomyelitis in adulthood in male, but not female, mice when a suboptimal disease-inducing immunization was used. Gestational bisphenol-A in male mice primed macrophages in adulthood and raised granulocyte-colony stimulating factor and neutrophil counts/activity postsuboptimal immunization. Neutralizing granulocyte-colony stimulating factor blocked susceptibility to disease in bisphenol-A mice. Early life exposure to bisphenol-A may represent an environmental consideration in multiple sclerosis.

Multiple sclerosis | bisphenol A | experimental autoimmune encephalomyelitis | innate immunity | gestational exposure

M ultiple sclerosis (MS) is a debilitating demyelinating disease of the central nervous system (CNS) that includes both inflammatory and neurodegenerative processes. A prominent model that has been used to examine immunopathogenesis and drug development in MS is experimental autoimmune encephalomyelitis (EAE) (1). As in MS, a number of immune cell subsets are recruited to the CNS in EAE, including CD4+ T lymphocytes, monocyte-derived macrophages (2), and neutrophils (3–5). Biological sex is influential, as females develop MS at a higher ratio than males (6), whereas male patients tend to acquire a more aggressive phenotype (7). MS risk has genetic and environmental determinants, and a number of environmental factors such as infections and inadequate UVB exposure have been associated with the disease (8, 9). Bisphenol-A (BPA) has not been associated with MS, but it is a known endocrine-disrupting chemical with effects on immune cells (reviewed in ref. 10), including CD4+ T lymphocytes, macrophages, and neutrophils. Moreover, the incidence of MS has risen in the past 50 y (6), coinciding with the introduction of BPA into widespread industrial use. Much of the exposure to BPA comes from drinking water in heavily industrialized regions; however, there is also significant intake from food and drink containers (11) and other sources, such as thermal receipts (12). We therefore determined whether exposure to BPA alters development of EAE in mice.

**Results**

**Gestational Exposure to BPA Increases Susceptibility of Adult Male Mice to EAE When a Suboptimal Immunization Was Used.** We began our investigations in adult female 8–10-wk-old C57BL/6 mice, a strain that shows no sex susceptibility, but in which pertussis toxin (PTx) facilitates the break of tolerance for initiation of EAE (1). We exposed mice to oral 1 or 3 mg/kg BPA, doses that are guided from previous literature and were estimated to result in serum BPA concentrations equivalent to previously published levels in human sera (13–15).

Mice were gavaged daily for 18 d before immunization with a “standard” EAE protocol (16), consisting of myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 emulsified in complete Freund’s adjuvant (CFA), and with PTx administered intraperitoneally at days 0 and 2. Treatment with daily oral BPA continued for 22 d postimmunization, and mice were monitored until day 47. BPA exposure did not markedly alter EAE clinical severity from that of vehicle treatment (Fig. 14).

Early life exposure to endocrine-disrupting chemicals such as BPA represents a time of increased vulnerability to lasting changes (17). We therefore exposed adult female mice on mating to daily oral gavage with 1 or 3 mg/kg BPA or vehicle; an additional control pregnant group did not receive daily handling. BPA is thought to cross the placental barrier and has been detected in human placenta, neonatal blood, and breast milk (18). On birth, the treatment of mothers was stopped. We found no differences in the frequency of female or male offspring as a result of gestational BPA exposure (Fig. S14).

Early life BPA exposure has been linked to increased risk of being overweight (19). We examined whether mice gestationally exposed to BPA showed significant weight elevations when left undisturbed to adulthood. At 8–10 wk of age, no significant ANOVA post hoc difference in weight was found in mice when they were gestationally exposed to BPA or vehicle and compared with offspring from unmanipulated mothers (Fig. S1 B and C for female and male mice, respectively).

We left pups undisturbed until 8–12 wk of age, whereupon they were immunized with the “standard” EAE protocol (i.e., with PTx). All the offspring developed EAE, with no differences encountered in disease frequency or severity (Fig. S1 D and E), despite the gestational exposure.

We reasoned that a “standard” EAE protocol may activate robust immune responses that masked subtle intrinsic differences across groups. Continuing with the gestational BPA treatment, subsequent adult 8–12 wk mice were exposed to the MOG/CFA

**Significance**

Gestational bisphenol-A exposure increases susceptibility to autoimmunity in a mouse model of multiple sclerosis; the disease manifested in adult male, but not female, mice when a suboptimal insult was applied. Bisphenol-A may be an environmental factor encountered during embryonic life that changes the immune system, leading to a lowered threshold for development of multiple sclerosis in later life.


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immunization procedure, but without PTx (Fig. 1B). We found that although offspring from unmanipulated mothers largely did not develop EAE (0.1% of males and females; 1 of 17 mice) in the absence of PTx, as predicted (1), a small number of mice from gestational vehicle exposure succumbed to EAE (18.6% of both sexes; 8 of 43 mice), likely because of handling of the mothers during pregnancy. Notably, male mice gestationally exposed to BPA had an increased incidence (68.6%; 24 of 35 mice in both the 1 and 3 mg/kg groups) of EAE [Fig. 1 C and D; P < 0.05, Log-Rank (Mantel-Cox) test]. Unexpectedly, despite a higher prevalence of MS in the female sex, as noted earlier, an increased incidence of EAE development was not observed in gestational BPA-exposed female mice [Fig. 1 E and F; 32.4% (12 of 37 mice) in both BPA groups compared with 17.4% (4 of 23 vehicle mice); P = 0.12, Log-Rank (Mantel-Cox) test]. In gestational BPA-exposed male and female mice that succumbed to EAE clinical signs, the severity of their disease did not differ from those of vehicle controls. Histological (hematoxylin/eosin and Luxol fast blue) and immunofluorescence (CD45 leuкоcytes in venules detected by laminin α basement membranes) analyses confirm that mice that succumbed to EAE clinical signs had neuropathology in their spinal cord and cerebellar white matter, representative of EAE (Fig. 1G). Taken together, these data show that gestational exposure to BPA lowers the threshold or requirements for induction of EAE in male, but not female, mice.

We note that another group found no increased susceptibility to adult EAE after gestational BPA exposure in mice (20). However, these authors injected MOG/CFA twice, 7 d apart, in C57BL/6 mice, and the dual insult likely produced massive immune changes that masked the BPA response.

As our initial investigations with oral adult exposure to BPA were in female mice (Fig. 1A), and given the male susceptibility to gestational BPA intake, we treated previously unmanipulated adult male mice to oral 1 or 3 mg/kg BPA from day of immunization with MOG/CFA without PTx and continued with daily exposures. No significant differences were observed in incidence of EAE in response to BPA or vehicle, affirming that gestational exposure is necessary for subsequent alterations in EAE susceptibility in male mice.

Gestational BPA Exposure Alters Responses of Macrophages Cultured from Adult Male Mice. We investigated the mechanisms by which gestational BPA exposure increased future susceptibility in adult male mice to EAE. We began with gestational-exposed but nonimmunized adult male mice at 8–12 wk of age (Fig. 2A), a period in which we would normally otherwise induce EAE. In these nonimmunized mice, we found no differences in the circulating levels of monocytes, neutrophils, or lymphocytes by automated differential cell counts of blood by a hospital laboratory facility (Fig. 2B) or by flow cytometry (Fig. 2C).

Next, given the importance of CD4+ T cells in EAE (1), we examined transcripts in splenic-sorted CD4+ T lymphocytes (Fig. 2C) or mixed splenocyte populations (Fig. S3). Analyses of the expression of Ifnx21, gata3, rorc, and foxp3, key transcription factors for T helper (Th), Th2, Th17, and regulatory T cells (Tregs), respectively, did not reveal significant differences in cells from gestational BPA-exposed compared with vehicle-exposed animals, with the exception of decreased expression of rorc in sorted CD4+ T lymphocytes (Fig. 2C). We then examined receptor systems linked to immune alterations and found no significant differences in transcripts encoding peroxisome proliferator-activated receptor-γ, aryl hydrocarbon receptor, estrogen receptor α, or androgen receptor in CD4+ T lymphocytes (Fig. 2D) or mixed splenocytes (Fig. S3). These results did not provide support for obvious perturbations of CD4+ T lymphocytes in the mechanisms by which gestational BPA exposure leads to elevated autoimmunity in adult mice.

Circulating hormones have been demonstrated to alter the phenotype of immune cells and are thought to play a role in sex bias in susceptibility to MS (21). We therefore examined levels of circulating estradiol and testosterone in male and female mice that had been gestationally exposed to BPA and were now at 8–12 wk of age (Fig. 2A) and found no differences compared with their vehicle-exposed controls (Fig. S4). The levels that we recorded were similar to those previously published by others for

Fig. 1. Gestational BPA exposure increases EAE incidence in adult male, but not female, suboptimally immunized mice. (A) Adult female mice fed BPA daily do not display a markedly altered EAE course compared with vehicle. Bars represent mean ± SEM. (8) Scheme of experiments in gestationally exposed mice. Male (C and D), but not female (E and F), mice have higher incidence of EAE in adulthood when gestationally exposed to BPA (P < 0.05, Log-Rank (Mantel-Cox) test). EAE controls are mice immunized with the same MOG/CFA regimen, but without gestational perturbations. The horizontal axis in C and E refers to when the mice first manifested signs of EAE. (D and F) Each dot represents an animal that developed EAE (+) or not (−). (G) Representative (of n = 3 animals per group) spinal cord histology of MOG-immunized (no PTx) adult mice exposed to vehicle (i) or 3 mg/kg BPA (ii and iii at higher magnification of hematoxylin/eosin and Luxol fast blue; iv, Iba1). Lesions also occur in the cerebellum (v and vi) of 3 mg/kg BPA gestationally exposed mice in adulthood after MOG immunization [laminin (red)/CD45 (green)]. The number of mice in experiments is indicated in parentheses.
male (testosterone) and female (estradiol) mice (22, 23). Our results do not support obvious hormonal modifications as a mechanism by which gestational BPA increases EAE susceptibility in adult male mice.

Monocyte-derived macrophages are instrumental in the development of EAE, as their depletion in blood by use of agents such as clodronate liposomes (24) prevents the manifestation of EAE. We harvested macrophages (Fig. 2E) from the peritoneum of male mice gestationally exposed to either doses of BPA or vehicle and unmanipulated controls. Cells were then stimulated with lipopolysaccharide (LPS) for 24 h, and their cell-conditioned medium was collected for ELISA and multiplex Luminex analyses. The results show that stimulated macrophages from mice gestationally exposed to BPA produce significantly more TNF-α (Fig. 2F), granulocyte-colony stimulating factor (G-CSF), and other cytokines and chemokines in vitro (Fig. S5). Thus, gestational exposure to BPA primes macrophages that increase their inflammatory response when subsequently activated.

Altered G-CSF and Neutrophil Immunity Are Observed Early Postsuboptimal Immunization in Gestationally BPA-Exposed Adult Male Mice. We reasoned that the macrophage phenotype may be uncovered further in a disease setting after MOG/CFA (without PTx) immunization. The innate immune system is activated early in EAE, so we investigated mice 6 d postimmunization (Fig. 3A). Plasma from gestational BPA-exposed mice taken for Luminex analyses revealed an increase in levels of G-CSF (Fig. 3B, by ELISA), IL-6, IP-10 (CXCL10), and keratinocyte chemo-attractant (KC) (CXCL1) (Fig. S6); other cytokines/chemokines measured in the multiplex Luminex were not altered between groups (Fig. S6). Intriguingly, the elevated G-CSF was found in male, but not female, mice gestationally exposed to BPA (Fig. 3B), correlating with EAE susceptibility. Interestingly, G-CSF administration produces relapses in patients with MS (25, 26). In mice, G-CSF is important for the mobilization and maturation of neutrophils (27), and neutrophil recruitment to the CNS is critical for EAE development (3–5) through proposed effector mechanisms such as the disruption of the blood–brain barrier and the maturation of antigen-presenting cells (3). In parallel, neutrophils in patients with MS are found to be more numerous and in a primed state (28) with elevated levels of the CD11b integrin (29), and greater propensity to form neutrophil extracellular traps particularly in male compared with female patients with MS (30). In addition, levels of systemic neutrophil chemokines correlate with indices of brain lesion formation in MS (5), and neutrophils have been observed in areas of blood–brain barrier disruption in early lesions of MS (31).
The elevation of neutrophils and their effect on the blood–brain barrier in male mice that are gestationally BPA-exposed likely replaces the requirement of PTx for EAE induction in C57BL/6 mice, as PTx is thought to disrupt blood–brain barrier functions (1).

Although we did not observe elevation of TNF-α in the plasma of gestational BPA-exposed male mice at 6 d postimmunization (Fig. S6), our analyses by PCR of spleen RNA samples from these mice demonstrate a significant increase in TNF-α transcript compared with mice gestationally exposed to vehicle (Fig. 3C); this supports the hypothesis of a primed macrophage phenotype, a prominent source of TNF-α in spleen, resulting from gestational BPA exposure, as suggested by the in vitro peritoneal macrophage phenotype observed earlier (Fig. 2F). Thus, the primed macrophage phenotype resulting from BPA appears to be in at least the peritoneal and spleen compartments; we did not evaluate dendritic cells. In addition, we noted the elevation of transcripts encoding CXCR2, a chemokine receptor important for neutrophil chemotaxis that is expressed by mature neutrophils (32), in the spleen RNA samples from gestational BPA-exposed mice (Fig. 3C).

The above results suggest that the mechanism by which gestational BPA exposure increases the future risk for autoimmunity could be through priming macrophages that produce G-CSF on activation, and that the G-CSF then mobilizes neutrophils as effector cells. To support the involvement of neutrophils, blood cell counts from day 6 mice after MOG/CFA (and without PTx) showed that male, but not female, mice gestationally exposed to BPA had an increased number of neutrophils compared with animals with vehicle treatment (Fig. 3 D and E). The neutrophil population isolated from the bone marrow at the same point showed an increased percentage of activated neutrophils by CD11b expression, as well as increased surface expression of CD11b on these neutrophils (Fig. 3F), suggesting a greater activation state.

G-CSF plays an important role in activating and maturing neutrophils (27); furthermore, it has been previously demonstrated that IL-17 increases the production of G-CSF, and thus the number of neutrophils in circulation in mice (33). Previous data in EAE have demonstrated that when Th17 cells were adoptively transferred to naïve mice, there was increased expression of G-CSF in lesions (34). As this suggests an intimate relationship of G-CSF and IL-17-producing Th17 cells, we measured IL-17 in plasma of day 6 immunized male mice, but found no increase of this cytokine in samples with G-CSF elevation as a result of gestational BPA exposure (Fig. S6). We also found no differences of IL-17 transcripts in the lymph node of gestational BPA- or vehicle-exposed mice at day 6 postimmunization in adult animals (Fig. S7). These results indicate that the increased G-CSF in male mice gestationally exposed to BPA was not driven by IL-17 production.

Neutralizing G-CSF in Adult Male Mice Mitigates EAE Susceptibility Caused by Gestational BPA Exposure. To address the pathogenic relevance of the increase of circulating G-CSF and neutrophils in gestational BPA-exposed mice, we blocked G-CSF signaling using a function blocking monoclonal antibody. Male mice gestationally exposed to 3 mg/kg BPA were immunized with MOG/CFA (without PTx; Fig. 4A) and randomly assigned 1 d after, a time shown to have high levels of G-CSF in circulation in EAE (5), to treatment with the G-CSF function blocking antibody or control IgG. We found that the BPA-exposed mice developed EAE at a significantly decreased incidence when receiving the G-CSF antibody compared with those treated with control IgG [Fig. 4A; P < 0.05, Log-Rank (Mantel-Cox) test]. Moreover, the mean clinical score of EAE was significantly lower in the group receiving anti-G-CSF compared with control IgG (Fig. 4B). These results are in concordance with previously published
observations, in which mice genetically lacking G-CSF signaling had reduced EAE development compared with wild-type mice (5), but we now extend this to relevance in BPA pathophysiology.

Discussion

The current results raise several questions that are left to be resolved in future studies. First, what is the mechanism by which gestational BPA exposure leads to the primed macrophage phenotype in adulthood in mice? Our attempts to address this using a microRNA library scan have been unsuccessful. Second, why are male mice preferentially targeted, and how does this inform on MS, where there is a higher bias toward susceptibility in the female biological sex? We do not know the reasons for the male vulnerability to gestational BPA, but neutrophils may be more vulnerable to insults in males, and there tends to be a higher prevalence of primary progressive MS in the male sex; male patients with MS also appear to progress more rapidly than female patients with MS (7). Moreover, we do not suggest that BPA accounts for all cases of MS, other than it may increase future risk when the factors for autoimmunity in some genetically predisposed male individuals are encountered. Third, the period of exposure to BPA in our mouse study raises questions about how gestational BPA lowers the threshold for autoimmunity in adulthood. Epidemiological studies in patients with MS have suggested that early life environmental insults are encountered in MS. Early life environmental changes imprint future susceptibility to the disease. For instance, studies looking at month of birth as a marker for early life exposure to protective vitamin D have reported an inverse association of adequate vitamin D or ambient UV radiation in the first and second trimester to future development of MS (35, 36), although this association has been questioned (37). Fourth, as BPA exposure is widespread, and if BPA elevates risk for MS, why is MS not more common in the human population? Here, it is likely the case that several factors have to converge to succumb to disease, including, but not limited to, genetics, the presence of protective factors such as vitamin D that abort the initiation of detrimental immune responses, individual variability to alterations of neutrophil and G-CSF activity, and complex interactions between BPA and endocrine biology. Finally, there are several BPA replacements introduced in recent times that are meant to lower the risk for BPA exposure; whether these replacements, such as bisphenol-S, are also proinflammatory remain to be investigated.

We note a corroborative report that SJL mice exposed to BPA during gestation and through the mother’s milk during lactation had accelerated onset of clinical signs after infection at 4 wk of age with Théier’s virus, which produces CNS inflammation (38). At 20 wk postinfection, the degree of inflammation in the spinal cord was higher in BPA-exposed compared with vehicle treatment: no sex differences in the degree of inflammation were noted in the BPA mice.

In summary, we report that gestational exposure to BPA increases the future susceptibility of male mice to develop EAE when suboptimal disease-manifesting conditions are encountered. The lowered threshold of disease induction conferred by gestational BPA exposure appears to be a result of primed macrophages that produce more proinflammatory cytokines, particularly G-CSF, that then mobilize circulating neutrophils to promote disease development. The mechanisms that link gestational BPA exposure in male mice to a primed macrophage phenotype remain to be elucidated, but do not appear to be a result of obvious hormonal alterations. Moreover, the involvement of G-CSF as an effector cytokine should be more thoroughly investigated in future experiments using G-CSF conditional knockout mice; as well, the roles of other cytokines such as TNF-α should be examined. Overall, we suggest that gestational BPA exposure is an environmental factor to be considered for the development of autoimmunity in the human male population.

Materials and Methods

Animal Husbandry. All animal protocols were in accordance with the guidelines of the University of Calgary Animal Care Committee, protocol A12-0181, in line with the guidelines of the Canadian Council of Animal Care. All animals were housed in polyethylene cages (Techniplast), and mice were fed standard chow. Only paper enrichments were used in housing units; no plastic enrichment was added. C57BL/6 mice bred in-house or received from Charles River were administered BPA oral gavage. For EAE experiments in which BPA was orally administered by gavage, either to adult mice for EAE assessment or to pregnant mice as a gestational exposure for their pups, C57BL/6 mice were treated with 100 µL assigned treatment (to achieve 1 or 3 mg/kg BPA) or vehicle per day. Gestational exposure breedings were carried out at the University of Calgary.

Chemical Preparation. For administration by oral gavage, BPA (Sigma-Aldrich) was prepared by dissolution in 100% ethanol and then diluting at 0.5% ethanol (vol/vol) in corn oil to achieve treatment doses of 1 or 3 mg/kg BPA. BPA solutions in ethanol/corn oil were prepared fresh daily. Vehicle mice received 100 µL 0.5% ethanol/corn oil (vol/vol).

EAE. EAE was induced in 8–12-wk-old mice using 50 µg MOG35–55 peptide emulsified in CFA (Difco), supplemented with Mycobacterium tuberculosis (Difco) (16). Ptx (List Biologics; 300 ng) was given on day 0 and day 2 postimmunization in experiments using Ptx. For G-CSF blockade experiments, rμ-CSF (R&S Systems) or control IgG (R&S Systems) were administered i.p. at 25 µg/mouse. Mice were administered μ-CSF or control IgG at day 1 postimmunization, and every 3 days thereafter.

Mice were scored and weighed daily, using a 15-point scoring scale previously described (16). In brief, the tail is scored out of 2, with 0 being normal function, 1 weakness, and 2 complete tail paralysis. Each limb received a score out of 3, where 0 is normal; 1 is weakness in walking or inability to right themselves; 2 is increased weakness, but some movement still present; and 3 is a completely paralyzed limb. A score of 15 represents the death of an animal.

Preparation of Splenocytes and CD4+ T Lymphocytes. Spleens were isolated from 8–12-wk-old C57BL/6 mice and dissociated by applying pressure by hand and filtration through a 70-µm culture filter (Falcon) in PBS. Splenocytes were subjected to ficoll-plaque IGE Healthcare) gradient centrifugation for 30 min at 680 × g. The "buffy layer" containing a mixed splenocyte preparation was isolated and washed in 10 mL PBS and centrifuged for 10 min at 300 × g. Mixed splenocytes were either placed into a Trizol (Life Technologies) solution for subsequent RNA isolation or were further...
processed using an EasySep negative selection kit (STEMcell) according to the manufacturer’s protocol to isolate CD4+ cells.

**Peritoneal Macrophages.** Mice were anesthetized with a combination of 200 mg/kg ketamine and 10 mg/kg xylazine. Five milliliters ice-cold HBSS (Gibco) was injected into the peritoneal cavity, and the area was gently massaged for 3–5 min to loosen cells from the wall of the peritoneum. Cells were pelleted and resuspended in 10 mL complete DMEM, supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 1% sodium pyruvate, and 1% MEM nonessential amino acids. Cells were plated on a 10-cm dish for 24 h, after which the adhered cells were collected by scraping and plating into wells of a 96-well flat-bottom plate. Medium was replaced with DMEM containing 1% FBS and the supplements mentioned earlier for experiments. Cells were allowed to rest in new medium for 1 h and were activated with 10 ng/mL LPS.

**Cytokine Analysis.** TNFα (Invitrogen), G-CSF (Thermo Fisher), estradiol (Calbiotech), and testosterone (Calbiotech) quantification was conducted by ELISA, as directed by the manufacturer. Luminex-multiplex analysis was conducted by Eve Technologies according to their protocol for mouse cytokine array/chemokine array 31-plex.

**Analysis of Circulating Leukocytes.** Blood collected via cardiac puncture of anesthetized mice was added to EDTA-coated tubes (BD Microtainer) and analyzed by Calgary Lab Services.

**Flow Cytometry Analysis of Circulating Monocytes and Neutrophils.** Blood collected through cardiac puncture was diluted with HBSS, exposed to Fc block (c Guinea/32, BD Biosciences), and the following antibodies were used to stain monocytes: CD45-PerCP (BD Biosciences), CD11b-FITC (BD Biosciences), Ly6C-APC (BD Biosciences), and CD115-PE (eBiosciences; all at 1:50). For staining neutrophils, CD45-PerCP (BD Biosciences) and Ly6G-APC (BD Biosciences) were added. After incubation for 30 min, red blood cell lysis buffer (BD Pharm Lyse, 1x) was added briefly, cells were washed and fixed in 1% buffered formalin for flow cytometry analysis. Data were analyzed using FlowJo 10.0.8 (FlowJo).

**PCR Analysis.** Cells for PCR were homogenized in TRIzol and maintained at –80 °C until time of RNA isolation, using an RNeasy minikit (Qiagen). RNA was converted to cDNA for PCR analyses, where amplifications were performed using iCycler thermocycler (Bio-Rad). Primers used were rorc (QT0019772), tbx21 (QT00129822), gata3 (QT00170828), foxp3 (PM0549F2-200), pparγ (QT00100296), ahr (QT00174251), eno (QT00132021), tnfα (QT0014006), and ccr2 (QT00283696). All analyses were compared with the same housekeeping gene (gapdh (PM0296E)). All primers were acquired from Qiagen.

**Statistical Analysis.** Analyses were conducted using Prism 6 for Mac OS X (GraphPad Software Inc). The particular statistical test used is described in the legend to figures. All data sets were analyzed with an alpha of 0.05.

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