Prenatal Exposure to Unconventional Oil and Gas Operation Chemical Mixtures Altered Mammary Gland Development in Adult Female Mice

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Unconventional oil and gas (UOG) operations, which combine hydraulic fracturing (fracking) and directional drilling, involve the use of hundreds of chemicals, including many with endocrine-disrupting properties. Two previous studies examined mice exposed during early development to a 23-chemical mixture of UOG compounds (UOG-MIX) commonly used or produced in the process. Both male and female offspring exposed prenatally to one or more doses of UOG-MIX displayed alterations to endocrine organ function and serum hormone concentrations. We hypothesized that prenatal UOG-MIX exposure would similarly disrupt development of the mouse mammary gland. Female C57Bl/6 mice were exposed to ~3, ~30, ~ 300, or ~3000 μg/kg/d UOG-MIX from gestational day 11 to birth. Although no effects were observed on the mammary glands of these females before puberty, in early adulthood, females exposed to 300 or 3000 μg/kg/d UOG-MIX developed more dense mammary epithelial ducts; females exposed to 3 μg/kg/d UOG-MIX had an altered ratio of apoptosis to proliferation in the mammary epithelium. Furthermore, adult females from all UOG-MIX–treated groups developed intraductal hyperplasia that resembled terminal end buds (*i.e.,* highly proliferative structures typically seen at puberty). These results suggest that the mammary gland is sensitive to mixtures of chemicals used in UOG production at exposure levels that are environmentally relevant. The effect of these findings on the long-term health of the mammary gland, including its lactational capacity and its risk of cancer, should be evaluated in future studies. (***Endocrinology*** **159: 1277–1289, 2018)**

Unconventional oil and gas (UOG) operations combine hydraulic fracturing (fracking) and directional drilling. These techniques were developed to collect deposits of oil and natural gas found in deep underground shale beds in low-permeability geologic formations (1). During the fracking process, a mixture of water and chemicals is pumped deep into the shale bed under high pressure, fracturing the reservoir rock, and releasing deposits of gas and/or oil, which can then be recovered at the surface. More than 1000 different chemicals are reportedly used during UOG operations for a range of purposes, including compounds that act as bactericides, stabilizers for the clay in the ground, chemicals that alter friction and fluid viscosity, and others, although each individual site typically uses only 12 to 24 of these compounds. During UOG operations, up to several million gallons of water are injected per well, and a mixture of injected fluids and target formation water are collected throughout the life of the producing well. With >17 million Americans living within 1 mile of an oil and gas well (2), concerns have been raised about the possibility of contamination of surface and groundwater by the released oil and gas, the numerous inorganic compounds that are liberated from target geologic layers (*e.g.*, trace metals, radioactive isotopes, minerals), and the chemicals used in well injection (3, 4).

More than 1000 chemicals have been identified in hydraulic fracturing fluids and waste water and/or have

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Abbreviations: AR, androgen receptor; EDC, endocrine-disrupting chemical; ER, estrogen receptor; PND, postnatal day; TEB, terminal end bud; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; UOG, unconventional oil and gas; UOG-MIX, 23-chemical mixture of unconventional oil and gas compounds.

been reported to be used by the industry (5, 6). Many of these chemicals are known developmental and reproductive toxicants (7). Furthermore, recent evaluations found that >100 of these chemicals are known or suspected endocrine-disrupting chemicals (EDCs) (4, 8–10) (*i.e.*, compounds that interfere with hormone action) (11). Water samples collected in drilling-dense or UOG wastewater-affected areas of the United States have exhibited disruption of the estrogen, androgen, progesterone, glucocorticoid, and thyroid receptors (4, 8, 12).

In 2015, Kassotis *et al.* (13) evaluated 24 chemicals that were reported by industry as commonly used and/or produced by UOG operations to determine whether they displayed endocrine-disrupting properties. With cell-based reporter gene assays, their study revealed antiandrogenic, antiestrogenic, antiprogestogenic, antithyroidogenic, and antiglucocorticogenic activities for many of these compounds. When evaluated as mixtures, additive and, in some cases, synergistic antagonism of these receptors was also observed. Kassotis *et al.* (13) then evaluated the effects of a 23-chemical mixture of UOG chemicals (UOG-MIX) on male mice. This mixture included the 24 chemicals originally assessed, absent bisphenol A, a well-characterized EDC that has been evaluated at length previously (14, 15) but that is not directly used in UOG extraction, as reported by the industry (16). Only one of the chemicals included in this list (Table 1), benzene, was evaluated in a review of 216 chemicals for carcinogenic effects in the mammary gland, highlighting the general lack of knowledge on these chemicals (17). Male mice exposed to environmentally relevant doses of UOG-MIX during prenatal development displayed an increased testicular weight before puberty and, in adulthood, decreased sperm counts, increased serum testosterone concentrations, and alterations to the weight of additional organs, including the heart and thymus (13). A second study revealed the effects of developmental exposures to UOG-MIX on the female siblings (18). Exposed females had alterations to the number and developmental stages of ovarian follicles measured before puberty and in adulthood. The weight of several organs, including the uterus, ovary, and heart, was also affected. Furthermore, the serum concentrations of several hormones were disrupted in these exposed females. These results suggest the possibility that other hormone-sensitive organs might be disrupted by exposure to UOG chemical mixtures.

The mouse mammary gland has proved to be an excellent model to study the effects of EDCs (19–21). The development of the mammary gland is dependent on estrogen, progesterone, prolactin, testosterone, and growth hormone, making it an integrated biological endpoint that is sensitive to the agonists and antagonists of different hormone receptors (22). The estrogen receptor (ER) is expressed in the mesenchymal compartment of the fetal gland; however, its expression shifts to the epithelial compartment in the adult gland (23). Although ERα knockout mice have mammary glands that are indistinguishable from those of wild-type controls before puberty, they are visibly stunted compared with controls once puberty begins, highlighting the importance of estrogen for growth of the gland (24, 25). The androgen receptor (AR) is also expressed in the mesenchyme of the fetal mammary gland, and the testosterone produced by the testes in male fetuses causes the mesenchyme to condense around the epithelium, detaching the epithelium from the skin. Thus, the male mice will typically not have nipples (26, 27). Antiandrogenic compounds can lead to nipple retention in exposed males (28). Testosterone is likely to have a physiological role during postnatal development of the female gland because the mammary glands of female AR knockout mice show impaired ductal growth in postnatal life, indicating a role in ductal elongation (29). A wide range of EDCs, including several pharmaceutical compounds, naturally occurring EDCs, and industrial compounds with varied modes of action, have been demonstrated to alter the development of the

Table 1. Chemicals in UOG Mixture and Hormone Receptor Antagonist Activity, as Listed in (13)

	Receptor Antagonist Activity				
Chemical Name	**ER**	**AR**	**PR**	**TR**	**GR**
1,2,4-Trimethylbenzene					
2-(2-Methoxyethoxy) ethanol	X	X			X
2-Ethylhexanol	X	X	X		X
2-Methyl-4-isothiazolin-3-one	X	X			X
Acrylamide		X			
Benzene	X	X			
Bronopol	X	X	X		X
Cumene	X	X	X		
Diethanolamine	X	X	X		
Ethoxylated nonylphenol	X	X	X	X	X
Ethoxylated octylphenol	X	X	X	X	X
Ethylbenzene	X	X			
Ethylene glycol	X	X	X	X	X
Ethylene glycol monobutyl ether	X	X		X	
Naphthalene	X	X	X	X	X
N,N-dimethylformamide	X	X	X		
Phenol	X	X	X		
Propylene glycol	X				
Sodium tetraborate decahydrate	X	X			
Styrene	X			X	X
Toluene	X	X			
Triethylene glycol	X	X			

X indicates the presence of receptor antagonist activity as measured via transiently transfected reporter gene assays in human cells.

Abbreviations: GR, growth receptor; PR, progesterone receptor; TR, thyroid receptor.

mammary gland, with effects that typically manifest at puberty and in adulthood, when endogenous hormones induce its growth (20, 30). The characteristics that are commonly evaluated to determine the level of development and the disruption of development by EDCs include a number of branching points, extension of the ductal epithelium into the stroma, the number and size of terminal end buds (TEBs) (*e.g.,* highly proliferative epithelial structures present during puberty), and the presence of alveolar buds and lobuloalveolar units (*e.g.,* structures that will produce milk in lactating females), among others (31).

Based on the hormone receptor antagonism of the 23-chemical mixture and the effects this mixture induced in the endpoints relevant to the hypothalamic-pituitary-gonadal axis in exposed mice, we hypothesized that prenatal exposure to the UOG-MIX would disrupt development of the mouse mammary gland. We evaluated the mammary glands in female mice after gestational exposure to one of four doses of the UOG-MIX. Consistent with our hypothesis, we characterized the substantial effects of this mixture on mammary gland morphology in adulthood and documented the presence of hyperplastic lesions.

Materials and Methods

Animal husbandry and chemical administration

C57BL/6J mice were housed in sterile polysulfone cages under temperature- and light-controlled (12-hour light, 12-hour dark) conditions in a barrier animal facility. The mice were fed LabDiet 5053 and provided acidified water *ad libitum* from glass bottles. Ten-week-old mice were mated, and the day of vaginal plug formation was denoted as gestational day 0. On gestational day 11, the dams were randomized to the treatment groups and provided with experimental treatment in their drinking water. The test concentrations included a 0.2% ethanol vehicle; flutamide, a known antiandrogenic pharmaceutical agent (mechanistic antiandrogen control, 167 μg/mL); and four concentrations of the 23-chemical mixture (UOG-MIX), with each individual chemical present at 0.01, 0.10, 1.0, and 10 μg/mL. The composition of the chemical mixture and hormone receptor antagonist activity are listed in Table 1. Water intake was monitored by weighing the drinking water bottle every day of the experiment. Based on intake, the chemical exposure was estimated at 3, 30, 300, or 3000 μg/kg body weight/d for the mixtures. These treatment groups are referred to as MIX-3, MIX-30, MIX-300, and MIX-3000, respectively. Intake of the antiandrogenic control was estimated at 50 mg flutamide/kg body weight/d. Water intake did not differ in experimental groups relative to the vehicle control (data not shown). Experimental treatment was provided until birth; the dams were then reverted to standard acidified water when the pups were first detected. Litters with <2 males and females were removed from the analyses owing to concerns of gestational hormone exposure. Litters with >2 males and females were left unaltered, because culling within litters has been shown to alter the feeding, behavior, and physiology of the remaining pups (32).

All experimental procedures were performed according to an approved University of Missouri Animal Care and Use Committee protocol and were in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals*.

Tissue collection

All mice were euthanized by carbon dioxide asphyxiation and cardiac puncture, and the mammary tissues were excised. One randomly selected female pup from each litter underwent necropsy on either postnatal day (PND) 21 or PND85. At both ages, one fourth of the inguinal mammary gland was whole mounted on a glass slide, fixed in neutral-buffered formalin overnight, and processed for carmine staining using protocols described previously (33). Whole mounts were preserved in K-pax sealed bags with methyl salicylate. The contralateral fourth inguinal mammary gland was formalin-fixed for 24 hours, washed in phosphate-buffered saline, and stored in 70% ethanol for histological evaluation.

Morphological analysis of whole mount mammary glands

Whole mount specimens were imaged using a Zeiss dissecting microscope with AxioCam HRc digital camera. For analyses of the prepubertal mammary gland (PND21), images were captured of the full ductal tree and its position relative to the central lymph node. The following measurements were taken using methods developed previously (33): total ductal area, measured by quantifying the area subtended by the ducts; ductal extension, quantified as the furthest growth of the ductal tree measured from the center of the lymph node; and the number of branching points, counted throughout the entire gland. No mice had visible TEBs; thus, these structures were not quantified.

For analyses of the adult gland, two photographs were taken, one of the entire mammary gland (at ×3 magnification) and one anterior to the central lymph node (at ×13.5 magnification); the former was used to evaluate the presence of TEB-like structures and the latter for unbiased stereological evaluations using ZEN imaging software (Zeiss). In brief, to quantify the volume fraction of epithelial structures, a 130-point grid was superimposed over each anterior photograph. The structure present on each crosshair was counted; the individual epithelial structures that were evaluated included ducts (when the crosshair hit the middle of a duct), terminal ducts/terminal ends (when the crosshair hit a blunt end of a duct), and alveolar buds (34). The volume fraction of each type of structure was calculated by counting the number of crosshairs that hit each structure divided by the total number of crosshairs hitting the mammary tissue (typically, 130). Volume fraction of all epithelium was calculated by summing all epithelial structures (*i.e.,* ducts, terminal ends, and alveolar buds).

Excision of mammary tissue from whole mounts

For whole mounts that displayed unusual or TEB-like structures, small areas of the gland, including these abnormal structures, were excised using a scalpel and dissecting microscope. The remainder of the whole mount was then rebagged using methyl salicylate. The excised parts of the whole mounts were washed, processed through a series of alcohols, embedded in paraffin, and sectioned using the methods described in the

following sections. Additional areas from the whole mount specimens with a normal appearance were also excised for use as control tissues. All excised tissues were coded such that additional analyses could be conducted by experimenters who were unaware of their origin (*e.g.*, unusual vs normal).

Histological evaluation

One mammary gland from each sample was processed through a series of dehydrating alcohol washes and embedded in paraffin under vacuum; 5-μm sections were produced using a rotary microtome and placed on Superfrost positively charged slides (Fisher Scientific). This process was also used to evaluate excised tissue from whole mount mammary glands. For the histological evaluations, the sections were deparaffinized in xylene and rehydrated in a series of alcohol washes. They were then stained using Harris hematoxylin and eosin (Fisher Scientific), dehydrated through an alcohol series, washed in xylene, and mounted using a permanent mounting medium (Fisher Scientific). The slides were examined using a Zeiss Observer Z1 inverted light microscope at ×40 magnification. Images were captured using an AxioCam HRc digital camera and evaluated with ZEN imaging software (Zeiss) for the presence of hyperplastic ducts.

Immunohistochemistry

Immunohistochemical analysis for ERα and Ki67, a marker of proliferation, was performed as previously described (34). Primary antibodies were used at 1:1000 (ERα; catalog no. 06-935; Millipore; Ki67; catalog no. 9106-S1; Thermo Fisher Scientific; Table 2). For feasibility, the samples were examined in the control, MIX-3, and MIX-3000 groups only; these groups were selected to evaluate a wide range of doses. The samples were visualized using a Zeiss Observer Z1 inverted light microscope at ×40 magnification, and the images were captured using an AxioCam HRc digital camera. The images were analyzed using ZEN imaging software (Zeiss). For each sample, two or three fields of view were selected arbitrarily (depending on the size of the epithelial ducts) and imaged at ×40; the expression of each marker was quantified in all ducts within these images. At least 200 epithelial cells were assessed for each antigen; each cell was counted as either positively expressing the marker of interest (brown owing to diaminobenzidine, the colorimetric reaction used to visualize immunohistochemical reactions), or no expression (blue; hematoxylin counterstain).

Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling assay

The Trevigen TACS 2 TdT-DAB *in situ* apoptosis detection kit was used for detection of apoptotic cells in the mammary tissue sections. All samples were counterstained with Harris hematoxylin, dehydrated, mounted with a permanent mounting medium, and imaged with a Zeiss Observer Z1 inverted light microscope at ×40 magnification. Quantification of terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) was completed in two or three fields of view selected arbitrarily; all ducts within these images were evaluated. At least 200 epithelial cells were assessed; each cell was counted as either TUNEL-positive (brown owing to diaminobenzidine, the colorimetric reaction used to visualize the TUNEL reactions) or no expression (blue; hematoxylin counterstain).

Statistical analysis

The SPSS statistical software package, version 22 (IBM Corp.), was used for all statistical analyses. Analysis of variance followed by Fisher *post hoc* tests, was used to assess for differences between the control and UOG-MIX treatment groups for each age (PND21 and PND85). Independent samples *t* tests were used to compare the control and flutamide-treated groups (35). A χ^2 test was performed to compare the incidence of hyperplasia in the mammary gland epithelium of control and UOG-MIX–exposed mice. To account for litter effects, only 1 mouse was selected from each litter for each age evaluated. For all statistical tests, the results were considered statistically significant at $P < 0.05$. All results are presented as the mean ± standard error of the mean and were collected and analyzed by experimenters who were unaware of the treatment.

Results

Prepubertal mammary gland morphology was not altered by developmental exposure to UOG-MIX

The mice were exposed to vehicle or one of four doses of a 23-chemical mixture (UOG-MIX) during gestation. To determine the effects of the UOG chemicals on the female prepubertal mammary gland, morphological evaluations were first conducted at PND21. TEBs were not present in any glands from any treatment group, consistent with the prepubertal stage of mammary gland development (Fig. 1A; data not shown). Morphometric evaluations revealed an inverse association between treatment and total ductal area (*e.g.*, smaller epithelial trees in UOG-MIX–treated females), although no statistically significant differences were found in this growth parameter between controls and the UOG-MIX treatments (Fig. 1B). Flutamide-treated females had significantly smaller ductal trees compared with the controls

Table 2. Antibody Table

Peptide/ Protein Target	Antigen Sequence (if Known)	Name of Antibody	Manufacturer; Catalog No.	Species Raised in; Monoclonal or Polyclonal	RRID	Dilution Used
ERα		Anti-ERα (C1355)	Millipore; 06-935	Rabbit; polyclonal	AB_310305	1:1000
Ki67		Ki67	Thermo Fisher Scientific; RM-9106-S1	Rabbit; monoclonal	AB_149792	1:1000
Secondary		Biotinylated goat anti-rabbit IgG	Abcam; ab64256	Goat; polyclonal	AB_2661852	Ready to use (5 μg/mL)

Abbreviations: IgG, immunoglobulin G; RRID, Research Resource Identifier.

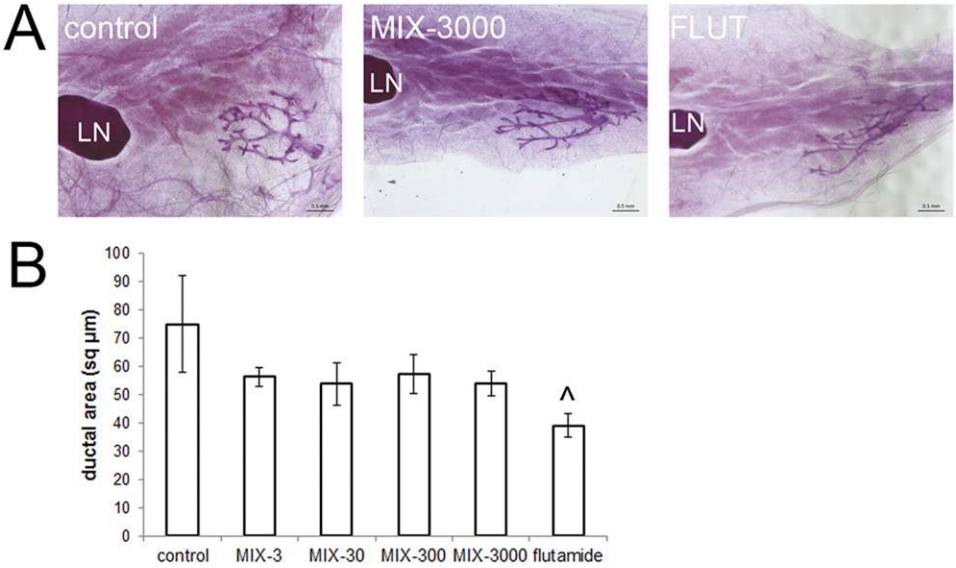

Figure 1. No substantial effects from the developmental exposure to fracking mixtures were observed on the morphology of the prepubertal mammary gland. (A) Examples of whole mount mammary glands from vehicle, MIX-3000, and flutamide (FLUT)-treated female mice. Scale bar = 0.5 mm. (B) Quantification of ductal area suggested an inverse relationship between UOG-MIX treatment and ductal area, although this difference was not statistically significant. The flutamide-treated female mice had significantly smaller ductal trees. ^$P < 0.05$, independent samples *t* test, comparing control and flutamide-treated female mice. LN, lymph node.

($P < 0.05$; independent samples *t* test; Fig. 1B). Ductal extension and the total number of branching points were not different in any treatment group, including the flutamide-treated females (Table 3). Collectively, these data suggest that prenatal exposure to the 23-chemical mixture did not alter the morphological features of the female mammary glands before puberty.

Adult mammary gland morphology was altered by developmental exposure to UOG chemical mixtures

Increased volume of ducts and epithelial compartment

Unbiased stereological methods revealed treatment-related effects on mammary gland morphology in early adulthood (PND85; Fig. 2A). Females exposed to MIX-300 had significantly more ducts compared with vehicle-treated controls ($P < 0.05$, Fisher *post hoc* test; Fig. 2A and 2B), and a trend for an increase was also seen in females exposed to MIX-30 and MIX-3000 ($P < 0.1$, Fisher *post hoc* test; Fig. 2A and 2B). The volume fraction of total mammary epithelium was significantly increased in females exposed to MIX-300 and MIX-3000 ($P < 0.05$, Fisher *post hoc* test; Fig. 2A and 2C). Neither of these parameters were significantly altered by flutamide treatment. Alveolar buds were not observed in females of any treatment group (Fig. 2A; data not shown).

Table 3. UOG-MIX Treatment Did not Alter Growth Parameters in Prepubertal Mammary Gland

Variable	Ductal Extension (mm)[a]	Number of Branching Points
Control (n = 6)	−13.64 ± 1.07	25.0 ± 2.2
MIX-3 (n = 8)	−14.19 ± 0.96	24.9 ± 2.1
MIX-30 (n = 5)	−12.96 ± 0.43	20.8 ± 2.3
MIX-300 (n = 6)	−15.74 ± 1.46	25.7 ± 2.5
MIX-3000 (n = 6)	−13.88 ± 0.34	26.2 ± 2.4
Flutamide (n = 9)	−14.03 ± 0.84	20.9 ± 1.4

[a]Negative values for ductal extension indicate ductal trees that have not yet grown past the central lymph node.

Increased proliferation/apoptosis ratio

The growth of the mammary epithelium is dependent on a balance of proliferation (to extend ductal structures into the mammary fat pad) and apoptosis (to produce hollow ducts capable of transporting milk) (36). To quantify apoptosis in the epithelium of the adult female mammary glands, TUNEL staining was used to compare the controls, MIX-3, MIX-3000, and flutamide-treated mice. No statistically significant changes in TUNEL incorporation were seen in MIX-3 and MIX-3000 females (Fig. 3A and 3B). A borderline statistically significant decrease was seen in TUNEL-positive cells in the flutamide-treated group ($P = 0.052$, independent samples *t* test; Fig. 3B).

We next evaluated proliferation in the mammary epithelium using antibodies for Ki67, a marker of proliferation. Although the proliferation levels were low, as expected for adult mammary glands, we observed statistically significant effects of the 23-chemical mixture on the number of cells expressing Ki67 in the MIX-3 group ($P < 0.05$; Fisher *post hoc* test; Fig. 3A and 3C). Females

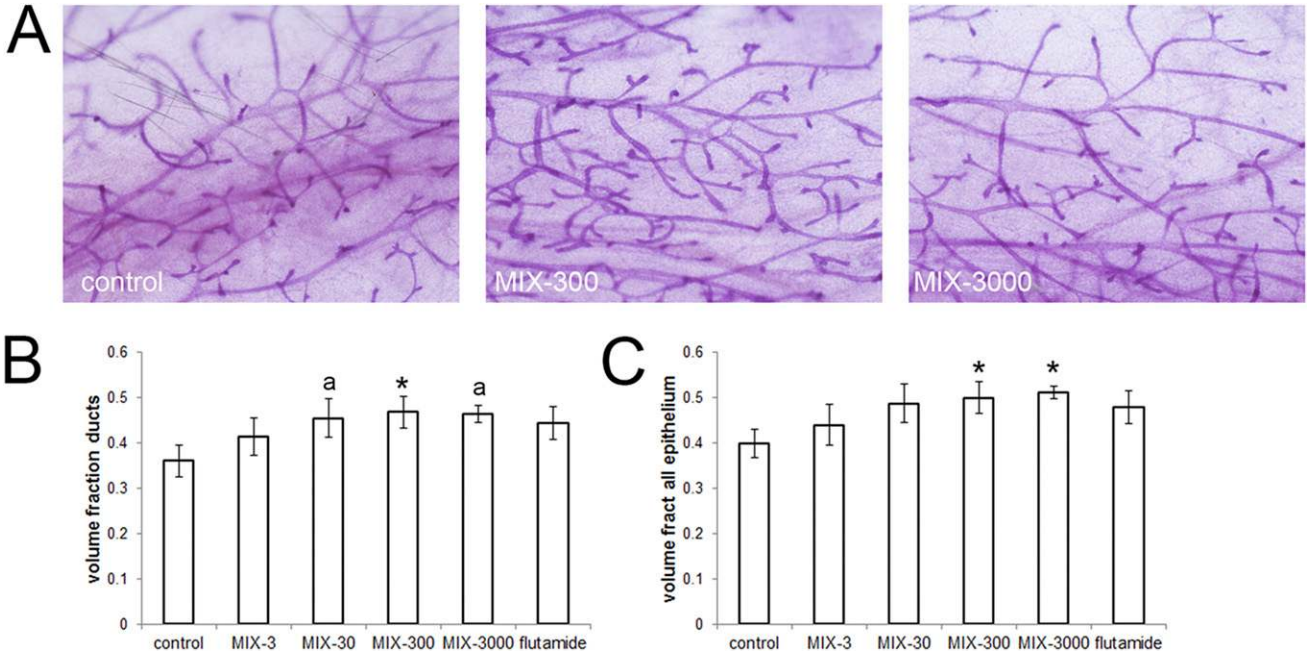

Figure 2. Prenatal UOG-MIX treatment induced increased epithelial density in female mammary glands at adulthood. (A) Example of whole mount mammary glands (magnification ×13) from control, MIX-300, and MIX-3000 treatment groups on PND85. Vehicle-treated females had the least dense mammary epithelium. Note that alveolar buds were not observed in any treatment group. (B) Volume fraction of ducts and (C) volume fraction of all epithelium was increased in UOG-MIX–treated groups. $^{*}P < 0.05$, Fisher *post hoc* test; $^{a}P < 0.1$, Fisher *post hoc* test.

from the MIX-3 group had 427% more Ki67-positive cells compared with the controls. The MIX-3000 treatment group had 54% more Ki67-positive cells compared with the controls, although these differences were not statistically significant. Ki67 expression was not altered by the use of flutamide (Fig. 3C).

We evaluated the ratio of proliferation/apoptosis in the control, MIX-3, MIX-3000, and flutamide-treated females. We found a striking 397% increase in the proliferation/apoptosis ratio in the MIX-3 treatment group ($P < 0.05$; Fisher *post hoc* test) and nonsignificant increases in the MIX-3000 group (131%) compared with the controls (Fig. 3D). No effect on the proliferation/apoptosis ratio was observed in the flutamide-treated females (Fig. 3D).

ERα expression tended to be associated with developmental UOG-MIX treatment

EDCs have been shown to not only bind to hormone receptors, but also to alter expression of hormone receptors in a dose-, age-, and tissue-specific manner (37). We next asked whether developmental exposure to fracking chemicals would alter expression of ERα in the mammary epithelium. We observed a general increase in ERα expression with an increasing UOG-MIX dose, although these differences were not statistically significant (Fig. 3A and 3E). Flutamide also did not affect the percentage of epithelial cells expressing ERα (Fig. 3E).

TEB-like intraductal hyperplasia in mammary glands after developmental mix treatment

A striking observation made during the morphological assessment of the adult (PND85) mammary glands was the appearance of structures that resembled TEBs (Fig. 4). These TEB-like structures were not observed in the control females (Table 4). The unusual structures were excised from whole mount mammary glands and further characterized using histological and immunohistochemistry tools (Fig. 5). Hematoxylin and eosin staining revealed ducts with excessive layers of epithelial cells, consistent with intraductal hyperplasia (Fig. 5A). These TEB-like lesions were highly proliferative: >40% of epithelial cells in lesions were Ki67-positive compared with <4% of epithelial cells in normal ducts collected from the same mice or ducts excised from control females (Fig. 5A and 5B). ERα expression was also greater in many, although not all, of the excised lesions (Fig. 5A and 5C). Collectively, these results suggest that the retained TEB-like structures are highly proliferative intraductal hyperplasias that are likely to be estrogen-responsive.

Discussion

We examined the effects of exposure to a mixture of 23 chemicals used in unconventional oil and gas extraction, most of which were previously shown to exhibit antagonistic properties on one or more hormone receptors [(13) and Table 1], including the ER, progesterone

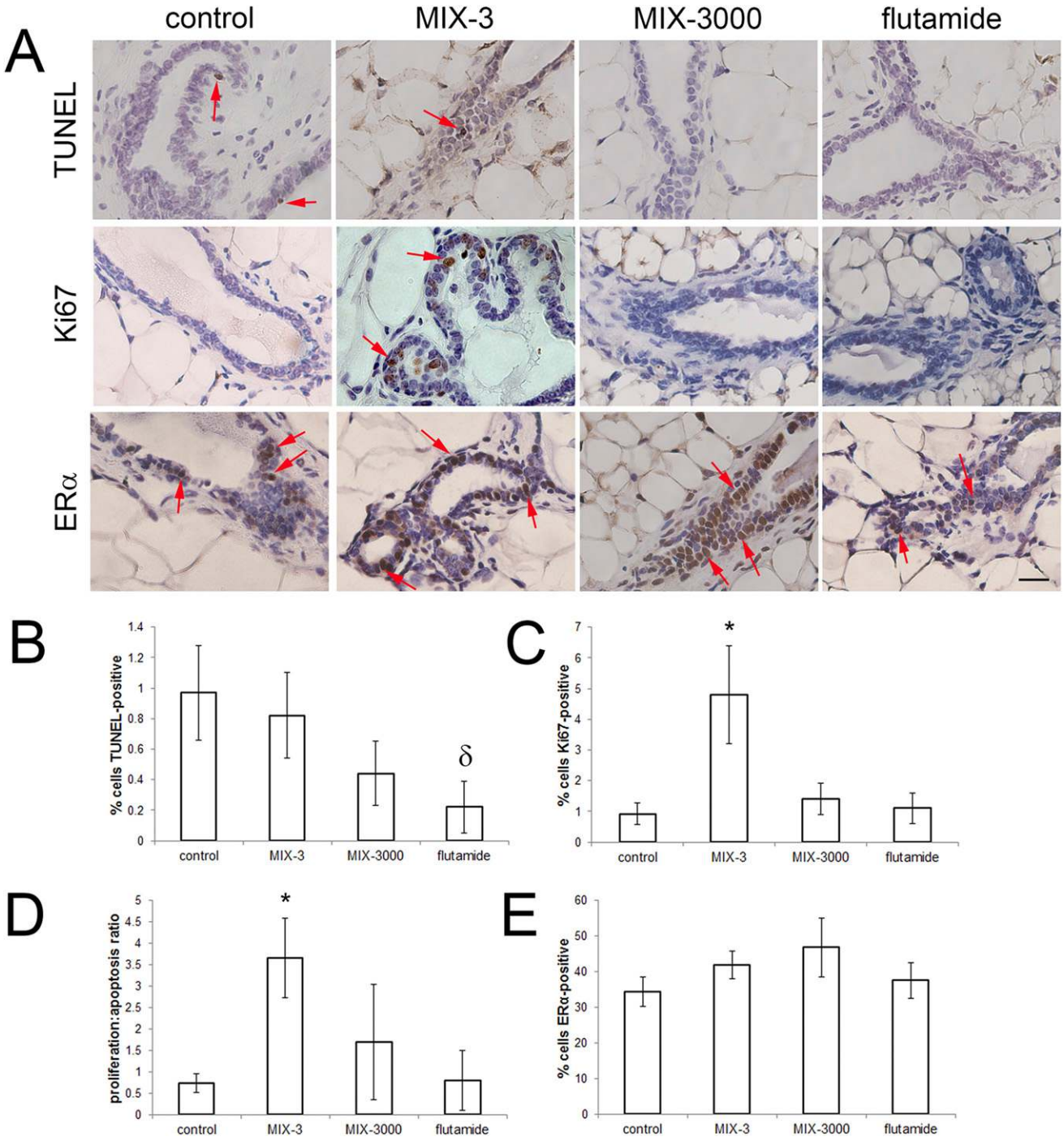

Figure 3. Apoptosis, proliferation, and proliferation/apoptosis ratios were altered in UOG-MIX– and flutamide-treated females. (A) Examples of TUNEL and immunohistochemistry testing for Ki67 and ERα in mammary gland sections from control and MIX-3–, MIX-3000–, and flutamide-treated females. Positive cells indicated by red arrows. Scale bar = 20 μm. (B) Quantification of TUNEL incorporation in epithelium from vehicle-, MIX-3–, MIX-3000–, and flutamide-treated females revealed a dose-dependent decrease in the percentage of epithelial cells undergoing apoptosis. (C) Quantification of Ki67, a marker of proliferation, in epithelial cells. (D) Substantial alterations to the ratio of cell proliferation and cell death were observed in both MIX-3 and MIX-3000 treatment groups and the flutamide-treated group. (E) Quantification of the percentage of mammary epithelial cells expressing ERα. $^*P < 0.05$, Fisher *post hoc* test; $\delta P < 0.1$, independent samples *t* test, comparing control and flutamide-treated females.

receptor, and AR. Also, 21, 20, and 11 of these chemicals were previously demonstrated to antagonize human ER, AR, and PR, respectively, in a reporter gene assay in human endometrial cells (summarized in Table 1). To the best of our knowledge, we have shown for the first time that the mouse mammary gland is sensitive to developmental UOG-MIX exposure, with dose-specific effects on tissue morphology (after exposure to MIX-300 and MIX-3000), cell proliferation (after exposure to MIX-3), and the induction of unique intraductal hyperplasias

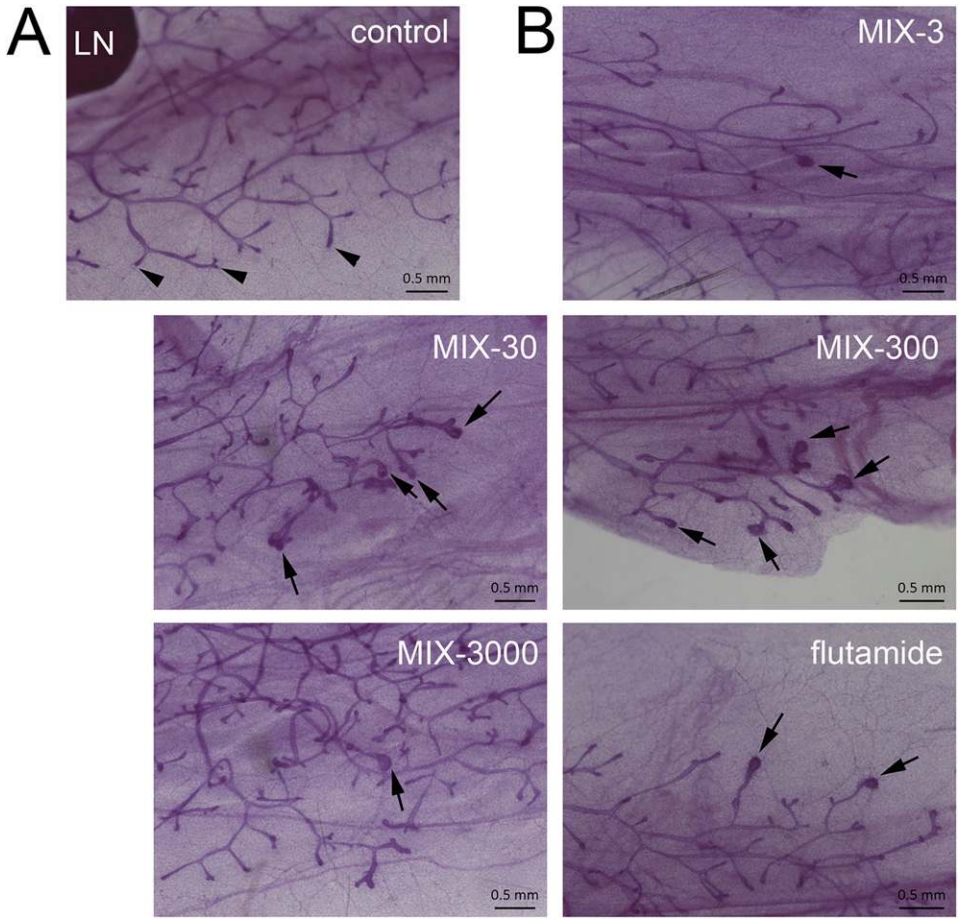

Figure 4. TEB-like structures observed in whole mount mammary glands from prenatally UOG-MIX–treated females on PND85. (A) Micrograph of whole mount mammary gland from a control mouse with typical terminal ducts. These blunt ends (arrowheads) are the normal structures observed at the ends of ducts. (B) These examples illustrate the TEB-like structures excised from whole mounts from UOG-MIX– and flutamide-treated females. Arrows indicate abnormal structures. Treatment groups for the individual female mice are indicated. LN, lymph node.

(observed in all MIX groups). The effects observed in the present study occurred at low doses; the two lowest dose groups (MIX-3 and MIX-30) are equivalent to the concentrations measured in drinking water in regions experiencing drilling and the highest dose group (MIX-3000) is equivalent to the concentrations of many UOG-MIX components measured in industry wastewater (4, 13, 18, 38). In addition, the concentrations for several of the 23 chemicals in the UOG-MIX used in the present study have not yet been determined in either drinking water or wastewater.

Table 4. Presence of TEB-Like Structures in Control or UOG-MIX–Treated Females

Variable	TEB-Like Structures, %
Control	0 (n = 9)
MIX-3	29 (n = 7)
MIX-30	17 (n = 6)
MIX-300	25 (n = 8)
MIX-3000	40 (n = 5)
Flutamide	22 (n = 9)

The mammary gland is a hormone-sensitive organ that is responsive to multiple endocrine inputs during early development. Testosterone has a unique role in establishing the sexually dimorphic development of the mouse mammary gland (26, 27) but is not thought to play a role in the female or postnatally. AR expression remains high in the mammary stroma until birth (26, 39), suggesting that UOG-MIX exposure could affect mammary development via actions at the AR. Although some endpoints were similarly affected (*e.g.*, retention of TEBs), other effects of UOG-MIX exposure we observed in the present study were distinct from the effects of flutamide, suggesting that the UOG mixture might not working through an antiandrogenic mechanism as we originally hypothesized. To date, few studies have evaluated the effects of prenatal exposures to antiandrogenic chemicals on the female mammary gland; most studies of antiandrogens have examined their effects on male offspring, including nipple retention and other disruptions to mammary gland morphology [reviewed in (40–42)]. These studies suggested that evaluations of mammary glands in male mice

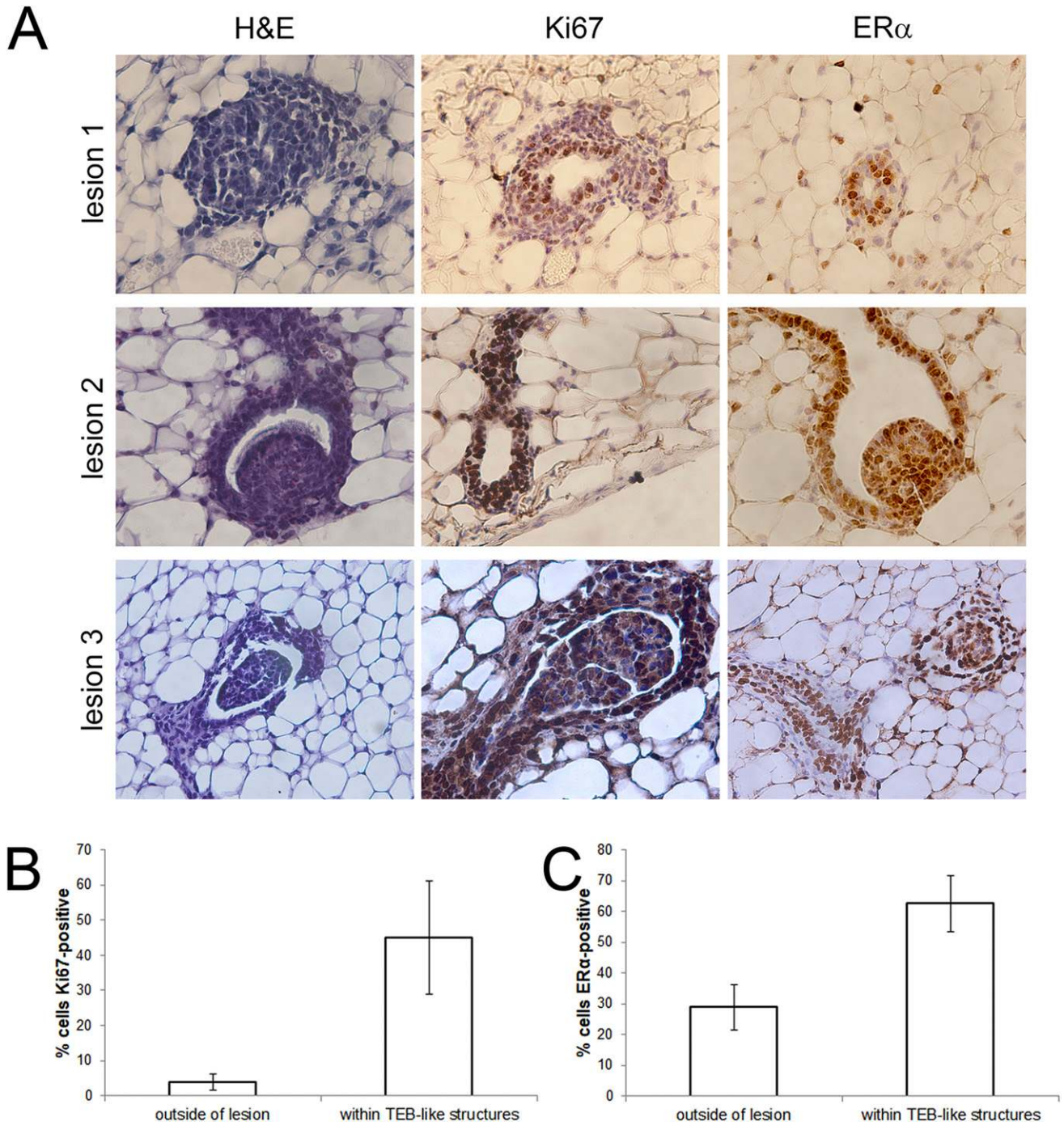

Figure 5. Histological and immunohistochemical evaluation of TEB-like structures excised from UOG-MIX–treated females. (A) Representative lesions from three MIX groups (lesion 1, MIX-30; lesion 2, MIX-3; lesion 3, MIX-300) were evaluated using hematoxylin and eosin (H&E) staining and immunohistochemistry for Ki67 (a marker of proliferation) and ERα. (B) Quantification of Ki67 in excised TEB-like structures compared with Ki67 expression in other regions of the same glands. (C) Quantification of ERα in excised TEB-like structures compared with ERα expression in other regions of the same glands.

exposed to UOG mixtures are a priority. A better mechanistic understanding of the effects of AR antagonists on the female mammary gland, including a wider range of exposure to flutamide, is also needed.

Previous studies of other EDCs have shown few effects on the female gland before puberty (33, 43), suggesting that the most obvious effects of EDCs manifest visibly only after the onset of ovarian hormone production. In support of this, we observed striking effects of developmental exposure to UOG chemical mixtures in the adult female mammary gland (at PND85). Not only were mammary glands more developed in the UOG-MIX–treated adult females, as indicated by the increased volume fraction of epithelium (Fig. 2), but UOG-MIX

treatment also altered the ratios of proliferation and apoptosis (Fig. 3), cell parameters important for dictating the growth and function of the mammary gland (33, 44, 45). Normal growth of the mammary epithelium is dependent on a balance of proliferation (to extend ductal structures into the mammary fat pad) and apoptosis (to produce hollow ducts capable of transporting milk) (36); thus, disruptions to these cellular features could predispose animals to mammary gland diseases (*e.g.,* cancer) or abnormal function (*e.g.,* disruptions to lactation). Additional studies are needed to evaluate these outcomes in UOG-MIX–treated females. The substantial alterations to the apoptosis/proliferation ratio suggest that the mammary glands from UOG-MIX–treated females might continue to manifest complex hyperplastic and preneoplastic lesions in later adulthood. Additional studies are needed to evaluate this possibility.

We were also surprised to see TEB-like hyperplastic lesions in the mammary glands collected from mice in the UOG-MIX–treatment groups, as well as in the flutamide-treated mice (Fig. 4). Other EDCs have also been shown to induce development of intraductal hyperplasias, although these typically will have a "beaded duct," rather than a TEB-like, appearance (34, 46, 47). TEBs are a characteristic structure of glands undergoing puberty (45, 48); these highly proliferative structures drive the growth of the mammary epithelium into the surrounding fat pad. Once the epithelial tree is fully formed, the TEB structures recede and are not seen in adult glands (19, 49). One possibility is that UOG-MIX exposure during early life delays the appearance of TEBs, and thus their presence in the glands of adult mice is indicative of a shift in the timing of puberty. Although the timing of vaginal opening and the age of first vaginal estrus were not affected by UOG-MIX exposure (18), the timing of pubertal growth in the mammary gland involves distinct events (50). Alternatively, it is possible that the timing of mammary puberty is unaffected by UOG-MIX exposure and that the retention of TEBs is indicative of failure to progress to blunt ductal ends. Thus, the presence of TEBs could be interpreted as diminished development of the mammary gland in UOG-MIX–treated groups, in contrast to the effects of these chemical mixtures on epithelial density, which are more consistent with advanced development of the gland (Fig. 2). EDCs have previously been shown to produce competing effects on growth parameters in the mammary gland, including some that advance one aspect of development and seem to delay other developmental landmarks (51). Studies of bisphenol A, for example, have shown that developmental exposure can both decrease ductal extension (growth) and increase the size of TEBs (33, 44, 52–54). These results are consistent with the two competing roles that hormones such as estrogen can have in the developing gland, including its ability to promote proliferation of some mammary epithelial cells and inducing apoptosis of other mammary epithelial cells inside the duct, allowing the lumen to form (55, 56). TEBs are one of the sites where cancers are thought to arise; thus, delays in TEB recession could increase the gland's sensitivity to carcinogens (51, 57). Future studies are needed to determine whether these TEBs are retained into later adulthood and whether prenatal UOG-MIX exposure increases the sensitivity of the gland to carcinogens.

The mammary gland provides an *in vivo* tool to evaluate the effects of EDCs with different modes of action, including chemicals administered as mixtures. To date, *in vivo* studies of EDC chemical mixtures have been limited, with even fewer studies examining mammals [examples include (58–60)]. The results from *in vitro* studies have suggested that compounds with a similar mode of action can have additive effects, including some xenoestrogen mixtures, which have been described as producing "something from nothing" (61–63). In the present study, the assessment of the mixture of 23 chemicals in human endometrial cells demonstrated synergistic responses for ER and thyroid receptor antagonism and less than additive effects for AR and glucocorticoid receptor antagonism (13). Other groups have found that the toxic effects of pesticides are compounded when examined as formulations compared with studying only the so-called active ingredient (64), suggesting that novel insights can be gained from examining chemical mixtures that would not be detected, or even anticipated, from examining the mixture's components individually. Concerns have been raised that any effects observed after exposure to mixtures will be difficult to evaluate mechanistically because they cannot be attributed to any single component of the chemical mixture unless all individual components are also evaluated. However, it is worth noting that humans are exposed to chemical mixtures, rather than single compounds; thus, the study of mixtures in laboratory animals might provide better understanding of the human condition (65).

To date, >1000 different chemicals have been reported for use during unconventional oil and gas extraction (5). Previous studies have shown that prenatal exposure to this same 23-chemical mixture induced adverse health outcomes in the male offspring (the brothers of the female mice examined in the present study) (13). Furthermore, other endocrine organs were affected in the female mice examined in the present study, in addition to alterations to serum hormone concentrations, including decreased levels of the pituitary hormones luteinizing hormone, prolactin, and follicle-stimulating hormone, among others (18). Decreased pituitary hormone

concentrations could influence mammary gland health, including the development of the gland during pregnancy, an endpoint that deserves future attention.

Determining whether mixtures of fracking chemicals affect human populations is an important goal, especially as the number of drilling sites continues to increase (66). A recent systematic review evaluating the strength of the data for the association between conventional oil and gas and UOG operations and human reproductive outcomes found moderate evidence for an increased risk of preterm birth, miscarriage, birth defects, decreased semen quality, and prostate cancer (67). The evidence for an association between UOG operations and breast cancer, or other diseases of the breast, remains inadequate. The results from our study suggest that longitudinal studies evaluating women exposed to UOG chemical mixtures during early life are needed to address this data gap.

Conclusion

Future studies are needed to evaluate the many additional chemicals used in, and produced by, UOG processes to better quantify the concentrations of these and other contaminants in environmental samples and to assess the effects of exposure during other sensitive windows of development, including pregnancy and lactation, puberty, and the aging female. Future studies should also evaluate whether developmental UOG-MIX treatments can sensitize animals to hormones or carcinogens, as would be expected in females with retained TEBs. For mechanistic insights, additional examination of some of the individual components in the fracking chemical mixture might be warranted.

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