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UOG chemical mixtures disrupt mouse mammary gland

## 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 operations (UOG), 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 exposures would similarly disrupt development of the mouse mammary gland. Female C57Bl/6 mice were exposed to approximately 3, 30, 300 or 3000  $\mu\text{g}/\text{kg}/\text{day}$  UOG-MIX from gestational day 11 to birth. Although no effects were observed on the mammary glands of these females prior to puberty, in early adulthood, females exposed to 300 or 3000  $\mu\text{g}/\text{kg}/\text{day}$  UOG-MIX developed more dense mammary epithelial ducts; females exposed to 3  $\mu\text{g}/\text{kg}/\text{day}$  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 unconventional oil and gas production, at exposure levels that are environmentally relevant. The impact 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.

Prenatal exposure to a mixture of chemicals used in unconventional oil and gas operations resulted in abnormal mammary morphology including intraductal hyperplasia resembling terminal end buds

### Introduction

Unconventional oil and gas operations (UOG) combines 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 one to two dozen 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 more than 17 million Americans living within one mile of a oil and gas well (2), concerns have

been raised about the possibility of contamination of surface and groundwater by the released oil and gas, numerous inorganic compounds that are liberated from target geologic layers (e.g. trace metals, radioactive isotopes, minerals) as well as the chemicals used in well injection (3,4).

More than 1000 chemicals have been identified in hydraulic fracturing fluids and waste water and/or are reported to be used by industry (5,6). Many of these chemicals are known developmental and reproductive toxicants (7). Furthermore, recent evaluations found that more than 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-impacted areas of the United States exhibited disruption of the estrogen, androgen, progesterone, glucocorticoid, and thyroid receptors (4,8,12).

In 2015, Kassotis et al. evaluated 24 chemicals that were reported by industry as commonly used and/or produced by UOG operations to determine if they displayed endocrine disrupting properties (13). With cell-based reporter gene assays, their study revealed anti-androgenic, anti-estrogenic, anti-progestogenic, anti-thyroidogenic and anti-glucocorticogenic 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. then evaluated the effects of a 23-chemical mixture of UOG chemicals (UOG-MIX) on male mice (13); this mixture was comprised of the 24 chemicals originally assessed, absent bisphenol A (BPA), a well-characterized EDC that has been evaluated at length previously (14), but which is not directly used in UOG extraction as reported by industry (15,16). 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 increased testes weight prior to 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 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 prior to puberty and in adulthood; weights of several organs including the uterus, ovary and heart were also affected. Further, serum concentrations of several hormones were disrupted in these exposed females. These results suggested the possibility that other hormone-sensitive organs may be disrupted by exposures to UOG chemical mixtures.

The mouse mammary gland has proven to be an excellent model to study the effects of EDCs (19-21). Development of the gland is dependent on estrogen, progesterone, prolactin, testosterone and growth hormone, making it an integrated biological endpoint that is sensitive to agonists and antagonists of different hormone receptors (22). Estrogen receptor (ER) is expressed in the mesenchymal compartment of the fetal gland, but its expression shifts to the epithelial compartment in the adult gland (23). Although ER $\alpha$  knockout mice have mammary glands that are indistinguishable from wildtype controls prior to puberty, they are visibly stunted compared to controls once puberty begins, highlighting the importance of estrogen for growth of the gland (24,25). Androgen receptor (AR) is also expressed in the mesenchyme of the fetal mammary gland, and testosterone produced by the testes in male fetuses causes the mesenchyme to condense around the epithelium, detaching the epithelium from the skin; thus, male mice typically do not have nipples (26,27). Anti-androgenic 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 mammary gland, with effects that typically manifest at puberty and in adulthood, when endogenous hormones induce its growth (20,30). Characteristics that are commonly evaluated to determine the level of development – and the disruption of development by EDCs – include 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 endpoints relevant to the hypothalamic-pituitary-gonadal axis in exposed mice, we hypothesized that prenatal exposures to the UOG-MIX would disrupt development of the mouse mammary gland. Here, mammary glands were evaluated in females after gestational exposure to one of four doses of UOG-MIX. Consistent with our hypothesis, we have characterized significant effects of this mixture on mammary gland morphology in adulthood and document the presence of hyperplastic lesions.

## Materials and Methods

### Animal husbandry & Chemical Administration

C57BL/6J mice were housed in sterile polysulfone cages under temperature- and light-controlled (12 h light, 12 h dark) conditions in a barrier animal facility. 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 was denoted as gestational day 0. On gestational day 11, dams were randomized to treatment groups and provided with experimental treatments in their drinking water. Test concentrations included a 0.2% ethanol vehicle; flutamide, a known anti-androgenic pharmaceutical (mechanistic anti-androgen 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 described in Table 1. Water intake was monitored by weighing the drinking water bottle every day of the experiment, and based on intake, chemical exposures were estimated at 3, 30, 300, or 3000 µg/kg body weight/day for the mixtures. These treatment groups will be referred to as MIX-3, MIX-30, MIX-300 and MIX-3000, respectively. Intake of the anti-androgenic control was estimated at 50 mg flutamide/kg body weight/day. Water intake did not differ in experimental groups relative to the vehicle control (data not shown). Experimental treatments were provided until birth; dams were then reverted to standard acidified water when pups were first detected. Litters with <2 males and females were removed from analyses due to concerns of gestational hormone exposure; litters with >2 males and females were left unaltered, as culling within litters has been shown to alter feeding, behavior, and physiology of 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 animals were euthanized by carbon dioxide asphyxiation and cardiac puncture, and mammary tissues were excised. One randomly selected female pup from each litter was necropsied on either postnatal day (PND) 21 or PND85. At both ages, one fourth 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 mounts were imaged using a Zeiss dissecting scope with AxioCam HRc digital camera. For analyses of the pre-pubertal mammary gland (PND21), images were captured of the full ductal tree and its position relative to the central lymph node. The following measurements were made using methods developed previously (33): total ductal area, measured by quantifying the area subtended by ducts; ductal extension, quantified as the furthest growth of the ductal tree measured from the center of the lymph node; and number of branching points, counted throughout the entire gland. No animals had visible terminal end buds (TEBs) so these structures were not quantified.

For analyses of the adult gland, two pictures were taken, one of the entire mammary gland (at 3x magnification) and one anterior to the central lymph node (at 13.5x magnification); the former was used to evaluate the presence of TEB-like structures and the latter image was used for unbiased stereological evaluations using ZEN imaging software (Zeiss). Briefly, to quantify the volume fraction of epithelial structures, a 130 point grid was superimposed over each anterior photo. The structure that fell on each crosshair was counted; 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 mammary tissue (typically 130). Volume fraction of all epithelium was calculated by summing all epithelial structures (ducts, terminal ends, and alveolar buds).

### **Excision of Mammary Tissue from Whole Mounts**

For whole mounts which displayed unusual or TEB-like structures, small areas of the gland including these abnormal structures were excised using a scalpel and dissecting scope. The remainder of the whole mount was then re-bagged using methyl salicylate. Excised parts of whole mounts were washed, processed through a series of alcohols, embedded in paraffin, and sectioned using the methods described below. Additional areas from whole mounts with normal appearances were also excised for use as control tissues. All excised tissues were coded so that additional analyses could be conducted by experimenters blind to their origin (e.g., unusual / 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  $\mu$ m 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 histological evaluations, 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). Slides were examined using a Zeiss Observer Z1 inverted

light microscope at 40x 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 estrogen receptor (ER) $\alpha$  and Ki67, a marker of proliferation, were performed as previously described (34). Primary antibodies were used at 1:1000 (ER $\alpha$ , Millipore, Cat #06-935; Ki67, ThermoFisher Scientific, Cat#9106-S1; see Table 2). For feasibility reasons, samples were examined in the control, MIX-3 and MIX-3000 groups only; these groups were selected to evaluate a wide range of doses. Samples were visualized using a Zeiss Observer Z1 inverted light microscope at 40x magnification and images captured using an AxioCam HRc digital camera. 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 epithelial ducts) and imaged at 40x; 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 due to diaminobenzidine, the colorimetric reaction used to visualize immunohistochemical reactions) or no expression (blue, hematoxylin counterstain).

### TUNEL Assay

The Trevigen TACS 2 TdT-DAB *in situ* apoptosis detection kit was used for detection of apoptotic cells in 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 40x magnification. Quantification of 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 due to diaminobenzidine, the colorimetric reaction used to visualize TUNEL reactions) or no expression (blue, hematoxylin counterstain).

### Statistical Analysis

The SPSS statistical software package v22 was used for all statistical analyses. ANOVA followed by Fisher's post hoc tests were used to assess differences between control and UOG-MIX treatment groups for each age (PND21 and PND85). Independent-samples T-tests were used to compare control and flutamide-treated groups (35). A chi square test was performed to compare the incidence of hyperplasia in the mammary gland epithelium of control and UOG-MIX-exposed animals. To account for litter effects, only one animal was selected from each litter for each age evaluated. For all statistical tests, results were considered significant at  $p < 0.05$ . All results are presented as mean  $\pm$  S.E.M. and they were collected and analyzed by experimenters blind to treatment.

### Results

**Prepubertal mammary gland morphology was not altered by developmental exposure to UOG-MIX**  
Mice were exposed to vehicle or one of four doses of a 23-chemical mixture (UOG-MIX) during gestation. To determine the effects of 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 pre-pubertal stage of mammary gland development (Figure 1A and 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 there were no statistically significant differences in this growth

parameter between controls and UOG-MIX-treatments (Figure 1B). Flutamide-treated females had significantly smaller ductal trees compared to controls ( $p < 0.05$ , Independent Samples T-test, Figure 1B). Ductal extension and the total number of branching points were not different in any treatment groups including the flutamide-treated females (Table 3). Collectively, these data suggest that prenatal exposure to the 23-chemical mixture did not alter morphological features of female mammary glands prior to 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, Figure 2A). Females exposed to MIX-300 had significantly more ducts compared to vehicle-treated controls ( $p < 0.05$ , Fisher's posthoc test, Figure 2A,B) and a trend for an increase was also seen in females exposed to MIX-30 and MIX-3000 ( $p < 0.1$ , Fisher's posthoc test, Figure 2A,B). Volume fraction of total mammary epithelium was significantly increased in females exposed to MIX-300 and MIX-3000 ( $p < 0.05$ , Fisher's posthoc test, Figure 2A,C). Neither of these parameters were significantly altered by flutamide treatment. Alveolar buds were not observed in females of any treatment group (Figure 2A and data not shown).

#### *Increased proliferation:apoptosis ratio.*

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 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 (Figure 3A,B). There was a borderline significant decrease in TUNEL-positive cells in the flutamide treated group ( $p = 0.052$ , Independent Samples T-test, Figure 3B).

We next evaluated proliferation in the mammary epithelium using antibodies for Ki67, a marker of proliferation. Although 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's posthoc test, Figure 3A,C); females from the MIX-3 group had 427% more Ki67 positive cells compared to controls. The MIX-3000 treatment group had 54% more Ki67 positive cells compared to controls, although these differences were not significant. Ki67 expression was not altered by flutamide (Figure 3C).

We evaluated the ratio of proliferation:apoptosis in 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's posthoc test), and non-significant increases in the MIX-3000 group (131%) compared to controls (Figure 3D). No effect on the proliferation:apoptosis ratio was observed in flutamide-treated females (Figure 3D).

#### *ER $\alpha$ 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 exposures to fracking chemicals would alter expression of ER $\alpha$  in the mammary epithelium. We observed a general increase in ER $\alpha$  expression with increasing UOG-MIX dose, although these differences were not statistically significant (Figure 3A,E). Flutamide also did not affect the percentage of epithelial cells expressing ER $\alpha$  (Figure 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 (Figure 4). These TEB-like structures were not observed in control females (Table 4). The unusual structures were excised from whole mount mammary glands and further characterized using histological and immunohistochemistry tools (Figure 5). H&E staining revealed ducts with excessive layers of epithelial cells, consistent with intraductal hyperplasia (Figure 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 animals or ducts excised from control females (Figure 5A,B). ER $\alpha$  expression was also higher in many, although not all, of the excised lesions (Figure 5A,C). 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 that are used in unconventional oil and gas extraction, the majority of which were previously shown to exhibit antagonistic properties on one or more hormone receptors [(13) and Table 1] including the estrogen, progesterone, and androgen receptors. Notably, 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 (See Table 1 for summary). Here, we show 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 (observed in all MIX groups). Importantly, the effects that were observed in this 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 while the highest dose group (MIX-3000) is equivalent to the concentrations of many UOG-MIX components measured in industry wastewater (4,13,18,38). It should be noted that concentrations for several of the 23 chemicals in the UOG-MIX used here have not yet been determined in either drinking water or wastewater

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 exposures could affect mammary development via actions at this receptor. Importantly, although some endpoints were similarly affected (e.g., retention of TEBs), other effects of UOG-MIX exposures we observed here were distinct from the effects of flutamide, suggesting that the UOG mixture may not be working through an anti-androgenic mechanism as we originally hypothesized. To date, few studies have evaluated the effects of prenatal exposures to anti-androgenic chemicals on the female mammary gland; most studies of anti-androgens have examined their effects on male offspring including nipple retention and other disruptions to mammary gland morphology [see for example (40-42)]. These studies suggest that evaluations of mammary glands in male mice 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 exposures to flutamide, is also needed.

Previous studies of other EDCs have shown few effects on the female gland at pre-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 UOG-MIX-treated adult females, as indicated by the increased volume fraction of epithelium (Figure 2), but UOG-MIX treatment also altered ratios of proliferation and apoptosis (Figure 3), cell parameters that are 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 functions (e.g., disruptions to lactation). Additional studies are needed to evaluate these outcomes in UOG-MIX treated females. The significant alterations to the apoptosis: proliferation ratio suggest that the mammary glands from UOG-MIX treated females may continue to manifest complex hyperplastic and pre-neoplastic lesions in later adulthood; additional studies are needed to evaluate this possibility.

We were also surprised to see TEB-like hyperplastic lesions in mammary glands collected from mice in UOG-MIX-treatment groups as well as the flutamide-treated animals (Figure 4). Other EDCs have also been shown to induce development of intraductal hyperplasias, although these typically have a ‘beaded duct’ rather than 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, 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 exposures, but the retention of TEBs is indicative of failure to progress to blunt ductal ends. Thus, the presence of TEBs may be interpreted as diminished development of the mammary gland in UOG-MIX-treated groups, in contrast with the effects of these chemical mixtures on epithelial density, which are more consistent with advanced development of the gland (Figure 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 while seeming to delay other developmental landmarks (51). Studies of BPA, for example, have shown that developmental exposures 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 while also 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 may increase the gland’s sensitivity to carcinogens (51,57). Future studies are needed to determine if these TEBs are retained into later adulthood, and if prenatal UOG-MIX exposures increase 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 examining mammals [examples include (58-60)]. *In vitro* studies have suggested that compounds with a similar mode of action can have additive effects, including some xenoestrogen mixtures that have been described as producing “something from nothing” (61-63). Assessment of the mixture of 23 chemicals assessed herein 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 rather than when studying only the so-called active ingredient (64), suggesting that novel insights may be gained from examining chemical mixtures that would not be detected, or even anticipated, from examining the mixture components individually. Concerns have been raised that any effects that are observed after exposures to mixtures are difficult to evaluate mechanistically because they cannot be attributed to any single component of the chemical mixture unless all individual components are also evaluated. Yet, it is worth noting that humans are exposed to chemical mixtures rather than single compounds, and thus the study of mixtures in laboratory animals may provide better understanding of the human condition (65).

To date, more than 1000 different chemicals have been reported for use during unconventional oil and gas extraction (5). Prior studies have shown that prenatal exposure to this same 23-chemical mixture induced adverse health outcomes in the male offspring (the brothers of the females examined in the current study) (13). Furthermore, other endocrine organs were affected in the females examined in this study as well as alterations to serum hormone concentration including decreased pituitary hormones LH, prolactin, and FSH, among others (18). Decreased pituitary hormone concentrations could influence mammary gland health, including 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, particularly 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 and unconventional oil and gas 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.

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 exposures during other sensitive windows of development including pregnancy/lactation, puberty, and in 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 may be warranted.

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**Figure 1. No significant effects of developmental exposures to fracking mixtures were observed on morphology of the pre-pubertal mammary gland.** A) Examples of whole mount mammary glands from vehicle, MIX-3000 and flutamide-treated females. Lymph node is indicated by (LN). Scale bar = 0.5 mm. B) Quantification of ductal area suggests an inverse relationship between UOG-MIX-treatment and ductal area, although this difference was not statistically significant. The flutamide-treated females had significantly smaller ductal trees. <sup>^</sup> p<0.05, Independent Samples T-test, comparing control and flutamide-treated females. *Authors request online publication in color, print publication in B&W.*

**Figure 2. Prenatal UOG-MIX-treatment induces increased epithelial density in female mammary glands at adulthood.** A) Example whole mount mammary glands (13x magnification) from control, MIX-300 and MIX-3000 treatment groups on PND85. Vehicle-treated females have 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's posthoc test, <sup>a</sup> p<0.1, Fisher's posthoc test. *Authors request online publication in color, print publication in B&W.*

**Figure 3. Apoptosis, proliferation, and proliferation:apoptosis ratios are altered in UOG-MIX- and flutamide-treated females.** A) Examples of TUNEL and immunohistochemistries for Ki67 and ER $\alpha$  in mammary gland sections from control, MIX-3, MIX-3000 and flutamide-

treated females. Positive cells are indicated by red arrows. Scale bar (bottom right panel) = 20  $\mu\text{m}$ . B) Quantification of TUNEL incorporation in epithelium from vehicle, MIX-3, MIX-3000 and flutamide-treated females reveals a dose-dependent decrease in the percent of epithelial cells undergoing apoptosis. C) Quantification of Ki67, a marker of proliferation, in epithelial cells. D) Significant 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 ER $\alpha$ . \* $p < 0.05$ , Fisher's posthoc test;  $\delta p < 0.1$ , Independent Samples T-test, comparing control and flutamide-treated females. *Authors request online and print publication in color.*

**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 animal with typical terminal ducts. These blunt ends (indicated by arrowheads) are the normal structures observed at the ends of ducts. The lymph node is indicated by 'LN'. B) These examples illustrate the TEB-like structures that were excised from whole mounts from UOG-MIX- and flutamide-treated females. Arrows indicate abnormal structures. Treatment groups for the individual females are indicated on each panel. *Authors request online publication in color, print publication in B&W.*

**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 and lesion 3:MIX-300) were evaluated using H&E staining and immunohistochemistry for Ki67 (a marker of proliferation) and ER $\alpha$ . B) Quantification of Ki67 in excised TEB-like structures compared to Ki67 expression in other regions of the same glands. C) Quantification of ER $\alpha$  in excised TEB-like structures compared to ER $\alpha$  expression in other regions of the same glands *Authors request online and print publication in color.*

Table 1. Chemicals in the UOG mixture and hormone receptor antagonist activity, as demonstrated in (13)

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

Table 2. Required Table of Information about Antibodies

Peptide/ Protein Target	Antigen Sequence (If Known)	Name of Antibody	Manufacturer, Catalog No., or Name of Source	Species Raised in Monoclonal or Polyclonal	RRID	Dilution Used
ER $\alpha$		Anti-Estrogen Receptor $\alpha$ (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_266185 2	Ready to use (5 $\mu$ g/ml)

Table 3. UOG-MIX treatment did not alter growth parameters in the pre-pubertal mammary gland

	Ductal extension (mm) <sup>a</sup>	Number of branching points
Control (n=6)	-13.64 $\pm$ 1.07	25.0 $\pm$ 2.2
MIX-3 (n=8)	-14.19 $\pm$ 0.96	24.9 $\pm$ 2.1
MIX-30 (n=5)	-12.96 $\pm$ 0.43	20.8 $\pm$ 2.3
MIX-300 (n=6)	-15.74 $\pm$ 1.46	25.7 $\pm$ 2.5
MIX-3000 (n=6)	-13.88 $\pm$ 0.34	26.2 $\pm$ 2.4
Flutamide (n=9)	-14.03 $\pm$ 0.84	20.9 $\pm$ 1.4

<sup>a</sup> Negative values for ductal extension indicate ductal trees that have not yet grown past the central lymph node

Table 4. Presence of TEB-like structures in control or UOG-MIX-treated females

	% with TEB-like structures (n)
Control	0% (9)
MIX-3	29% (7)
MIX-30	17% (6)
MIX-300	25% (8)
MIX-3000	40% (5)
flutamide	22% (9)