Understanding the Villain: DMBA-Induced Preantral OVotoxicity Involves Selective Follicular Destruction and Primordial Follicle Activation through PI3K/Akt and mTOR Signaling

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7,12-Dimethylbenz-[a]anthracene (DMBA) is an environmental carcinogen which has a potent ovotoxic affect on rat and mouse ovaries, causing complete follicular depletion resulting in premature ovarian failure. Although the overall effects of DMBA on ovarian folliculogenesis are well known, little is known about the exact molecular mechanisms behind its ovotoxicity. In this study, we characterized the mechanisms behind DMBA-induced ovotoxicity in immature follicles. Microarray analysis of neonatal mouse ovaries exposed to DMBA in vitro revealed a multilayered mechanism of DMBA-induced neonatal ovotoxicity involving a distinct cohort of genes and ovarian signaling pathways primarily associated with follicular atresia, tumorigenesis, and follicular growth. Histomorphological and immunohistological analysis supported the microarray data, showing evidence of primordial follicle activation and preantral follicle atresia both in vitro and in vivo. Further immunohistological analysis identified increased Akt1 phosphorylation, mTOR activation, and decreased FOXO3a expression in DMBA-treated primordial oocytes. Our results reveal a novel mechanism of DMBA-induced preantral ovotoxicity involving selective immature follicle destruction and primordial follicle activation involving downstream members of the PI3K/Akt and mTOR signaling pathways.

Key Words: DMBA; fertility; ovary; primordial follicle; PI3K/Akt/mTOR signaling.

The irreplaceable primordial follicle represents the basic unit of female fertility, serving as the primary source of all developing oocytes in the ovary. In order to maintain a steady supply of developing follicles throughout the mammalian female reproductive lifespan, the majority of these finite follicles must be maintained in a quiescent state (McGee and Hsueh, 2000). Once recruited into the growing pool of follicles, <1% will reach ovulation, the rest undergo an apoptotic process known as atresia (Hirshfield, 1991). Menopause, or ovarian senescence, occurs when the pool of primordial follicles has become exhausted (McGee and Hsueh, 2000). The delicate balance between primordial follicle quiescence, activation, and follicular atresia is dependent on the expression and activation of a number of growth factors promoting follicular growth, differentiation, and atresia. Although we do not know exactly why certain follicles are selected for growth and atresia, recent research has identified a number of cellular signaling pathways, including PI3K/Akt-mediated and caspase pathways, involved in these processes (McLaughlin and McIver, 2009; Reddy et al., 2010).

Premature ovarian failure (POF; or early menopause) is an ovarian defect characterized by the early loss of primordial follicles before the age of 40. Approximately 1–4% of the female population suffers from this condition, making POF a major cause of female infertility (Coulam et al., 1986). There is now a growing body of evidence which suggests that foreign chemical compounds (or xenobiotics), which target immature follicles are capable of causing POF by inducing premature follicular atresia (Borman et al., 2000; Hoyer and Sipes, 1996). Recent studies conducted by ourselves and others have also found that these ovotoxic xenobiotics might induce POF through dysfunctional primordial follicle activation (Keating et al., 2010, Sobinoff et al., 2010). The discovery of a xenobiotoxically perturbed developmental process not directly associated with follicular destruction is intriguing and has led to new approaches in studying xenobiotoxically ovotoxicity.

The polycyclic aromatic hydrocarbon 7,12-dimethylbenz-[a]anthracene (DMBA) is an environmental carcinogen used in the induction of many tumors, including ovarian, in rodent models of cancer (Hoyer et al., 2009). Sources of human DMBA exposure in the environment include cigarette smoke, charbroiled foods, and automobile exhaust (Gelboin, 1980). In addition to its carcinogenic nature, DMBA has also been shown to disrupt folliculogenesi, causing the depletion of all follicle populations leading to POF (Mattison and Schulman,...
In the ovary, DMBA is converted into a 3,4-diol 1,2-epoxide by members of the cytochrome P450 family of oxidases and the microsomal epoxide hydrolase enzyme (Igawa et al., 2009). This bioactive metabolite binds to both dAdo and dGuo residues in DNA, forming DMBA-DNA adducts which result in follicular atresia (Vericat et al., 1989). Recent evidence suggests that DMBA does not cause POF through increased follicular activation, consequently resulting in accelerated atresia in both primordial and primary follicles in rodents (Keating et al., 2010), but instead causes direct follicle atresia.

Although there are a number of studies which demonstrate the overall effects of DMBA on ovarian folliculogenesis, little is known about the exact molecular mechanisms behind its ovotoxicity. Therefore, the objective of this study was to use a genomic approach to better understand the molecular mechanisms behind DMBA-induced ovotoxicity by examining its effects on the ovarian transcriptome of cultured neonatal mouse ovaries. Microarray analysis confirmed via qPCR revealed a complex mechanism of DMBA-induced neonatal ovotoxicity involving a distinct cohort of genes and ovarian signaling pathways primarily associated with follicular atresia, tumorigenesis, and follicular growth. Interestingly, histomorphological and immunohistochemical analysis revealed no evidence supporting the hypothesis that DMBA causes primordial follicle atresia, instead revealing a consistent mechanism of DMBA-induced primordial follicle activation and developing follicle atresia. In addition, we also provide evidence of increased Akt phosphorylation, mTOR activation, and decreased nuclear FOXO3a localization, all of which have been associated with increased primordial follicle activation/survival.

MATERIALS AND METHODS

Reagents. DMBA (> 95% purity) and custom designed primers were purchased from Sigma Chemical Co. (St Louis, MO) and were of molecular biology or research grade. Mouse monoclonal anti-Proliferating Cell Nuclear Antigen antibody (anti-PCNA, NA03T) was obtained from Merck KGaA (Darmstadt, Germany). Rabbit polyclonal anti-active Caspase 3 antibody (anti-Casp3, ab13847), Rabbit polyclonal anti-active Caspase 2 antibody (anti-Casp2, ab2251), Rabbit monoclonal anti-Akt1 (phospho S473) (anti-pAkt1 (S474), ab81283), Rabbit monoclonal anti-Akt1 (phospho T308) (anti-pAkt1 (T308), ab5626), and Rabbit polyclonal anti-Foxo3a (anti-Foxo3a, ab47409) were obtained from Abcam (Cambridge, MA). Mouse monoclonal anti-human hormone antibody (anti-AMH, MCA2246) was obtained from AbD Serotec (Kidlington, U.K.). Rabbit polyclonal anti-Akt (anti-Akt, #9272) was obtained from Cell Signaling Technologies (Beverly, MA). Alexa Fluor 594 goat anti-rabbit IgG (A11012), Alexa Fluor 594 goat anti-mouse IgG (A11005), fetal bovine serum, t-glutamine, and insulin-transferrin-selenium (ITS) were purchased from the Invitrogen Co. (Carlsbad, CA). t-ascorbic acid was obtained from MP Biomedicals (Solon, OH). Rabbit polyclonal anti-phospho-mTOR (phosphor S2446) (anti-pmTOR (S2448), 09-2185PS), Rabbit polyclonal anti-phospho-mTOR (phosphor T2446) (anti-pmTOR (T2446), 09-345SP), and 0.4 μm culture plate inserts were purchased from Milipore (Billerica, MA). All culture dishes and cell culture plates were obtained from Greiner Bio-One (Mooroe, NC). Oligo(dT)15 primer, RNasin, dNTPs, M-MLV-Reverse Transcriptase, RQ1 DNase, GoTaq Felix, MgCl2, GoTag qPCR master mix, and Proteinase K were purchased from the Promega Corporation (Madison, WI).

Animals. All experimental procedures involving the use of animals were performed with the approval of the University of Newcastle’s Animal Care and Ethics Committee (ACEC). Inbred Swiss mice were obtained from a breeding colony held at the institute’s central animal facility and maintained according to the recommendations prescribed by the ACEC. Mice were housed under a controlled lighting regime (16L:8D) at 21°C–22°C and supplied with food and water ad libitum.

Animal dosing. Female Swiss neonatal mice (day 4; 6–10 animals per treatment group) were weighed and administered (ip) 7 daily consecutive doses of either sesame oil containing vehicle control (<10 μL/kg/daily acetone) or sesame oil containing DMBA (1mg/kg/daily). The dosage, routes of administration, and dosing time courses were based on previous studies and were chosen with the intention of inducing ovotoxicity with minimal cytotoxicity (Borman et al., 2000). Animals were observed daily for symptoms of toxicity and mortality. Treated animals were culled by CO2 asphyxiation 24 h after the last injection.

Ovarian culture. Ovaries from days 3 to 4 Swiss neonatal mice were cultured described previously (Sobinoff et al., 2010). Briefly, Swiss neonates were sacrificed by CO2 inhalation followed by decapitation. Ovaries were excised, trimmed of excess tissue, and placed on culture plate inserts in 6-well tissue culture plate wells floating atop 1.5 ml Dulbecco’s modified Eagle’s medium/F12 medium containing 5% (vol/vol) fetal calf serum, 1 mg/ml bovine serum albumin (BSA), 50 μg/ml ascorbic acid, 27.5 μg/ml ITS, 2.5mM glutamine and 5 U/ml penicillin/streptomycin. Media were supplemented with 40 ng/ml basic fibroblast growth factor, 50 ng/ml leukemia inhibitory factor, and 25 ng/ml stem cell factor. A drop of medium was placed over the top of each ovary to prevent drying. Ovaries were cultured for 4 days at 37°C and 5% CO2 in air, with media changes every 2 days. Ovaries were treated with vehicle control medium (0.01% acetone) or DMBA (50nM). The DMBA culture concentration was determined by pilot studies performed in our laboratory with the intention of inducing overt ovotoxicity.

Histological evaluation of follicles. Following in vitro culture/in vivo dosing, ovaries were placed in Bouin’s fixative for 4 h, washed in 70% ethanol, paraffin embedded, and serially sectioned (4 μm thick) throughout the entire ovary, with every 4th slide counterstained with hematoxylin and eosin. Healthy oocyte-containing follicles were then counted in every hematoxylin and eosin-stained section. Follicles with eosinophilic (pyknotic) oocytes were considered as degenerating or atretic and so were not counted. Primordial follicles were classified as those with a single layer of squamous granulosa cells. Activating follicles were identified as those which contained one or more cuboidal granulosa cells in a single layer. Primary follicles were classified as those which contained more than four cuboidal granulosa cells in a single layer. Secondary follicles were identified as those with two layers of granulosa cells and preantral follicles were classified as those with more than two layers of granulosa cells. Both in vitro and in vivo treated ovaries did not contain follicles beyond the preantral stage.

Immunohistochemistry. Ovaries for immunohistochemistry were fixed in Bouin’s and sectioned 4 μm thick. PCNA, active Casp2, active Casp3, AMH, Akt, pAkt1, and Foxo3a were stained using the same protocol with the exception of the primary antibody. Slides were deparaffinized in xylene and rehydrated with subsequent washes in ethanol. Antigen retrieval was carried out by microwaving sections for 3 × 3 min in Tris buffer (50mM, pH 10.6). Sections were then blocked in 3% BSA/Trits buffer saline (TBS) for 1.5 h at room temperature. The following solutions were diluted in TBS containing 1% BSA. Sections were incubated with either anti-PCNA (1:80), anti-Casp2 (1:200), anti-Casp3 (1:200), anti-AMH (1:200), anti-Akt (1:100), anti-pAkt1 (1:100), or anti-Foxo3a (1:200) for 1 h at room temperature. After washing in TBS containing 0.1% Triton X-100, sections were incubated with the appropriate fluorescent conjugated secondary antibodies (Alexa Fluor 594 goat anti-rabbit.
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IgG, Alexa Fluor 594 goat anti-mouse IgG; 1:200 dilution) for 1 h. Slides were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min, mounted in Mowiol, and observed on an Axio Imager A1 fluorescent microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) under fluorescent optics and pictures taken using an Olympus DP70 microscope camera (Olympus America, Center Valley, PA). Protein staining was quantified according to Cy-5 intensity in primordial follicle oocytes using ImageJ software (NCBI).

**Terminal deoxynucleotidyl transferase dUTP nick end labeling analysis.** Bouin’s fixed sections were deparaffinized and rehydrated as mentioned previously. Sections were then boiled in Tris buffer (50mM, pH 10.6) for 20 min and treated with 20 μg/ml Proteinase K for 15 min in a humidified chamber. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis was then performed using an In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics Pty Ltd., Dee Why, Australia) according to the manufacturer’s instructions. Slides were then counterstained with DAPI for 5 min, mounted in Mowiol, and observed using an Axio Imager A1 epifluorescent microscope (Carl Zeiss) and images captured using an Olympus DP70 microscope camera (Olympus).

**RNA extraction.** Total RNA was isolated from ovaries using two rounds of a modified acid guanidinium thiocyanate-polytron-chloroform protocol (Chomczynski and Sacchi, 1987): washed cells resuspended in lysis buffer (4M guanidinium thiocyanate, 25mM sodium citrate, 0.5% sarkosyl, 0.72% β-mercaptoethanol). RNA was isolated by phenol/chloroform extraction and isopropanol precipitated.

**Real-time PCR.** Reverse transcription was performed with 2 μg of isolated RNA, 500 ng oligo(dT)15 primer, 40 U of RNasin, 0.5mM dNTPs, and 20 U of M-MLV-Reverse Transcriptase. Total RNA was DNase treated prior to reverse transcription to remove genomic DNA. Real-time PCR was performed using SyBr Green GoTaq qPCR master mix according to manufacturer’s instructions on an MJ Opticon 2 (MJ Research, Reno, NV). Primer sequences along with annealing temperatures have been supplied as Supplementary data (Supplementary Table 3). Reactions were performed on cDNA equivalent to 100 ng of total RNA and carried out for 40 amplification cycles. SYBR Green fluorescence was measured after the extension step at the end of each amplification cycle and quantified using Opticon Monitor Analysis software Version 2.02 (MJ Research). For each sample, a replicate omitting the reverse transcription step was undertaken as a negative control. Reverse transcription reactions were verified by β-actin PCR, performed for each sample in all reactions in triplicate. Real-time data were analyzed using the equation $C(t) = C(t - 1)$, where $C(t)$ is the cycle at which fluorescence was first detected above background fluorescence. Data were normalized to cyclophilin, beta-2-microglobulin, and beta-glucuronidase and are presented as the average of each replicate normalized to an average of the reference genes ($±$SEM).

**Microarray analysis.** Total RNA (approximately 3 μg) was isolated from DMBA-cultured neonatal ovaries and prepared for microarray analysis at the Australian Genome Research Facility (AGRF) using an Illumina Sentrix Mouse ref8v2 Beadchip. Labeling, hybridizing, washing, and array scanning were performed by the AGRF using the Illumina manual on an Illumina BeadArray Reader and normalized according to the quantile normalization method using GenomeStudio version 1.6.0 (Illumina, Inc., San Diego, CA). All experiments were performed in triplicate with independently extracted RNAs. Statistically significant genes with more than a twofold difference in gene expression ($p < 0.05$) determined through the use of a “volcano plot” were then analyzed using Ingenuity Pathways Analysis (Ingenuity Systems, Redwood City, CA) software to identify canonical signaling pathways influenced by DMBA exposure. The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE29263 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GC%20GSE29263).

**Statistics.** Comparisons between the control and treatment groups were performed using one-way analysis of variance (ANOVA) and Tukey’s Honestly Significant Difference test. The assigned level of significance for all tests was $p < 0.05$.

**RESULTS**

**Effects of DMBA Exposure on the Neonatal Ovarian Transcriptome**

DMBA exposure caused a statistically significant change in gene expression for a small cohort of 110 genes, representing 0.4% of all the genes present on the array, suggesting a specific response in neonatal ovarian gene expression (Fig. 1A). These significantly altered genes were analyzed for their roles in networks and molecular functions using Ingenuity Pathway Analysis software. In accordance with its reported ovotoxicity/carcinogenicity, DMBA-altered genes were identified as components of molecular networks involving cancer, cell death, genetic disorder, and organismal injury (Fig. 1B). Interestingly, DMBA also significantly altered the expression of a number of genes belonging to other functional groups including cellular growth and proliferation, immunological disease, inflammatory disease, and cellular development (Table 1; Supplementary Table 1). This suggests that, despite apparent selective gene expression modulation, DMBA-induced ovotoxicity may involve a variety of other mechanisms apart from follicular atresia.

**Canonical Pathways Significantly Upregulated by DMBA Exposure**

In order to gain a further understanding of, and confirm a multilayered mechanism of DMBA-induced ovotoxicity, differentially expressed genes were also classified according to signaling pathways (Fig. 2; Supplementary Table 2). DMBA exposure influenced two signaling pathways involved in the immune response, suggesting a potential immunological like mechanism of follicular destruction (antigen presentation, complement system). DMBA also influenced four pathways associated with follicular growth and development (mTOR, integrin-linked kinase (ILK), EIF2, and vascular endothelial growth factor signaling) and one pathway associated with amino acid synthesis (methionine metabolism). Interestingly only one pathway out of the top 10 influenced by DMBA was associated with follicular atresia and DNA repair (protein ubiquitination). This was surprising, given DMBA’s well-known ability to form DNA adducts resulting in apoptosis and suggests that the observed ovarian response to DMBA might not be limited to follicular atresia but involve a number of other biological processes.

**qPCR Validation of Microarray Results**

Validation of microarray results was performed by examining the levels of expression for 10 different genes using qPCR (Table 2). Similar upregulated gene expression patterns were observed for all targets measured by qPCR when compared with the results of the microarray gene expression study.
Consistent with a role in ovotoxicity/carcinogenesis, three of these genes were associated with cell cycle arrest and apoptosis (Cdkn1a, Ddx5, and Foxn3), three were associated with increased tumorigenesis (Srsf5, Ddx5, and Gbas), and one with repressed tumorigenesis (Foxn3). Interestingly, four genes were also associated with cell cycle progression and follicular development (Srsf5, Hspa8, Dnaja6, and Ccnd1). The two remaining genes, Anapc5 and Calr, are both associated with a variety of cellular processes, including translational repression and cell cycle progression. These results further implicate DMBA-induced ovotoxicity with other cellular mechanisms apart from apoptosis, including follicular development.

<table>
<thead>
<tr>
<th>Molecular and cellular function</th>
<th>Upregulated</th>
<th>Downregulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell death</td>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td>Cellular growth and proliferation</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>Cancer</td>
<td>27</td>
<td>4</td>
</tr>
<tr>
<td>Immunological disease</td>
<td>17</td>
<td>7</td>
</tr>
<tr>
<td>Inflammatory disease</td>
<td>17</td>
<td>13</td>
</tr>
<tr>
<td>Cell-to-cell signaling and interaction</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>Genetic disorder</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>Cellular development</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>Tissue morphology</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Connective tissue disorders</td>
<td>18</td>
<td>5</td>
</tr>
</tbody>
</table>

Note. Genes were analyzed using ingenuity pathways analysis (ingenuity systems) for molecular and cellular functions. Only those genes exhibiting a greater than twofold change in expression were categorized (p < 0.05), note that some genes are listed in multiple functional groups.

**DMBA Causes Primordial Follicle Activation and Immature Follicle Atresia**

To consolidate the data obtained from our microarray analysis and further characterize the ovarian response to DMBA, cultured neonatal ovaries were fixed and stained by immunohistochemistry for markers associated with follicular development and atresia. Actively proliferating granulosa cells and activated primordial oocytes were identified by probing for PCNA, a developmental marker of primordial follicle activation. Follicles destined for follicular atresia were identified by staining for activated Casp2 and Casp3, which are early markers of apoptosis in oocytes and granulosa cells, respectively. Follicles undergoing the final stages of atresia were...
identified by using TUNEL, a technique used to detect DNA strand breaks indicative of DNA degradation.

PCNA staining was detected in both the granulosa cell and the oocyte nuclei of DMBA-treated primordial follicles and absent in the control, suggesting increased levels of primordial follicle activation (Fig. 3A). Casp2, Casp3, and TUNEL staining were detected in the majority of primary and secondary follicles present in DMBA-cultured ovaries, suggesting follicular atresia. However, all three markers of cell death were absent in DMBA-treated primordial follicles, suggesting a selective mechanism of follicular destruction.

To confirm the observed effects of neonatal xenobiotic exposure on follicular activation and atresia in vivo, female Swiss neonatal mice (PND4) were administered daily injections of DMBA over 7 days. Follicle counts determined a significant reduction in the percentage of primordial follicles, mirrored by a comparable increase in activating primordial follicles in DMBA-treated animals (Fig. 3B). In addition, DMBA also caused a significant increase in the percentage of primary (~twofold) and secondary (~threefold) follicles compared with the control. Analysis of the average number of follicles per section demonstrated a significant decrease in follicular number for DMBA-treated animals (~50% of the control). These results suggest an excessive stimulation of follicular activation and development combined with increased follicular atresia in DMBA-treated animals.

Effect of DMBA Exposure on Akt, pAkt1 (S473), and pAkt1 (T308) Proteins

To explore the mechanisms associated with DMBA-induced primordial follicle activation, cultured neonatal ovaries were probed for Akt, pAkt1 (S473), and pAkt1 (T308) (Fig. 4). Total Akt protein staining was detected in both the oocyte and the granulosa cells of primordial, primary, and secondary follicles. In the oocyte specifically, it was localized in both the cytoplasm and the nucleus, with no distinguishable difference in the level of staining observed between control- and DMBA-cultured oocytes. Similarly, pAkt1 (S473) and pAkt1 (T308) staining were detected in both the oocyte and the granulosa cells of primordial, primary, and secondary follicles. However, there was an observable increase in the levels of pAkt1 expression in DMBA-cultured ovaries compared with the control; with pAkt1 expression being significantly enriched (~twofold) in DMBA-cultured primordial follicles. These results suggest DMBA caused a significant increase in Akt1 phospho activation, implicating the PI3K/Akt pathway in DMBA-induced follicular activation.

Effect of DMBA Exposure on pmTOR (S2448), pmTOR (T2446), and Foxo3a Proteins

Given DMBA’s significant affect on the phosphorylation status of Akt1, we next observed the protein levels of downstream targets pmTOR (S2448), pmTOR (T2446), and Foxo3a in primordial follicle oocytes (Fig. 5). pmTOR (S2448) expression was found to have significantly increased (~2.5 fold), whereas pmTOR (T2446) expression had significantly decreased (~fivefold) in DMBA-treated primordial oocytes. Additionally, pmTOR (S2448) expression was localized within the cytoplasm and nucleus, whereas pmTOR (T2446) expression was localized to the oocyte nucleus. Foxo3a protein staining was detected in the granulosa and oocyte cell nuclei of both control- and DMBA-cultured primordial, primary, and secondary follicles. Staining was predominantly localized to the oocyte nucleus, with a significant decrease in expression (78% of the control) observed in DMBA-treated primordial follicles.

DISCUSSION

In this study, we examined the effects of the potent ovotoxic agent DMBA on the ovarian transcriptome of neonatal mice in vitro. Microarray analysis revealed a composite mechanism of DMBA-induced ovotoxicity involving a subset of genes involved in cancer, follicular growth/development, and atresia.
This is the first time DMBA has been linked with developmental pathways in addition to apoptosis in the ovary, suggesting an ovarian response separate from follicular atresia. Canonical pathway analysis also identified a number of significantly ($p < 0.05$) upregulated pathways involved in follicular growth/development (Fig. 2). In particular, DMBA upregulated genes involved in mTOR signaling, which has been shown to induce primordial follicle activation (Adhikari et al., 2010), and ILK signaling, which has been previously implicated in cell survival and proliferation (Reddy et al., 2010). Conversely, protein ubiquitination was the only significantly upregulated pathway directly associated with follicular atresia.

**TABLE 2**

qPCR Validation of Microarray Results for Select Transcripts Upregulated by DMBA-Cultured Neonatal Ovaries

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Summary of function</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdkn1a</td>
<td>Cyclin-dependent kinase inhibitor 1A</td>
<td>Regulator of cell cycle progression at G1 phase; implicated in DNA damage repair, increased expression associated with DMBA-induced ovarian neoplasm (Kim et al., 2003)</td>
<td>3.77 ± 0.3</td>
</tr>
<tr>
<td>Anapc5</td>
<td>Anaphase-promoting complex subunit 5</td>
<td>Forms part of the APC; regulates sister chromatid segregation and mitotic exit, regulates cell cycle through ubiquitination. Acts independently as a negative regulator of translation (Baker et al., 2007; Kolotova-Levine et al., 2004)</td>
<td>2.03 ± 0.2</td>
</tr>
<tr>
<td>Hspa8</td>
<td>Heat shock protein 8</td>
<td>Stress-related chaperone; regulates cyclin D1 and cyclin D1-dependent protein kinase accumulation (Dielhl et al., 2003)</td>
<td>1.93 ± 0.2</td>
</tr>
<tr>
<td>Dnajb6</td>
<td>DnaJ (Hsp40) homolog, subfamily B, member 6</td>
<td>Stress-related chaperone; regulates gene expression in response to stress through Class II Histone Deacetylase Recruitment (Dai et al., 2005)</td>
<td>2.85 ± 0.5</td>
</tr>
<tr>
<td>Ddx5</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 5</td>
<td>RNA helicase; stimulates cell proliferation, coactivator of p53 signaling, overexpression associated with estrogen receptor alpha tumorigenesis (Nicol and Fuller-Pace, 2010)</td>
<td>1.71 ± 0.1</td>
</tr>
<tr>
<td>Srsf5</td>
<td>Serine/arginine-rich splicing factor 5</td>
<td>RNA-binding protein; involved in constitutive and alternative splicing of pre-mRNAs, implicated in cell cycle regulation, aberrant expression associated with tumorigenesis (Diamond et al., 1993; Huang et al., 2007)</td>
<td>1.85 ± 0.3</td>
</tr>
<tr>
<td>Foxn3</td>
<td>Forkhead box N3</td>
<td>Transcription factor; regulator of cell cycle progression at G2 phase, implicated in preventing tumorigenesis (Scott and Plon, 2005)</td>
<td>1.48 ± 0.2</td>
</tr>
<tr>
<td>Calr</td>
<td>Calreticulin</td>
<td>Ca$^{2+}$-binding chaperone involved in numerous cellular processes; major Ca2+-binding chaperone in oocytes, (Du et al., 2009; Vougas et al., 2008; Zhang et al., 2010)</td>
<td>1.41 ± 0.1</td>
</tr>
<tr>
<td>Gbas</td>
<td>Glioblastoma amplified sequence</td>
<td>Mitochondrial protein; involved in mitochondrial oxidative phosphorylation, expressed in 40% of glioblastomas (Martherus and Sluiter, 2010; Wang et al., 1998)</td>
<td>1.68 ± 0.2</td>
</tr>
<tr>
<td>Ccnd1</td>
<td>Cyclin D1</td>
<td>Promotes cell cycle progression from G1 to S phase; overexpression associated with breast/ovarian cancer (Hashimoto et al., 2011; Robker and Richards, 1998)</td>
<td>5.82 ± 0.5</td>
</tr>
</tbody>
</table>

Note. Total RNA was isolated from xenobiotic-cultured ovaries, reverse transcribed, and qpcr performed with primers specific for the cDNA of indicated genes as described in the “materials and methods” section. genes selected for validation were chosen from those most significantly altered by DMBA exposure as detected via microarray analysis. preference was given to those genes with the highest changes in gene expression. Fold change (mean ± se) and summary of function relating to folliculogenesis are included. all fold changes were statistically significant ($p < 0.05$). er, estrogen receptor.
In addition to a role in upregulating follicular growth/development and atresia, canonical pathway analysis also revealed DMBA upregulated immune response pathways (antigen presentation and the complement system). We suggest that the observed upregulation of the antigen presentation pathway may allude to a mechanism by which apoptotic DMBA-afflicted cells are signaled for destruction. Indeed, there is evidence of a capacity for antigen presentation in ovarian cells, and a mechanism by which nonimmune apoptotic cells directly present antigens to the immune system to signal their destruction (Barua and Yoshimura, 1999; Blachère et al., 2005). Upregulation of the complement system may also be another mechanism by which apoptotic DMBA follicles are targeted for destruction. A major role for the complement system is the removal of apoptotic cells through phagocytosis to prevent the release of intracellular contents (Nauta et al., 2003). In theory, this could limit the effects of DMBA and its toxic metabolites on other cells in the ovary.

Another significantly upregulated pathway was methionine metabolism. Methionine metabolism is an important aspect of cellular physiology, involved in preventing oxidative stress and cell cycle progression (Moskovitz et al., 1997). As DMBA metabolism causes elevated levels of reactive oxygen species in the ovary, its upregulation in DMBA-treated ovaries may be a mechanism utilized by the ovary to prevent DMBA-induced oxidative stress (Tsai-Turton et al., 2007).

In further support of a complex mechanism of DMBA-induced ovotoxicity, qPCR analysis confirmed the upregulation of 10 genes of interest involved in a variety of cellular processes (Table 2). One of these genes was Ddx5, a member of the DEAD-box RNA helicase family. In addition to its role in RNA splicing and microRNA processing, Ddx5 also acts as a coactivator for two highly regulated transcription factors, one of which is tumor suppressor p53 (Nicol and Fuller-Pace, 2010). Tumor suppressor p53 is upregulated in response to DNA damage and in conjunction with Ddx5 upregulates genes

FIG. 3. DMBA exposure causes immature follicular destruction and primordial follicle activation in vitro and in vivo. (A) Fluorescent immunohistological and TUNEL staining as visualized via fluorescent microscopy. Ovaries excised from neonatal mice (4 days old) were cultured in DMBA-treated medium for 96 h and processed for immunohistochemistry and TUNEL analysis as described in the “Materials and Methods” section. Ovarian sections were incubated with antibodies against PCNA, active caspase 2, and active caspase 3 or subjected to TUNEL analysis. Blue staining (DAPI) represents nuclear staining; red staining (Cy-5) represents specific staining for the protein of interest; green staining (Fluorescein) represents specific staining for degraded DNA (TUNEL). The results presented here are representative of n = 3 experiments. The percentage of labeled follicles per section is represented by the following scale: * = < 25%, ** = 25–50%, *** = 51–75%, **** = 76–100%. Thin arrow = primordial follicle; arrowhead = primary follicle; scale bar is equal to 100 μm. (B) Effect of xenobiotic exposure on ovarian follicle composition and number in vivo. Neonatal mice (4 days old) were dosed with DMBA over a 7-day period as described in the “Materials and Methods” section. Ovarian sections were stained with hematoxylin and eosin and healthy oocyte-containing follicles were classified and counted under a microscope. Ovarian follicle composition (left panel) and average number of follicles per counted section (right panel). Values are mean ± SE, n = 3–5 ovaries. The symbol ** represents p < 0.01 in comparison with control values. Note. See online version for color version.
involved in cell cycle arrest and apoptosis. Interestingly, one of these upregulated genes is Cdkn1a, a regulator of G1 cell cycle arrest and another gene of interest upregulated in DMBA-treated ovaries (Kim et al., 2003). Foxn3, a transcription factor involved in G2 phase cell cycle arrest in response to DNA damage, was also upregulated by DMBA (Pati et al., 1997). Given DMBA’s capacity to cause DNA damage through DNA adducts, the upregulation of Ddx5 and Foxn3 may be mechanisms by which DMBA induces follicular atresia.

In addition to their roles in cell cycle arrest, both Ddx5 and Foxn3 have been shown to induce and prevent tumorigenesis, respectively. Ddx5 acts a coactivator of estrogen receptor alpha, upregulating its expression and promoting growth/cell survival in cancer cell lines (Fuller-Pace and Moore, 2011). In contrast, Foxn3 has been shown to interact with SKIP, a transcriptional coregulator which represses genes important for tumorigenesis in response to cancer treatments (Scott and Plon, 2005). Given that DMBA has been shown to generate ovarian adenocarcinoma in rats, Ddx5 upregulation could be a mechanism by which DMBA induces tumorigenesis, whereas Foxn3 upregulation might be the ovaries way of combating this (Crist et al., 2005).

Two stress-related chaperones, Hspa8 and Dnajb6, were also upregulated by DMBA exposure. Hspa8 is a heat shock protein constitutively expressed within both the nuclear and the cytosolic compartments of the cell and is responsible for regulating protein maturation and function (Agashe and Hartl, 2000). Hspa8 has been shown to play a role in cyclin D1 maturation, another gene upregulated by DMBA exposure (Diehl et al., 2003; Muñiz et al., 2006). Cyclin D1 promotes cell cycle progression from G1 to S phase and is exclusively expressed within the theca cells of the ovary (Robker and Richards, 1998). As theca cells are only present in developing follicles, increased Cyclin D1 expression could represent increased primordial follicle activation. Recent evidence also suggests Cyclin D1 overexpression is associated with the overall prognosis in epithelial ovarian cancer patients (Hashimoto et al., 2011). Dnajb6 is also a heat shock protein which regulates gene
expression in response to stress by inhibiting nuclear factor of activated T cells (NFAT) transcriptional activity through the recruitment of class II histone deacetylase (Dai et al., 2005). NFAT is a transcription factor that has been shown to negatively regulate CDK4 and positively regulate PTEN expression (Baksh et al., 2002; Wang et al., 2011). PTEN is a known inhibitor of Akt1 phosphorylation, an essential event during PI3K/Akt signaling, which is responsible for primordial follicle activation (Reddy et al., 2009). It is therefore possible that the upregulation of Hspa8, Cyclin D1, and Dnajb6 by DMBA could lead to increased theca cell proliferation, tumorogenesis, and primordial follicle activation.

Other genes found to be upregulated by DMBA exposure were Anapc5, Srsf5, Calr, and Gbas. Anapc5 encodes a subunit which forms part of the anaphase-promoting complex (APC) which regulates cell cycle progression through ubiquitination (Baker et al., 2007). However, no other members of the APC were upregulated in the presence of DMBA, suggesting a role independent from the APC. Interestingly, Anapc5 has been shown to interact with poly(A) binding protein and inhibits mRNA circularization of mRNAs which rely on internal ribosome entry sites (IRES), repressing translation (Koloteva-Levine et al., 2004). Although most commonly used by viral mRNAs, IRES is also used for the alternative initiation of translation of several mammalian genes (Arnaud et al., 1999; Huez et al., 1998; Martineau et al., 2004). Srsf5 is an RNA binding protein involved in the constitutive and alternative splicing of premRNAs linked with cell cycle progression and increased tumorigenesis (Diamond et al., 1993; Huang et al., 2007). Calr is a Ca\textsuperscript{2+} storage protein required for multiple functions, including apoptosis and oocyte maturation and is overexpressed in many cancer cells, suggesting a role in the progression of tumorigenesis (Du et al., 2009; Vanoverberghe et al., 2003; Vougas et al., 2008; Zhang et al., 2010). Gbas is a mitochondrial protein thought to be involved in oxidative phosphorylation and is overexpressed in 40% of glioblastomas (Martherus and Sluiter, 2010; Wang et al., 1998).
We have compiled our microarray results into a preliminary model of DMBA-induced ovotoxicity which attempts to connect the observed changes in gene expression to possible effects on folliculogenesis (Fig. 6). The common themes seen in this model were apoptosis, tumorigenesis, and follicular growth/development. As mentioned before, the prospect of DMBA-inducing follicular growth/development was intriguing, as DMBA has been traditionally thought to cause indiscriminately follicular atresia (Mattison and Schulman, 1980). To investigate this, we probed DMBA-cultured ovaries for markers of follicular activation and atresia (Fig. 3A). PCNA, a marker of primordial follicle activation, was detected in the granulosa cells and oocytes of DMBA-treated primordial follicles. Markers of follicular atresia, Casp2, Casp3, and TUNEL, were all detected in developing primary and secondary follicles. Interestingly, all three markers of cell death were absent from DMBA-treated primordial follicles. Histomorphological analysis also detected increased levels of primordial follicle activation and follicular atresia in vivo (Fig. 3B) (Supplementary Fig. 2).

Collectively, these results support a mechanism of DMBA-induced ovotoxicity involving primordial follicle activation and developing follicle atresia. These results coincide with our previous study, in which we found three potent ovotoxicants also caused developing follicle atresia and primordial follicle depletion through increased primordial follicle activation (Sobinoff et al., 2010). We proposed that the increase in primordial follicle activation was due to a homeostatic mechanism of follicular replacement, leading to a vicious cycle of developing follicle atresia and primordial follicle activation. Indeed, primordial follicle activation is under negative control by the growing follicle population through the secretion of AMH, a member of the TGF-β superfamily (Reddy et al., 2010). As DMBA caused immature follicle atresia, we observed the level of, and distribution of, AMH, a negative regulator of follicular recruitment. AMH staining was localized to the granulosa cells of primary and secondary follicles in both the control- and the DMBA-cultured ovaries, with no apparent difference in the level of or number of follicles stained (Supplementary Fig. 1). This suggests that a homeostatic mechanism of follicular replacement may not be occurring in DMBA-induced ovotoxicity.

Our results demonstrating DMBA-induced primordial follicle activation are directly at odds with the current literature, which suggests DMBA-induced ovotoxicity is not promulgated by PI3K/Akt signaling, a molecular pathway responsible for primordial follicle activation (Keating et al., 2010). The study conducted by Keating and colleagues used the compound LY294002, a competitive inhibitor of PI3K activation. Therefore, we hypothesized that DMBA-induced primordial follicle activation might be initiated downstream of PI3K. In support of this hypothesis, we observed increased
levels of Akt1 T308 and S437 phosphorylation in DMBA-treated primordial follicle oocytes (Fig. 4). Akt1 is phosphorylated on its T308 residue by Pdk1, an event which is essential for primordial follicle activation and occurs directly downstream of PI3K signaling (Reddy et al., 2009). Although pAkt1 (T308) is an indirect measurement of PIK3 activity, other events could have led to its phosphorylation. As mentioned previously, DMBA increased the expression of Dnajb6, which could lead to increased pAkt1 through PTEN abolition, and Calr, which has also been shown to induce Akt1 phosphorylation (Du et al., 2009). Akt1 is also phosphorylated on its S473 residue by mTORC2, an event which coincides with, although which may be independent of, Akt1 T308 phosphorylation (Polak and Hall, 2006).

To provide further support of DMBA-induced primordial follicle activation, we investigated the levels of mTOR phosphorylation in primordial follicle oocytes. mTOR is a Ser/Thr protein kinase which functions as a key regulator of protein translation and cell growth. It is now known that mTOR signaling is responsible for primordial follicle activation, forming the central component of the multimeric kinase complex mTORC1, whose suppression leads to a reduction in nucleocytoplasmic shuttling, resulting in primordial follicle activation (Adhikari et al., 2010). mTOR itself is regulated via a phosphorylation-dependent molecular switch, whereby mTOR Ser2448 phosphorylation results in activation and mTOR Thr2446 phosphorylation results in inhibition (Cheng et al., 2004). We observed an increase in mTOR Ser2448 phosphorylation and a decrease in mTOR Thr2446 phosphorylation in DMBA-treated primordial follicle oocytes compared with the control, suggesting increased mTOR activity resulting in primordial follicle activation (Fig. 5). As mTOR activation is proposed to be synergistic to PI3K/Akt signaling in primordial follicle activation, these results further support DMBA-induced primordial follicle activation (Adhikari et al., 2010). Interestingly, mTOR Ser2448 phosphorylation was primarily cytoplasmic, whereas mTOR Thr2446 phosphorylation was nuclear. Given that mTOR functions predominantly within the cytoplasm, these results suggest a possible phosphorylation-dependent nucleocytoplasmic shuttling mechanism of mTOR activation within the primordial follicle.

Within oocytes, increased pAkt1 leads to Foxo3a nucleocytoplasmic shuttling, resulting in primordial follicle activation (Reddy et al., 2010). However, Foxo3a was not detected in the cytoplasm of both DMBA- and control-treated primordial follicles (Fig. 5). This suggests that pAkt1 was not interacting with Foxo3a to cause primordial follicle activation. However, there was a significant reduction in the level of Foxo3a staining in the nucleus of DMBA-treated primordial follicles. Although the mechanism behind DMBA-induced Foxo3a reduction remains unknown, it is reasonable to assume that this reduction may have contributed to the observed increase in primordial follicle activation through a downstream event following DMBA Akt1 and mTOR phosphorylation.

In summary, this study represents the first in vitro examination of the effect of DMBA exposure on ovarian gene expression at the transcriptome level. Our results describe a multilayered mechanism of DMBA-induced ovoxicity which is not limited to cell death, involving both selective developing follicle atresia and primordial follicle activation. In regards to primordial follicle activation, DMBA activates downstream members of the PI3K/Akt and mTOR signaling pathway via phosphorylation events which may be independent of PI3K activity.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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