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Original Research

7,12-Dimethylbenz[a]anthracene-Induced Malignancies in a Mouse Model of Menopause

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Ovarian cancer has a high mortality rate because there are few symptoms in early disease development. The incidence of ovarian cancer increases in women after menopause. Understanding early events in this disease can best be achieved by using animal models. Therefore, the objective of this study was to develop and track the onset of ovarian tumorigenesis in mice mimicking characteristics of postmenopausal epithelial cancer in women. Female B6C3F1 mice (age, 28 d) received 4-vinylcyclohexene diepoxide (VCD, 160 mg/kg IV daily for 20 d) to cause ovarian failure. Four months after VCD treatment, via surgical intervention, each mouse received a single injection of 7,12-dimethylbenz[a]anthracene (DMBA) or vehicle control (sesame oil) under the bursa of the right ovary to cause ovarian neoplasms. The experimental groups were untreated controls (Con–Con), DMBA-treatment only (Con–DMBA), VCD treatment only (VCD–Con), and VCD+DMBA-treated (VCD+DMBA) mice. At 3, 5, 7, and 9 mo after DMBA injection, ovaries were collected for histologic and immunohistochemical evaluation. No tumors developed in Con–Con mice. All VCD-treated mice (with or without DMBA) exhibited ovarian failure. Mice that received both VCD and DMBA exhibited tumors at 3 mo (50%), 5 mo (14%), 7 mo (90%), and 9 mo (57%) after DMBA treatment; 31% of the tumors were epithelial in origin. Our findings confirm that inducing ovarian tumors in mice by chemical means is an effective method for studying early stages of tumor development that may be relevant to epithelial ovarian cancers that arise in postmenopausal women.

Abbreviations: DMBA, 7,12-dimethylbenz[a]anthracene; VCD, 4-vinylcyclohexene diepoxide.

Ovarian cancer, the most deadly female reproductive malignancy, has a high mortality rate because high-grade cancers are thought to metastasize early prior to the development of symptoms in early stages of disease.[4,27,28] The risk of contracting ovarian cancer over a lifetime is about 1 in 70, so it is a relatively rare cancer.[28] Although more than 20 types of ovarian malignancies exist, about 90% of human ovarian cancers are epithelial in origin.[28] Most cases are diagnosed at stages when the disease has metastasized outside the ovary, hindering efforts to treat or cure the disease. In addition, few reliable detection methods exist for early diagnosis of this disease. The incidence of ovarian cancer increases 8- to 10-fold among women in the peri- to postmenopausal period when compared with younger women.[28] The generation of animal models of ovarian cancer has been attempted for decades. These models have included whole-body irradiation,[5-7] chemical induction,[13,15,17,21] genetic manipulation,[18,25] and xenograph development.[9,23] It was observed as early as 1936 that the removal of all follicles from a mouse ovary was followed by the appearance of benign tubular adenomas in the residual ovarian tissue.[6,7,21,27] These adenomas appear to originate at the surface epithelium and proceed to invaginate and spread throughout the ovary.

As women transition from peri- to postmenopause, circulating levels of estrogen and progesterone decrease, and the relative ratio of estrogens to androgens decreases in response to the decline of estrogen. In addition, gonadotropin (follicle stimulating hormone, luteinizing hormone) levels rise due to loss of negative feedback on the anterior pituitary and, thereafter, remain elevated.[26] One theory of ovarian carcinogenesis proposes that increased circulating gonadotropin levels after menopause contribute to the development of ovarian epithelial cancers by stimulating surface epithelium proliferation.[18] Women who have undergone a natural progression to menopause have lost ovarian function but retain residual ovarian tissue. Therefore, because ovarian cancers in women arise more frequently after than before menopause, models developed in animals that have undergone ovarian failure and retain residual ovarian tissue likely most closely resemble the disease in postmenopausal women.

Repeated daily dosing of mice with the ovotoxic chemical 4-vinylcyclohexene diepoxide (VCD) results in a gradual onset of ovarian failure.[24] Because VCD selectively targets primordial and primary follicles,[22] larger follicles remain and develop toward ovulation.[8] With the depletion of primordial and primary follicles, recruitment into the larger follicle pool eventually ceases, and a gradual onset of ovarian failure results. In VCD-treated mice, estrogen and progesterone concentrations decline and follicle-stimulating hormone levels rise after follicle depletion, similar to the scenario in postmenopausal women.[19] A recent mouse model that combined virally induced changes in genes within the ovary and treatment with VCD resulted in ovarian failure along with induction of tumors characterized as undifferentiated tumors

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with mixed epithelial and stromal components along with some features of sex cord stromal tumors.[18]

In a previous study, female Fisher 344 rats with VCD-induced ovarian failure developed ovarian tumors after treatment with 7, 12-dimethylbenz[a]anthracene (DMBA).[11] Specifically, 57% of the VCD+DMBA-treated rats developed ovarian tumors within 5 months after DMBA treatment. However, the tumors were all classified as Sertoli–Leydig cell type lesions, which are rare ovarian neoplasms in women and often much less aggressive than are their epithelial counterpart.[27,28] In another study,[3] female $B_6C_3F_1$ mice were treated in the same way as in the Fisher 344 rat study.[9] Similarly, all tumors that developed within 5 mo in treated mice (28%) were also Sertoli–Leydig cell type masses. Therefore, the present study was undertaken in $B_6C_3F_1$ mice to observe and classify DMBA-induced ovarian tumor development at later time points (7 and 9 mo after DMBA exposure) to determine whether epithelial tumors would develop and, if so, when.

Materials and Methods

Reagents. VCD, DMBA, and sesame oil were purchased from Sigma Chemical Company (St Louis, MO). A polyclonal antibody to a wide range of cytokeratins was used (catalog no. ab9377, Abcam, Cambridge MA). An antibody directed to the N-terminus of human α-inhibin was obtained from Abbiotech (catalog no. 251404; San Diego, CA). Ketamine (catalog no. 91050) and xylazine (catalog no. 5530) were from Western Medical Supply (Arcadia, CA). Tert-amyl alcohol and tribromoethanol were from Aldrich Chemical (Milwaukee, WI). All other chemicals were from Sigma Chemical Company.

Animals. Female $B_6C_3F_1$ mice (n = 116) were purchased from Harlan (Dublin, VA) as 21-d-old pups. Mice were housed in the conventional facility in clear plastic cages with plastic lids containing filter space. Temperature, humidity, and photoperiod were constant (22 °C, 50% to 70%, 12:12-h light:dark cycle). Mice acclimated to the facility for 7 d before the experiment began. They were allowed access to food and sterilized distilled water ad libitum; throughout the experiment, mouse housing and use were in accordance with NIH guidelines and the policies of the University of Arizona IACUC.[12] The University of Arizona animal facility is fully accredited by the NIH, USDA, and AAALAC. According to the vendor, mice were pathogen-free for ectromelia virus, Hantaan virus, K virus, lactic dehydrogenase elevating virus, lymphocytic choriomeningitis virus, minute virus of mice, adenovirsues types 1 and 2, cytomegalovirus, mouse hepatitis virus, mouse parvovirus, polyoma virus, rotavirus, thymic virus, norovirus, pneumonia virus of mice, respiratory enteric virus III, Sendai virus, Theiler murine enchpahalomyetlitis, *Bordetella bronchioseptica*, cilia-associated respiratory bacillus, *Citrobacter rodentium*, *Clostridium pilforme*, *Corynebacterium kutscheri*, dermatophytes, *Helicobacter bilis*, *Helicobacter hepaticus*, *Helicobacter* spp., *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Mycoplasma pulmonis*, *Pastuerella multocida*, *Pasturella pneumotropica*, *Pneumocystis murina*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Staphylococcus aureus*, *Streptobacillus moniliformis*, *Streptococcus pneumoniae*, *Streptococcus* spp. group B, ecto- and endoparasites, enteric protozoa, and *Encephalitozoon cuniculi*.

VCD treatment. Beginning on day 28 of age, mice were injected intraperitoneally daily for 20 d with sesame oil (2.5 mL/kg body weight) or VCD (160 mg/kg, 1.14 mmol/kg) in sesame oil (2.5 mL/kg body weight) as previously described.[3] The body weight of each mouse was recorded daily, and the volume of each injection adjusted appropriately.

DMBA treatment. Four months after VCD treatment, by using a surgical approach, mice received a single injection of sesame oil or DMBA dissolved in sesame oil (50 µg DMBA in 5 µL oil) under the bursa of the right ovary, as previously described.[3] The left ovary was not injected. Control mice were injected with oil (5 µL) only. For the surgical approach, anesthesia was induced by using a mixture of ketamine (0.16 µg/kg body weight) and xylazine (0.032 µg/kg body weight) in sterile saline injected intraperitoneally or by using avertin (a mixture of tert-amyl alcohol, 193 mL/kg body weight, and 2,2,2,-tribromoethanol, 0.4 gm/kg body weight, in sterile saline, intraperitoneally) followed by isofluorane gas. The right flank region of skin was shaved, treated to remove fur, and cleaned with a providone–iodine solution and 70% ethanol. The ovary was accessed by an incision through the skin and muscle layers. The organ was located within a fat pad near the kidney, exteriorized along with the attached uterine horn, injected under the bursa with the appropriate treatment, and then returned intact to the body cavity. The muscle layer was closed with 6-0 absorbable suture, and the skin was closed with small metal wound clips. This method has been described previously.[3] Analgesia was administered as sodium ibuprofen (0.2 mg/mL) in sterile distilled drinking water for 24 h prior to until 3 d after surgery.

Experimental design. Mice were assigned randomly to 1 of 4 experimental groups. The Con–Con group (n = 29) received sesame oil by intraperitoneal injection (2.5 mL/kg daily for 20 d) followed by a single ovarian injection of sesame oil vehicle (5 µL) 4 mo later. Mice in the Con–DMBA group (n = 26) received sesame oil by intraperitoneal injection (2.5 mL/kg daily for 20 d) followed by a single ovarian injection of DMBA. The VCD–Con group (n = 25) received VCD (160 mg/kg, 1.14 mmol/kg) in sesame oil (2.5 mL/kg body weight) intraperitoneally daily for 20 d followed by a single ovarian injection of sesame oil 4 mo after VCD treatment. Mice in the VCD+DMBA group (n = 28) received VCD and DMBA as described.[3] A total of 116 mice began the study. During the course of the experiment, 7 animals died, and 1 was removed from the study due to conditions unrelated to the experiment. Therefore, data were collected from a total of 108 mice that completed the study. At various times after DMBA injection (3, 5, 7, and 9 mo), mice from each of the 4 experimental groups were euthanized, and ovaries were collected and processed for histologic evaluation.

Histology and immunohistochemistry. Ovarian tissue was harvested at appropriate times, fixed in 4% neutral buffered formalin for 2 to 4 h, and then transferred to 70% ethanol. The tissues were processed through graded alcohols and xylene, embedded in paraffin blocks, and sectioned (thickness, 5 µm). Routine hematoxylin and eosin stains were performed on every 10th section after mounting on glass slides. In addition, 2 sections from each of 3 areas of every ovary were mounted on glass slides (treated with Super Frost Plus, catalog no. 48311-703, VWR, Tempe, AZ) as unstained sections for processing by immunohistochemistry. A wide-spectrum anticytokeratin antibody was used to identify cells that were of epithelial origin. An antiα-inhibin antibody was used to identify cells of granulosa cell origin.

Some of the tissue sections were processed on a Discovery XT Automated Immunostainer (Ventana Medical Systems, Tucson, AZ). All steps performed on this instrument used manufacturer-

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validated reagents, including deparaffinization, cell conditioning (antigen retrieval with a borate–EDTA buffer), primary antibody staining, detection and amplification by using a biotinylated streptavadin–horseradish peroxidase and diaminobenzidine system, and hematoxylin counterstaining. After staining on the instrument, slides were dehydrated through graded alcohols to xylene and coverslipped with mounting medium (catalog no. 4112, Richard–Allen, VWR). Other slides were processed by hand as follows: slides were heat-treated (62 °C for 1 h), deparaffinized with xylene, treated with methanol containing 0.6% H_2O_2, passed through graded alcohols (100% to 70%), rinsed in distilled water, treated with protease at 37 °C for 10 min, washed in PBS, blocked with 5% BSA in PBS, rinsed twice, and then treated overnight with primary antibody. The next day, the slides were treated with a biotinylated secondary antibody for 1 h at room temperature, washed with PBS, treated with streptavadin–horseradish peroxidase for 1 h, and washed, and the stain was visualized with freshly prepared diaminobenzidine substrate for 25 min. Slides were rinsed with water and counterstained with dilute hematoxylin, dehydrated with 2 rinses of 100% ethanol and 2 rinses of xylene, and mounted with coverslips and Cytoseal XYL (catalog no. 22050262, Fisher Scientific, Houston, TX).

Results

Figure 1 shows the incidence of tumors of all types for each of the 4 treatment groups across each of the 4 time points, as well as the total number of tumors developed over all time points within a treatment group (Figure 1, insert). Tumors collected from mice in this study were of 3 general types. The most common was sex cord stromal tumors resembling Sertoli–Leydig cell neoplasms (38%) similar to those seen in previous experiments conducted in rats[11] and mice.[3] Another sex cord–gonadal stromal type was granulosa cell tumors (19%). The granulosa cell and Sertoli–Leydig cell tumors were shown to be other than epithelial in origin due to their negative response to the wide-spectrum cytokeratin antibody and the positive reaction to an α-inhibin antibody (Figure 2 A through C). The second most common tumor was adenocarcinoma of epithelial origin (31%), as demonstrated by a positive reaction to the wide-spectrum polyclonal anticytokeratin antibody (Figure 2 D through F). These tumors occurred in mice treated with VCD only (1 of 7 mice) or VCD and DMBA (7 of 17 mice). Furthermore, adenocarcinomas developed only at the later time points (7 and 9 mo). In addition, Figure 2 shows a carcinoma of mixed origin (both epithelial and nonepithelial characteristics), as demonstrated by its areas of positive and negative staining for cytokeratin. This tumor type had scattered malignant cells within it that stained positive for α-inhibin. A total of 11% of tumors were uncharacterized.

A clear majority of mice that had undergone VCD-induced ovarian failure also developed nonmalignant tubular adenomas. The adenomas were seen in 88% of right ovaries and in 84% of left ovaries of mice treated with VCD alone. However, when mice had also been treated with DMBA in the right ovary, fewer (32%) of the right ovaries developed adenoma, whereas the contralateral left ovary within these same animals showed 82% with tubular adenomas. None of the Con–Con animals or Con–DMBA mice (25 animals) developed tubular adenoma.

Figure 3 shows an ovary from a Con-Con animal stained with hematoxylin and eosin (Figure 3A) or stained with hematoxylin and treated with anticytokeratin antibody (Figure 3B), demonstrating intense staining in a single layer of surface epithelium cells. In contrast, an ovary from a VCD–Con mouse demonstrates that, as a result of VCD treatment, tubular structures lined with epithelial cells (cytokeratin-positive), that is tubular adenomas, have developed throughout large areas of the ovary (Figure 3).

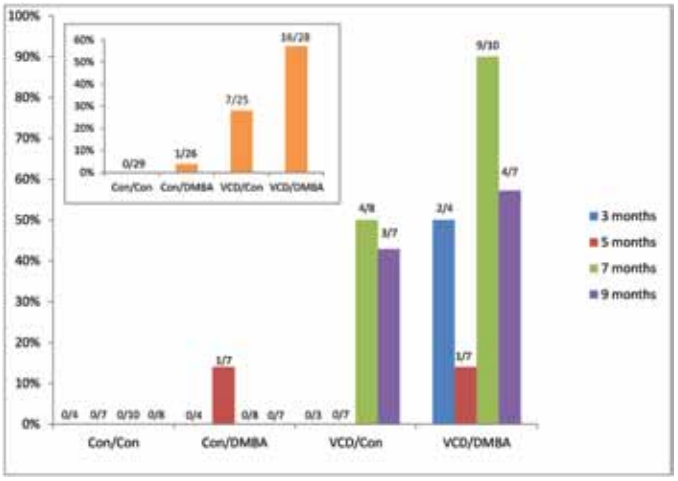

Figure 1. Incidence of tumor development. Female $B_6C_3F_1$ mice were treated daily with or without (control, Con) VCD and with or without DMBA as described in the Methods. Insert, The total number of tumors that developed in all animals over all time points by treatment group. Bars represent the percentage of mice that developed tumors in each group. Numbers over the bars indicate the number of mice with tumors compared with the total number of mice in that group.

Figure 4 shows a section of ovary from a VCD+DMBA-treated mouse at the 7-mo time point and stained with hematoxylin and eosin. This is an example of a granulosa cell tumor with a high mitotic index. Sections of this same ovary were stained for a variety of cytokeratins by using a wide-spectrum polyclonal antibody and antibody for α-inhibin. Whereas the cytokeratin stain is negative, the α-inhibin stain is positive, showing brown staining that is predominantly in the cellular cytosol.

Discussion

Ovarian cancer is the most lethal of all the reproductive malignancies in women. Even though many advances have recently been made in chemotherapy, surgery and the understanding of the genetics of ovarian cancers, little has changed with respect to mortality. In fact, 60% of women who contract this cancer die from it.[27] Most ovarian cancers occur in postmenopausal women. Whether this incidence is due to ovarian failure or aging is not well understood. Therefore, an animal model that approximates peri- and postmenopause at an age at which mice are still reasonably viable would be a helpful addition to understanding the development and progression of ovarian tumor formation.

Many ovarian cancer models have been proposed and developed, but only the method described in the current study and a few others in mice[3,18] and rats[11,14] have deliberately attempted to approximate in rodents the natural course of events that are associated with menopause in women. In the current study, ovarian failure, caused by repeated daily dosing of mice with VCD, serves as an animal model for menopause due to a gradual onset of follicular depletion with a concomitant loss of circulating progesterone and estradiol and a rise in circulating follicle-stimulating hor-

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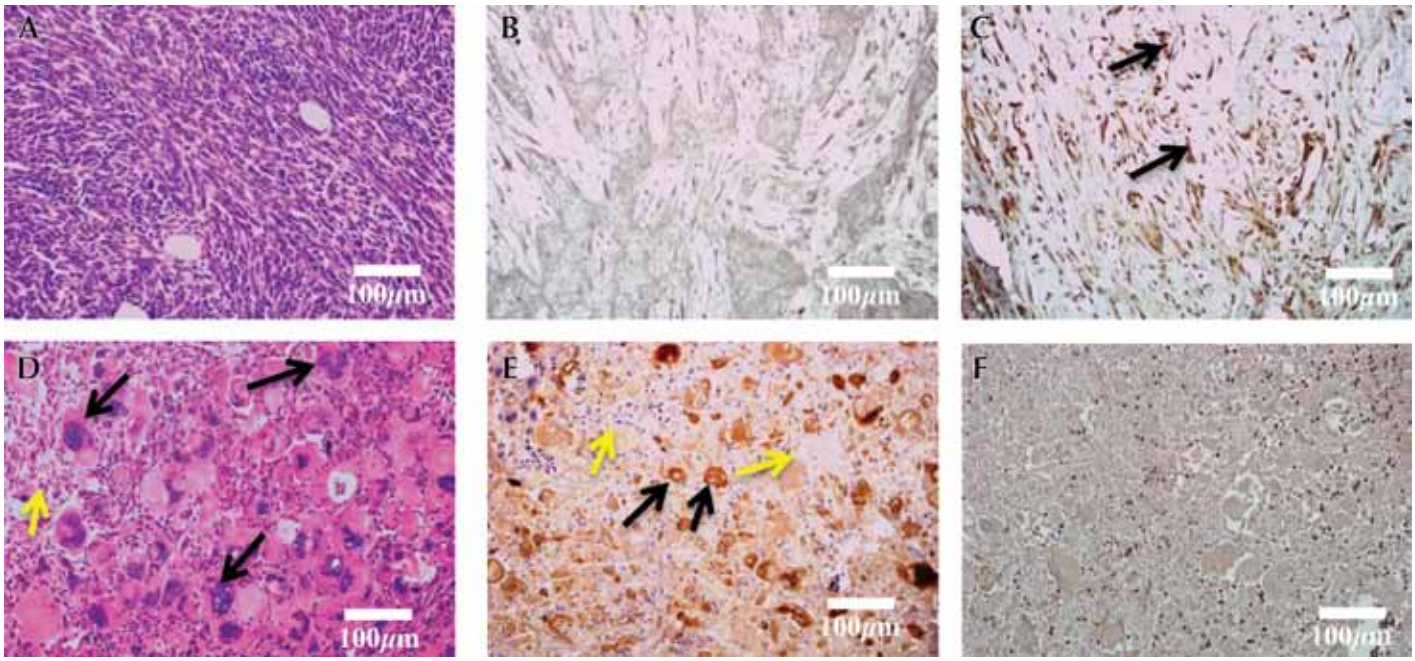

Figure 2. Histology of various tumor types. (A) Nonepithelial malignant tumor from a VCD+DMBA-treated mouse. It displays sheets of spindle-shaped cells and is likely a sarcoma. The ovary was collected 9 mo after DMBA injection; hematoxylin and eosin stain. (B) The same ovary as in panel A, but treated with a wide-spectrum cytokeratin antibody. (C) The same ovary as in panel A, but treated with an α-inhibin antibody (brown stain). (D) Malignant tumor containing epithelial (adenocarcinoma) and nonepithelial components from a VCD+DMBA-treated mouse. Black arrows indicate adenocarcinoma cells. Yellow arrow indicates an area of nonepithelial cells. The ovary was collected 9 mo after DMBA injection; hematoxylin and eosin stain. (E) The same ovary as in panel D but treated with a wide-spectrum cytokeratin antibody. Black arrows show positively stained cells (brown stain). Yellow arrows indicate areas of unstained cells. (F) The same ovary as in panel D but treated with an α-inhibin antibody, showing no staining. Scale bars, 100 μm.

mone.[19,24] Further, the mice retain residual ovarian tissue. This mouse model of menopause (VCD treatment) was combined with a known carcinogen (DMBA) to create animals with which to study the early stages of ovarian tumor development. The model for ovarian failure combined with tumor development used here was based on our earlier work.[3,11,14,19,20,24]

The present study was designed to identify an optimal time in which tumors induced by ovarian exposure to DMBA after chemical-induced ovarian failure in mice can be observed. Ovarian tumors mostly occurred in DMBA-treated mice that had undergone VCD-induced ovarian failure (VCD+DMBA, 57%). Furthermore, the majority of those tumors were noted at later (7 and 9 mo) as compared with earlier (3 and 5 mo) times after DMBA exposure.

In a recent study, ovarian failure was induced by VCD in the tg-CAG-LS-Tag mouse model of ovarian cancer.[18] In that study, no tumors were observed in mice 1 y after treatment with VCD only (without tumorigenic induction). The authors concluded that VCD did not have direct tumorigenic effects. However, in the present study, 28% of mice in the VCD–Con group developed tumors within a year of VCD exposure. Therefore, whether tumors in the VCD–Con group developed as a result of ovarian failure or due to direct effects of VCD is unknown. Future studies using the tg-CAG-LS-Tag model of ovarian cancer would be useful for making this distinction.

In women, the majority of ovarian cancers are thought to originate from the surface epithelium or the epithelium lining the fallopian tube.[16] With the onset of menopause, depletion of follicles results in ovarian failure with a loss of production of estrogen and progesterone. As a result of the loss of negative feedback on the pituitary, gonadotropin (follicle-stimulating hormone and luteinizing hormone) levels rise. In women, this situation results in surface invaginations containing epithelial cells (inclusion cysts), which are thought to be the tissues of origin in many ovarian cancers.[1] However, a new theory concerning the origin and pathogenesis of human ovarian cancer has been proposed recently. This theory states that many cancers formerly thought to be ovarian in origin may actually be from epithelial cells of fallopian tubes, uterine endometrium, or other pelvic organs.[16] In rats and mice, unlike humans, ovarian tumors are more commonly thought to have a somatic cell origin, because the majority of tumors are either of a granulosa or Sertoli–Leydig cell type.[3,11,26] Spontaneous ovarian tumors in mice are rarely epithelial in origin.

Therefore, another aim of the current study was to create tumors that were epithelial in origin. In mice, after VCD-induced ovarian failure, residual ovarian tissue contains nonmalignant tubular adenomas of epithelial cell origin.[3] Others have noted similar tubular adenomas in mice.[6,7,18,27] Because the number of epithelial cells in tubular adenomas is increased greatly after VCD-induced ovarian failure, we reasoned that this approach might expose more epithelial cells to the carcinogenic effects of DMBA, and thereby, increase the number of epithelial tumors. Accordingly, 31% of mice that developed tumors in the present study had malignancies with epithelial characteristics (positive for cytokeratin by using a polyclonal anticytokeratin antibody). Interestingly, epithelial tumors only developed at later time points (7 and 9 mo); those times had not been investigated in earlier studies in which only Sertoli–Leydig cell type tumors were observed[3,11] The previous studies used the identical dosing regi-

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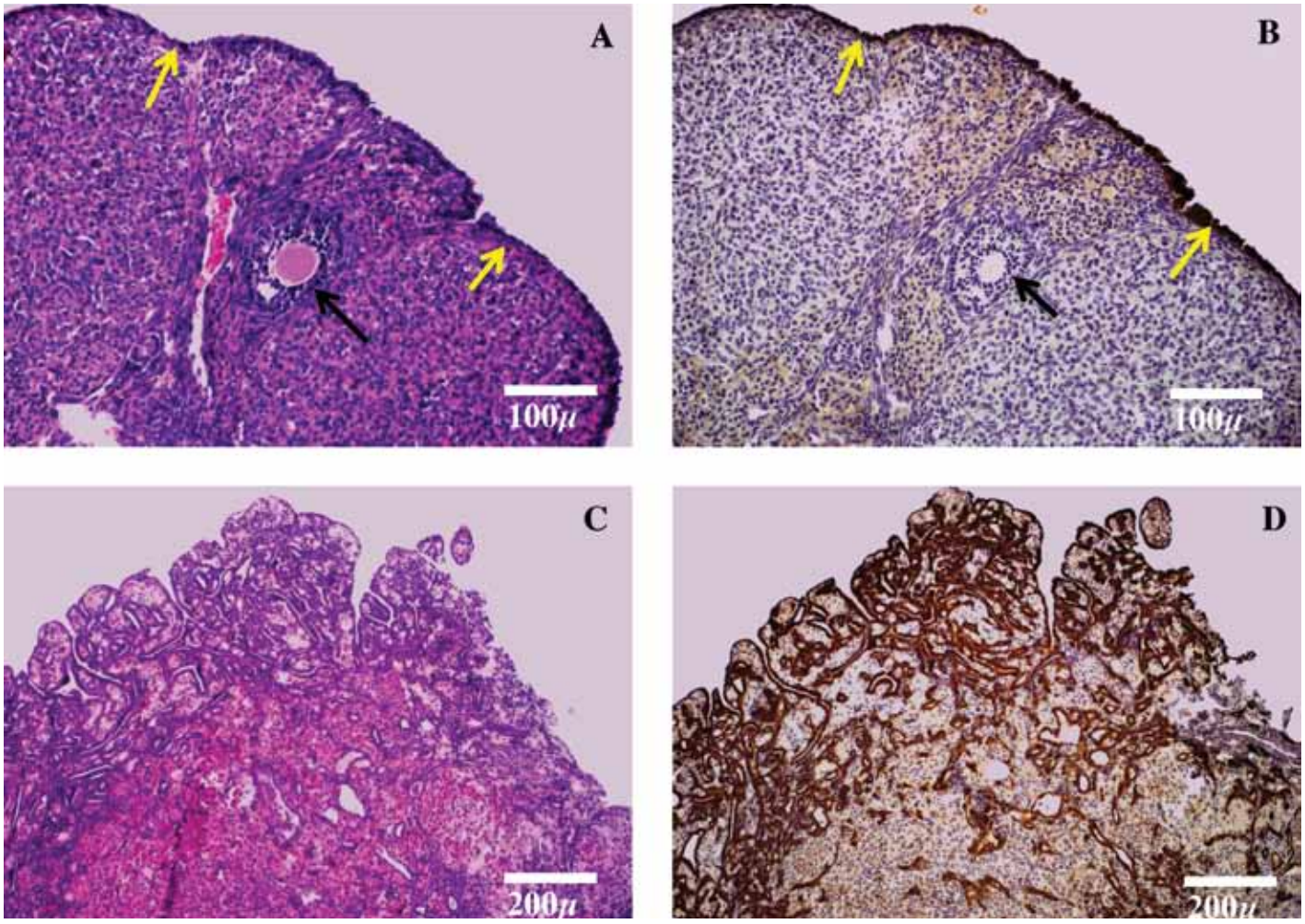

Figure 3. Histology of tubular adenomas. (A) Ovary from an untreated control (Con–Con) mouse. A developing antral follicle (black arrow) and the edge of the ovary (yellow arrows) containing a single layer of epithelial cells are indicated. The ovary was collected 9 mo after ovarian injection with vehicle; hematoxylin and eosin stain. (B) The same ovary as in panel A but treated with a wide-spectrum cytokeratin antibody (brown stain). The single layer of epithelial cells (yellow arrows) is now stained brown. Black arrow, antral follicle. Scale bar, 100 µm. (C) Ovary from an animal injected daily for 20 d with VCD only. The ovary was collected 9 mo after vehicle injection into the right ovary; hematoxylin and eosin stain. (D) The same ovary as in panel C but treated with a wide-spectrum cytokeratin antibody. Scale bar, 200 µm.

men with VCD and DMBA as in the current study, but ovaries were evaluated only at 3 and 5 mo after the single intraovarian injection with DMBA. Therefore, the current observation supports that extended time points can result in epithelial tumor development, and this approach may facilitate studying the pathophysiology of ovarian tumors and testing anticancer drugs. Despite this improvement, a method to more reliably produce epithelial tumors in the VCD model remains of interest. Such an improvement would provide a more consistent approach to be used for testing treatment modalities or investigating developmental signaling and early detection.

Recently, a transgenic mouse model of ovarian cancer (Tg-*MISIIR-TAg-DR26*) has been developed in which female mice express the large and small T-antigen genes of SV40. Expression is directed to the ovary because it is under the control of the 5′ upstream regulatory region of the promoter for Mullerian inhibitory substance receptor 2. The transgenic female mice develop ovarian neoplasms of epithelial origin with 100% penetrance within 10 to 12 wk of age.[10] Neoplasms develop spontaneously in these animals, are completely bilateral and epithelial in origin, and display characteristics of serous morphology (the most common subtype in women[27]). In addition, these neoplasms are often accompanied by malignant ascites and peritoneal spreading.[2] Therefore, future studies combining the VCD model with the Tg-MISIIR-TAg transgenic mice might provide an ideal approach for enhanced development of epithelial ovarian tumors in a 'postmenopausal' animal. During the course of the current study, mice that were dosed with VCD developed nonmalignant tubular adenomas lined with cells of an epithelial origin within 4 mo after the end of VCD dosing. Whereas more than 80% of ovaries from VCD-treated mice developed these adenomas, only 32% of right ovaries that had been injected with DMBA displayed them. The reason for this apparent effect of DMBA is unknown. However, DMBA may have caused a direct toxic effect on that tissue, which impaired its capacity for ordered cellular proliferation.

In summary, the results presented here demonstrate that 7 mo after DMBA treatment is the optimal time point for observing epithelial tumor development in the VCD–DMBA model. This

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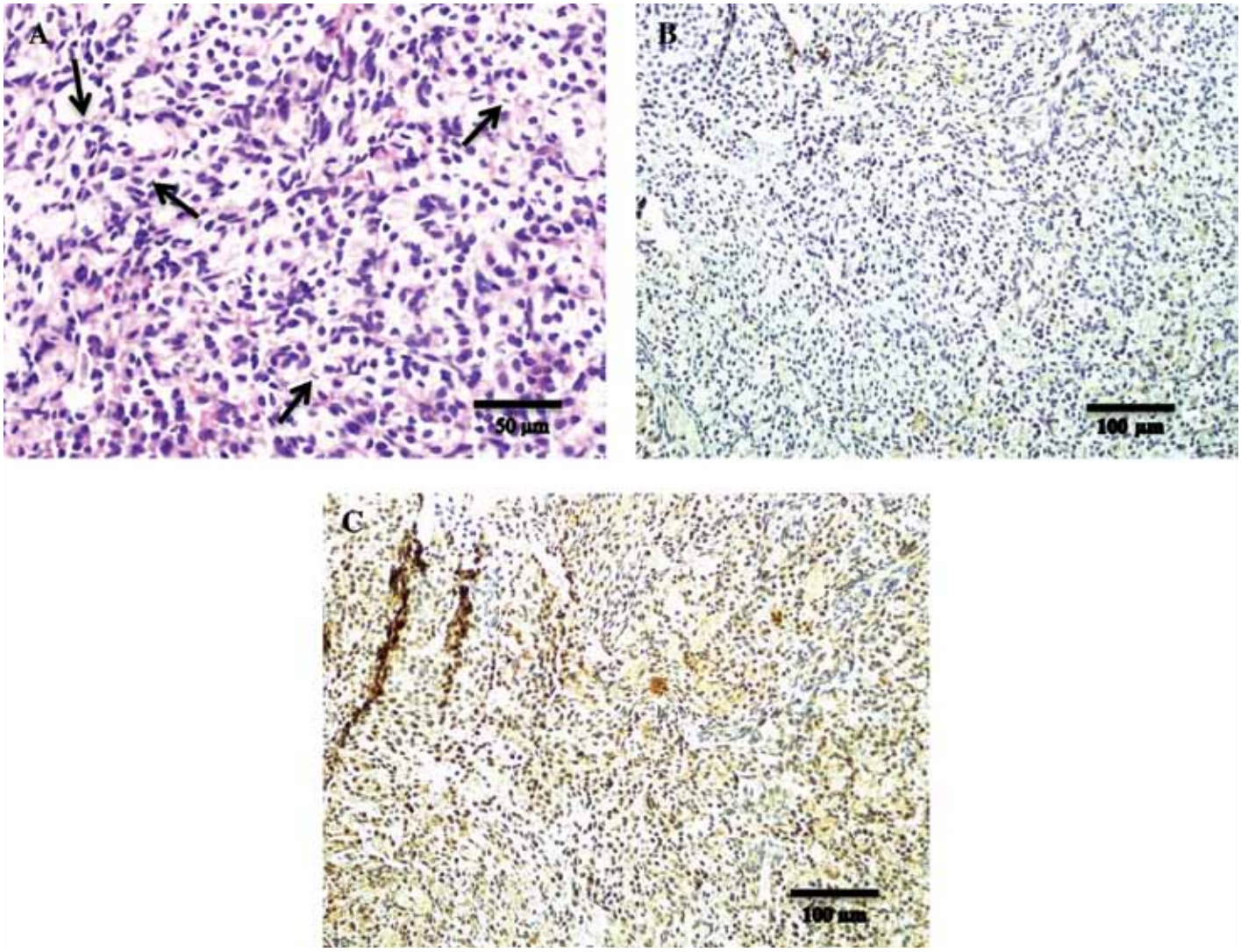

Figure 4. Histology of granulosa cell tumors. (A) Granulosa cell tumor from a VCD+DMBA-treated mouse. The ovary was collected 7 mo after DMBA injection. Black arrows, mitotic figures; scale bar, 50 µm. (B) The same ovary as in panel A but treated with a wide-spectrum anticytokeratin antibody. Scale bar, 100 µm. (C) The same ovary as in panel A but treated with an α-inhibin antibody (brown stain). Scale bar, 100 µm.

approach affords the ability to model the postmenopausal period for studying early stages of tumor development, with a substantial number being epithelial in origin. Future studies will include larger numbers of mice focused on the 7-mo time point and will explore improved approaches for the production of more extensive epithelial ovarian tumor development.

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