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## Distribution and responsiveness of rat anti-Müllerian hormone during ovarian development and VCD-induced ovotoxicity

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### Abstract

Anti-Müllerian hormone (AMH) is produced by granulosa cells in primary to small antral follicles of the adult ovary and helps maintain primordial follicles in a dormant state. The industrial chemical, 4-vinylcyclohexene diepoxide (VCD) causes specific ovotoxicity in primordial and small primary follicles of mice and rats. Previous studies suggest that this ovotoxicity involves acceleration of primordial to primary follicle recruitment via interactions with the Kit/Kit ligand signaling pathway. Because of its accepted role in inhibiting primordial follicle recruitment, the present study was designed to investigate a possible interaction between AMH and VCD-induced ovotoxicity. Protein distribution of AMH was compared in neonatal and adult F344 rat ovaries. AMH protein was visualized by immunofluorescence microscopy in large primary and secondary follicles of the adult ovary, but in small primary follicles in neonatal rat ovaries. In cultured postnatal day (PND) 4 F344 rat ovaries, VCD exposure (30  $\mu$ M, 2-8d) decreased ( $P<0.05$ ) AMH mRNA (d4-8) and protein (d6-8). Recombinant AMH (100-400 mg/ml) in PND4 ovaries cultured 8d  $\pm$  VCD (30  $\mu$ M) caused an increase ( $P<0.05$ ) in primordial, and a decrease ( $P<0.05$ ) in small primary follicles, supporting that AMH retarded primordial follicle recruitment. However, no concentration of AMH had an effect on VCD-induced ovotoxicity. Whereas, VCD caused a reduction in expression of AMH (d4-d8), it followed previously reported initial disruptions in Kit signaling induced by VCD (d2). Thus, collectively, these results do not support a mechanism whereby VCD causes ovotoxicity via generalized activation of primordial follicle recruitment, but instead provide further support for the specificity of other intracellular mechanisms involved in VCD-induced ovotoxicity.

### Keywords

anti-Müllerian hormone; Müllerian inhibiting substance; 4-vinylcyclohexene diepoxide; follicle; ovary

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### Conflict of Interest:

The authors declare that there are no conflicts of interest.

## Introduction

Female mammals are born with a finite pool of immature ovarian primordial follicles (Hirshfield, 1991). Primordial follicles are selected for activation, however, only a few (<1%) of the oocytes in these follicles will fully develop and be ovulated. Instead, the vast majority become activated and degrade by a process of atresia. Individual follicle activation can occur relatively early or much later in the life span of a female (Hirshfield, 1991). Factors coordinating this activation/recruitment are not clearly understood. It is thought that the rate of primordial follicle activation/recruitment is influenced in part by signaling from larger growing follicles (Adhikari and Liu, 2009).

Anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS), is a member of the transforming growth factor  $\beta$  (TGF $\beta$ ) superfamily and uses a bone morphogenic protein-like pathway for signal transduction. AMH was first recognized for its role in causing regression of Müllerian ducts during fetal development in males (Sinisi *et al.*, 2003). In females, AMH is expressed postnatally by granulosa cells of ovarian growing pre-antral and early antral follicles in which it decreases the responsiveness of growing follicles to FSH (Broekmans *et al.*, 2008). Additionally, AMH inhibits the recruitment of primordial follicles into the pool of growing follicles (Durlinger *et al.*, 2001, 2002a, 2002b). It is unclear, however, whether AMH has a direct effect on primordial follicles or provides the signaling indirectly. Previous studies in adult rats have shown that AMH is not expressed in primordial or small primary follicles, but is first expressed in granulosa cells of maturing large primary follicles and is at its maximal expression in the small preantral follicles (Baarends *et al.*, 1995). At the large antral follicle stage, AMH expression diminishes and ultimately becomes undetectable once FSH-dependent follicular growth is initiated (Durlinger *et al.*, 2001). Thus, it appears that AMH provides a mechanism by which larger follicles can indirectly influence and suppress primordial follicle development by having a direct effect on small follicles. However, the mechanisms involved are not clearly understood since the two receptors for AMH are located in the granulosa cells of secondary and antral follicles (Durlinger *et al.*, 2002b). As a result, the mechanism by which AMH inhibits primordial follicle recruitment is unknown. Further, AMH is not the only important factor for regulating follicle recruitment because AMH knockout mice still retain some primordial follicles at 13 months of age (Durlinger, *et al.*, 1999).

4-Vinylcyclohexene diepoxide (VCD) is a by-product of the chemical synthesis of rubber tires, insecticides, flame retardants, and plasticizers (Rappaport and Fraser, 1977). VCD selectively targets small pre-antral (primordial and small primary) follicles in the ovaries of rats and mice (Springer *et al.*, 1996a; 1996b). Extensive repeated exposure of rats and mice to VCD results in depletion of the follicular pool and premature ovarian failure (Mayer *et al.*, 2002; Devine and Hoyer, 2005). Thus, exposure of women to VCD is viewed as a potential health risk.

The neonatal rat ovarian culture system is an ideal model for studying VCD-induced ovotoxicity because those ovaries predominantly contain the earliest follicle stages, which are selectively targeted by VCD. Prior studies have characterized this model and demonstrated that ovaries cultured for up to 15 days remained healthy, and follicle development progressed to the secondary stage over time (Devine *et al.*, 2002). Further, incubation of cultured neonatal rat ovaries with 30  $\mu$ M VCD for 8d caused a significant loss of primordial and small primary follicles. However, VCD treatment for 8d did not induce visible signs of necrosis nor was follicle development or morphology grossly altered relative to controls.

Previous mechanistic studies using the ovary culture system demonstrated that VCD interacts with the Kit/Kit ligand signaling pathway. VCD exposure caused a decrease ( $P < 0.05$ ) in Kit mRNA expression in rat ovaries by both *in vivo* and *in vitro* exposure (Fernandez *et al.*, 2008). A time course of postnatal day (PND) 4 rat ovaries exposed *in vitro* to VCD (d2-8) observed a decrease in Kit mRNA on d4, prior to the observed follicle loss on d6 (Fernandez *et al.*, 2008; Keating *et al.*, 2009a). Additionally, exogenous Kit ligand was able to attenuate VCD-induced follicle loss (Fernandez *et al.*, 2008). These findings provided a possible mechanism by which the Kit/Kit ligand pathway is targeted in VCD-induced ovotoxicity.

Previous observations investigated an involvement of PI3kinase as a signaling molecule downstream of Kit which directs primordial follicle activation and recruitment (Liu *et al.*, 2006). LY294002, a competitive inhibitor of PI3kinase, was used to prevent the recruitment of primordial follicles into the primary follicle pool. Interestingly, this inhibition also prevented VCD-induced ovotoxicity in primordial follicles, but increased follicle loss in small primary follicles (Keating *et al.*, 2009a). AMH signaling has also been shown to play a significant role in primordial follicle recruitment; however, its potential ability to interact with VCD-induced ovotoxicity is unknown. Therefore, the present study was designed to further investigate the selectivity of VCD for the Kit/PI3K signaling pathway by determining whether inhibition of primordial follicle recruitment using exogenous AMH could also impact VCD-induced loss of primordial and/or small primary follicles. In addition, AMH mRNA and protein expression patterns are compared in neonatal and adult rat ovaries.

## Materials and Methods

### Reagents

4-vinylcyclohexene diepoxide (VCD; CAS # 106-87-6; >99% purity), bovine serum albumin (BSA), ascorbic acid (Vitamin C), and transferrin were purchased from Sigma-Aldrich Inc. (St Louis, MO). Dulbecco's Modified Eagle Medium: nutrient mixture F-12 (Ham) 1X (DMEM/Ham's F12), Albumax, penicillin/streptomycin (5000U/ml, 5000µg/ml, respectively), Hanks' Balanced Salt Solution (without CaCl<sub>2</sub>, MgCl<sub>2</sub>, or MgSO<sub>4</sub>) were obtained from Invitrogen Co. (Carlsbad, CA). Millicell-CM filter inserts were purchased from Millipore (Bedford, MA), and 48-well cell culture plates were obtained from Corning Inc. (Corning, NY). Anti-AMH antibody and donkey anti-goat secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). BCA protein quantification kits were obtained from Pierce Biotechnology (Rockford, IL). Cy-5-streptavidin was obtained from Vector (Burlingame, CA). YOYO-1 was purchased from Molecular Probes (Eugene, OR). Recombinant human AMH was purchased from R&D Systems (Minneapolis, MN).

### Animals

A breeding colony was established from Fischer 344 rats that were originally purchased from Harlan Laboratories (Indianapolis, IN) to use as a source of PND4 female rat pup ovaries for culture. All pregnant animals were housed singly in plastic cages, and maintained in a controlled environment ( $22 \pm 2^\circ\text{C}$ ; 12h light/ 12h dark cycles). The animals were provided a standard diet with *ad libitum* access to food and water, and allowed to give birth. All animal experiments were approved by the University of Arizona's Institutional Animal Care and Use Committee.

## Rat ovary collection

Ovaries were collected from sixteen week old adult cycling female F344 rats (n=3) in the breeding colony. Ovaries were collected on the day of estrus (determined by vaginal cytology). Ovaries were also collected from PND4 and PND6 rats. Rats were euthanized by CO<sub>2</sub> inhalation and the ovaries were removed. The oviduct and excess tissue was trimmed from the ovaries, which were snap frozen in liquid nitrogen, and stored at -80°C until processing for histological evaluation or for protein isolation.

## *In vitro* ovarian culture

Ovaries from PND4 F344 rats were cultured as described by Devine *et al.* (2002). Briefly, PND4 female F344 rats were euthanized by CO<sub>2</sub> inhalation followed by decapitation. Each ovary was removed, and after oviduct and excess tissue were trimmed, it was placed on a piece of Millicell-CM membrane floating on 250 µl of DMEM/Ham's F12 medium containing 1 mg/ml BSA, 1 mg/ml Albumax, 50 µg/ml ascorbic acid, 5 U/ml penicillin/5 µg/ml streptomycin, and 27.5 µg/ml transferrin per well in a 48-well plate previously equilibrated to 37°C. Using fine forceps, a drop of medium was placed to cover the top of the ovary to prevent drying. Plates containing ovaries were cultured at 37°C and 5% CO<sub>2</sub> in air. For those cultures lasting more than 2d, media were removed and fresh medium ± treatment were replaced every 2d. Ovaries were treated with vehicle control medium (1% DMSO), or VCD (30 µM) ± AMH (100, 200, 400 ng/ml) for 8d. The concentrations and conditions of VCD exposures were previously determined to cause follicle loss *in vitro* (Fernandez *et al.*, 2008). All cultured ovaries remained viable up through 8 days in culture; consistent with a previous report (Devine *et al.*, 2002).

## Histological evaluation of follicle numbers

Following incubation, ovaries were placed in Bouin's fixative for 1.5h, transferred to 70% ethanol, embedded in paraffin, serially sectioned (5 µm thick), and every 6<sup>th</sup> section was mounted. All ovarian sections were stained with hematoxylin and eosin. Healthy oocyte-containing follicles were classified and counted in every 12<sup>th</sup> section. Unhealthy follicles were distinguished from healthy follicles by granulosa cell content of pyknotic bodies and intense eosinophilic staining of oocytes (Devine *et al.*, 2002). Follicle population classification was according to the procedure of Flaws *et al.* (1994) with modifications. Briefly, primordial follicles contained the oocyte surrounded by a single layer of squamous-shaped granulosa cells, small primary follicles contained the oocyte surrounded by three to twenty cuboidal-shaped granulosa cells, large primary follicles contained the oocyte surrounded by greater than twenty cuboidal-shaped granulosa cells and secondary follicles contained the oocyte surrounded by multiple layers of granulosa cells. In cultured neonatal rat ovaries, no follicle development beyond the secondary stage was observed. Histological images were captured with an Olympus IX-70 inverted microscope.

## Immunofluorescence staining and confocal microscopy

Following *in vitro* culture or collection from 16 week old adult rats, ovaries were fixed in 4% buffered formalin for 2-4 h, transferred to 70% ethanol, embedded in paraffin, serially sectioned, and every 10<sup>th</sup> section was mounted. Sections were deparaffinized (approximately 10 sections/ovary) and incubated with primary antibody directed against AMH (1:200 dilution) 4°C overnight. Secondary biotinylated antibody was applied for 1 h, followed by CY-5-streptavidin (1 h; 1:100 dilution). Sections were treated with Ribonuclease A (100µg/ml) for 1 h, followed by staining with YOYO-1 (10 min; 5nM). Slides were repeatedly rinsed with phosphate buffered saline (PBS), cover-slipped, and stored in the dark (4°C) until visualization. Primary antibody was not added to immuno-negative ovarian sections. Immunofluorescence was visualized on a Zeiss (LSM 510 NLO-Meta) confocal microscope

with an argon and helium-neon laser projected through the tissue into a photomultiplier at  $\lambda = 488$  and  $633$  nm for YOYO-1 (green) and CY-5 (red), respectively. All images were captured using a 40 X objective lens. Multiple sections were imaged and analyzed throughout each ovary.

### RNA isolation

Following *in vitro* culture, ovaries treated with vehicle control or VCD (30  $\mu$ M) were stored in RNAlater at  $-80^{\circ}\text{C}$ . Total RNA was isolated ( $n=3$ ; 10 ovaries per pool) using an RNeasy Mini kit. Briefly, ovaries were lysed and homogenized using a motor pestle followed by applying the mixture onto a QIAshredder column. The QIAshredder column containing ovarian tissue sample was then centrifuged at 14,000 rpm for 2 min. The resulting flow-through was applied to an RNeasy mini column, allowing RNA to bind to the filter cartridge. Following washing, RNA was eluted from the filter, and concentrated using an RNeasy MinElute kit. Briefly, isolated RNA was applied to an RNeasy MinElute spin column, and after washing, RNA was eluted using 14  $\mu$ L of RNase-free water. RNA concentration was determined using an ND-1000 Spectrophotometer ( $\lambda = 260/280\text{nm}$ ; NanoDrop technologies, Inc., Wilmington, DE).

### First strand cDNA synthesis and real-time polymerase chain reaction (PCR)

Total RNA (0.5  $\mu$ g) was reverse transcribed into cDNA utilizing the Superscript III One-Step RT-PCR System. cDNA was diluted (1:25) in RNase-free water. Two microliters of diluted cDNA were amplified on a Rotor-Gene 3000 using Quantitect<sup>TM</sup> SYBR Green PCR kit and custom designed primers for AMH (forward primer: 5' GGA GAC CTA CCA AGC CAA CA 3'; reverse primer: 5' CAT TTT TAG CAG CAG CCA CCA; NCBI Genbank accession number NM\_007445) and  $\beta$ -actin (forward primer: 5' TCT ATC CTG GCC TCA CTG TC 3'; reverse primer: 5' ACG CAG CTC AGT AAC AGT CC 3'; NCBI Genbank accession number NM\_031144). The cycling program consisted of a 15 min hold at  $95^{\circ}\text{C}$  and 45 cycles of: denaturing at  $95^{\circ}\text{C}$  for 15s, annealing at  $58^{\circ}\text{C}$  for 15s, and extension at  $72^{\circ}\text{C}$  for 20s at which point data were acquired. Product melt conditions were determined using a temperature gradient from  $72^{\circ}\text{C}$  to  $99^{\circ}\text{C}$  with a  $1^{\circ}\text{C}$  increase at each step. There was no difference in  $\beta$ -actin mRNA between vehicle control and VCD-treated ovaries. Therefore, each sample was normalized to  $\beta$ -actin before quantification.

### Protein Isolation

Pools of whole ovarian protein homogenates (cultured ovaries, 10 per pool; 16 week old ovaries, 1 per pool) were prepared from cultured ovaries via homogenization in tissue lysis buffer as previously described (Thompson *et al.*, 2005). Briefly, homogenized samples were placed on ice for 30 min, followed by two rounds of centrifugation at 10,000 rpm for 15 min. Supernatant was aliquoted and stored at  $-80^{\circ}\text{C}$  until further use. Protein was quantified using a standard BCA protocol on a 96-well assay plate. Emission absorbance values were detected with a  $\lambda = 540\text{nm}$  excitation on a Synergy<sup>TM</sup> HT Multi-Detection Microplate Reader using KC4<sup>TM</sup> software (Bio-Tek<sup>®</sup> Instruments Inc., Winooski, VT). Protein concentrations were calculated from a BSA protein standard curve.

### Western Blot Analysis

SDS-PAGE (10%) was used to separate proteins in homogenates (25  $\mu$ g;  $n=3$ ) and subsequently transferred onto nitrocellulose membranes as previously described (Thompson *et al.*, 2005). Briefly, membranes were blocked for 1 h with shaking at  $4^{\circ}\text{C}$  in 5% milk in Tris-buffered saline with Tween-20 (TTBS). Membranes were incubated with primary antibody in 5% milk in TTBS overnight at  $4^{\circ}\text{C}$ . The antibody dilution used was AMH (1:200). Membranes were washed three times for 10 min each with TTBS. HRP-conjugated

secondary antibody (1:2000 dilution) was added for 1 h at room temperature. Membranes were washed three times for 10 min each in TTBS, followed by a single wash for 10 min in Tris Buffered Saline (TBS). Western blots were detected by chemiluminescence (using ECL plus chemiluminescence detection substrate) and exposed to X-ray film. Densitometry of the appropriate bands was performed using LabWorks™ software from a UVP Bioimaging system (UVP Inc., Upland, CA). There was no difference in  $\beta$ -actin protein between vehicle control and VCD-treated ovaries. Therefore, each sample was normalized to  $\beta$ -actin before quantification and expressed as the relative protein intensity.

### Statistical analysis

For Western blot data (Figure 3), a 2-way ANOVA with a Bonferroni post test was performed. All other data were analyzed using ANOVA with a Tukey post test.

Analysis was performed using GraphPad Prism version 5.02 (GraphPad Software, San Diego, CA). The assigned level of significance for all tests was  $P < 0.05$ .

## Results

### Localization of AMH protein in cultured neonatal and adult ovary

A comparison of AMH protein distribution was investigated in adult and neonatal F344 rat ovaries. Protein was observed in PND4 ovaries cultured for 2d or 6d to confirm AMH protein expression patterns at time points chosen for later experiments. In adult ovaries, immunofluorescence staining with confocal microscopy demonstrates AMH protein in cytoplasm of granulosa cells in large primary and secondary follicles, but not in primordial or small primary follicles (Figure 1A, 1B). Also, no AMH staining can be observed in theca cells, interstitium, or corpora lutea (data not shown). In PND4 ovaries there is weak staining for AMH protein (Figure 1C), however, AMH staining increases after 2d of culture (Figure 1E). In PND4 ovaries cultured for 2d, AMH staining is seen in cytoplasm of granulosa cells in small and large primary follicles (Figure 1E). Cultured ovaries exposed to control media for 6d demonstrate a decrease in AMH protein staining compared to d2 of culture (Figure 1E, 1F). AMH staining intensity was similar between PND6 ovaries and PND4 ovaries cultured for 2d (analogous time points; Figure 1D, 1E). All samples of *in vivo* and *in vitro* ovaries appeared healthy, with no signs of apoptosis or necrosis.

### Effect of VCD on AMH mRNA and protein levels

The effect of VCD (30  $\mu$ M) on ovarian *Amh* mRNA was measured using real-time RT-PCR. The relative abundance of *Amh* mRNA on d 2-8 following VCD (30  $\mu$ M) exposure is shown in Figure 2. Relative to control, *Amh* mRNA was decreased ( $P < 0.05$ ) beginning on d4 (4d:  $0.48 \pm 0.15$ , VCD/control; 6d:  $0.52 \pm 0.11$  VCD/control; 8d:  $0.73 \pm 0.07$  VCD/control).

The effect of VCD on relative amounts of total ovarian AMH protein in cultured neonatal ovaries was also measured using Western blotting followed by densitometric analysis. Ovaries were collected from F344 PND4 rats and cultured for 2-8 days  $\pm$  VCD (30  $\mu$ M, Fig 3A). AMH protein levels declined ( $P < 0.05$ ) over the time course of culture (d2-8), while  $\beta$ -actin protein levels remained consistent among samples. Thus,  $\beta$ -actin was used to normalize the intensity of AMH in each sample. Relative to the time-matched control, VCD exposure caused a decrease ( $P < 0.05$ ) in AMH protein on 6d and 8d of culture (Figure 3B).

### Effect of exogenous AMH on VCD-induced follicle loss

The effect of recombinant AMH on specific ovarian follicle populations on d8 of culture was investigated (Figure 4). At 200 and 400 ng/ml of AMH, there was an increase ( $P < 0.05$ ) in primordial follicle numbers relative to controls (Figure 4A). Furthermore, at 100 and 200

ng/ml of AMH, there was a decrease ( $P < 0.05$ ) in small primary follicles ( $P < 0.05$ ; Figure 4B). Relative to control, there was no effect of exogenous AMH alone on large primary follicles or secondary follicles (Figure 4C, 4D). Follicle loss ( $P < 0.05$ ) occurred on d8 with 30  $\mu\text{M}$  VCD in culture for primordial, and small and large primary follicles, relative to control incubations, with no effect ( $P < 0.05$ ) of VCD on secondary follicles. Exogenous AMH did not affect ( $P < 0.05$ ) VCD-induced ovotoxicity at any concentration measured (Figure 4A-D).

## Discussion

The present study was designed to characterize a potential involvement of AMH in primordial and primary follicle loss caused by VCD in the neonatal rat ovarian culture system. In order to more fully understand ovarian AMH expression, the distribution of AMH protein was compared in adult vs. neonatal rat ovaries. In an *in situ* study by Baarends *et al.*, AMH mRNA expression was in granulosa cells of healthy preantral and small antral follicles of adult (30-day-old) rat ovaries, with little or no expression in oocytes, theca cells, and interstitial cells (Baarends *et al.*, 1995). AMH staining was also not present in primordial, small primary, and large antral follicles, or corpora lutea. Consistent with that study, the present study in ovaries from adult cycling rats identified AMH protein localization in large primary and secondary follicles and not in primordial or small primary follicles, with its highest expression in the largest preantral follicles. In contrast to adult ovaries, PND4 and PND6 ovaries and PND4 ovaries cultured for either 2d or 6d showed a different distribution of AMH protein. There was weak staining for AMH protein in PND4 ovaries. However, in PND4 ovaries following 2 days of culture as well as in PND6 (analogous time points) ovaries, there was similar prominent staining intensity for AMH protein in small and large primary follicles, follicle types not stained in adult ovaries. Further, AMH staining of these follicles in cultured PND4 ovaries diminished after 6 days as compared with d2 *in vitro*. Interestingly, the distribution of AMH staining in primordial and small primary follicles became reduced as time in culture progressed to more closely resemble its distribution in the adult ovary. In addition, overall AMH protein levels were decreased with time in culture. This may reflect a biological response in which the AMH signaling system shifts in pattern of expression to larger follicles as the ovary becomes more functionally established. Although cultured ovaries remained fully viable throughout the culture period in all experiments (8d), this may reflect selective effects in response to culturing or nonphysical changes in the culture that make the ovaries different from those that develop *in vivo*.

Conversely, decreased ovarian AMH with time in culture may reflect that the age of the rat plays an important part in determining the ovarian distribution of AMH expression. AMH has been shown to play a vital role in primordial follicle activation and recruitment by maintaining primordial follicles in a dormant state, thus inhibiting their recruitment (Visser and Themmen, 2005). It is tempting to speculate that during fetal development and neonatal primordial follicle formation in the rodent, AMH serves a critical role in preventing activation of those small follicles as they are being formed. Based on studies in genetically altered mice (Amh  $-/-$ , FOXO3  $-/-$ , Akt  $-/-$ ), it is known that uncoordinated global activation and recruitment of primordial follicles results in premature ovarian failure and reduced reproductive lifespan (Visser *et al.*, 2007; Castrillon, *et al.*, 2003; Brown, *et al.*, 2010). Thus, tightly regulated maintenance of newly formed primordial follicles in a dormant state could serve as a protective measure against premature ovarian senescence. Subsequently, once the ovary has become fully populated with primordial follicles, expression of AMH and its role in regulating follicle recruitment may shift to larger preantral and early antral follicles. This hypothesis is difficult to address directly in the ovarian culture system because larger follicles do not develop under the conditions used here.

The Kit/Kit ligand pathway has been shown to be targeted by VCD to mediate its ovotoxic effects. When PND4 rat ovaries were exposed to VCD, relative to controls there was a decrease in mRNA encoding the pro-survival gene *Kit* on d4 and an increase in mRNA encoding its ligand, Kit ligand (*Kitl*) on d6 (Fernandez *et al.*, 2008). When exogenous Kit ligand was added to cultured PND4 ovaries, VCD-induced ovotoxicity on d8 was attenuated. Importantly, other exogenous survival growth factors, including growth and differentiation factor 9 (GDF9) and bone morphogenic protein 4 (BMP4), had no effect on follicle loss caused by VCD (Fernandez *et al.*, 2008). This observation provided additional support for the specificity of the Kit/Kit ligand pathway as a target for VCD.

Since the prevention of primordial follicle recruitment by inhibition of PI3kinase has been shown to be important for protection from VCD-ovotoxicity, and AMH has also been shown to prevent the recruitment of small primary follicles from the primordial follicle pool, one goal of the present study was to determine if exogenous AMH could also protect against VCD-induced ovotoxicity. The increase in primordial and decrease in small primary follicle numbers following culture of ovaries with exogenous AMH supported a previously proposed role for AMH in retarding primordial follicle activation and recruitment (Nilsson *et al.*, 2007). However, AMH did not prevent VCD-induced ovotoxicity at any concentration. This finding provides further support for the specificity of VCD targeting the Kit/PI3kinase pathway. Because VCD caused earlier decreases in signaling in the Kit/Kit ligand pathway (pAkt; d2 in culture; Keating *et al.*, 2009b) it is likely that the reduced AMH expression caused by VCD is the result of, rather than the initiator of VCD-induced ovotoxicity.

The effect of VCD on ovarian expression of AMH mRNA and protein was also investigated using a time course in the PND4 ovary culture. VCD specifically targets small pre-antral follicles (primordial and small primary) in ovaries of rats and mice by enhancing the natural process of atresia (Springer *et al.*, 1996a). Further, VCD-induced ovotoxicity has been proposed to result from the activation of primordial follicle recruitment (Keating *et al.*, 2009a). In the present study VCD exposure caused a reduction in *Amh* mRNA on d4-8 and a decrease in AMH protein on d6 and 8 of culture. Because AMH is thought to inhibit primordial follicle recruitment, a VCD-induced decrease in AMH expression (d4, mRNA; d6, protein) might serve to facilitate the increase in primordial follicle recruitment being caused by VCD.

In summary, results of the present study demonstrate that the ovarian distribution of AMH expression may shift from small pre-antral to large pre-antral/early antral follicles as the neonatal ovary matures. Additionally, whereas, AMH-mediated inhibition of primordial follicle recruitment had no effect on VCD-induced ovotoxicity, reduced AMH expression that follows VCD exposure may accelerate primordial follicle recruitment thought to be caused by VCD. Finally, these results provide further evidence of the Kit/Kit ligand pathway as the specific target of VCD for induction of ovotoxicity. The specificity of that mechanism is currently under further investigation.

#### Summary sentence

Expression of AMH in neonatal rat ovaries and its lack of involvement in VCD-induced ovotoxicity provides further evidence for direct VCD effects on the Kit/Kit ligand signaling pathway.



## Acknowledgments

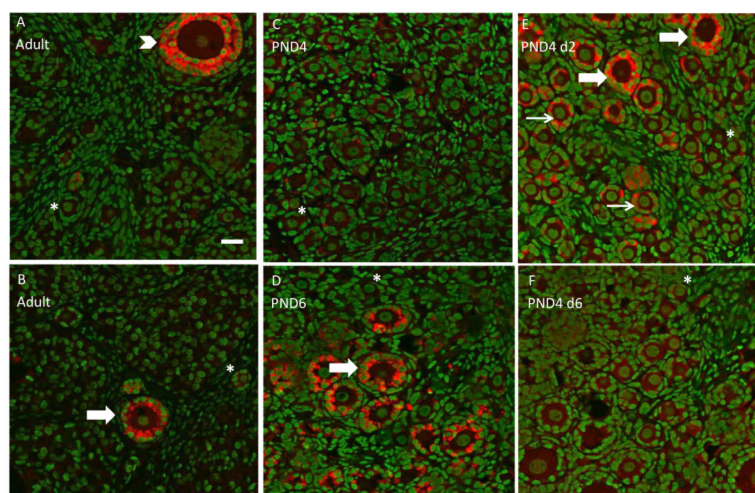
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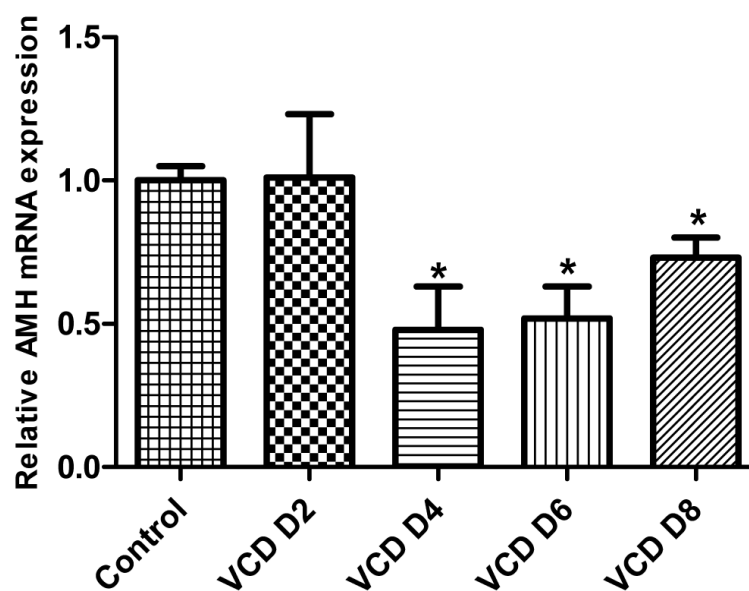
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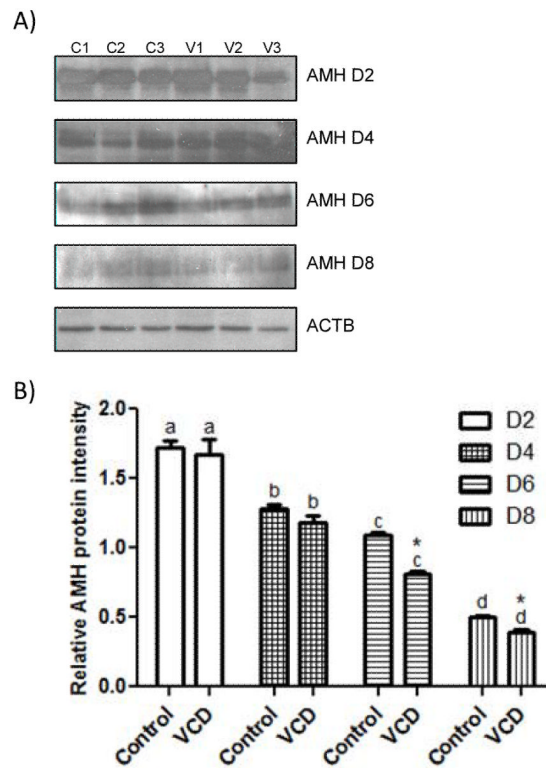


**Figure 1.** Ovarian AMH protein staining. *In vivo* ovaries were collected from adult F344 rats (16 weeks; A and B), PND4 (C) or PND6 (D) rats. PND4 ovaries were cultured *in vitro* with control medium for 2 (E) or 6 days (F). All ovaries were processed for confocal microscopy as described in materials and methods. All panels are a combined overlay of AMH (Cy-5 red stain) and genomic DNA (green YOYO stain) at 40X magnification. Thin arrow, small primary follicle; thick arrow, large primary follicle; arrowhead, secondary follicle; asterisk, primordial follicle. Scale-bar equal to 25  $\mu$ M.



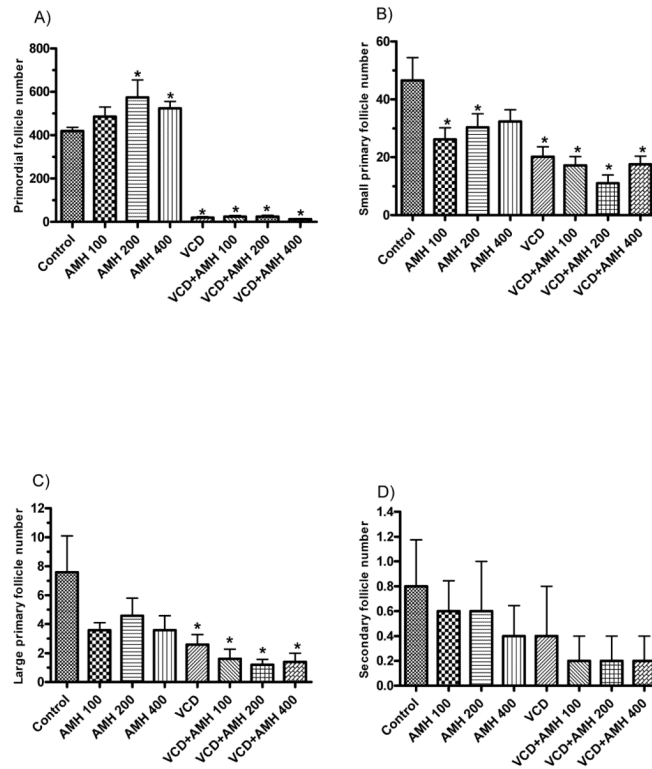
**Figure 2.**

Effect of VCD on expression of mRNA encoding *Amh*. Ovaries from PND4 F344 rats were cultured with control medium  $\pm$  VCD (30  $\mu$ M) for 2-8 days. (labeled VCD D2-8). Total RNA was extracted, and relative amounts of mRNA encoding *Amh* were determined using quantitative real-time PCR as described in Materials and Methods. Values indicate the mean fold change (normalized to  $\beta$ -actin)  $\pm$  SEM; n = 3; 10 ovaries/pool; \*P < 0.05, different from control.



**Figure 3.**

Effect of VCD on expression of AMH protein. Ovaries from PND4 F344 rats were cultured with control medium  $\pm$  VCD (30  $\mu$ M) for 2-8 days. (labeled D2-8) (A) Representative Western blots for AMH and  $\beta$ -actin (ACTB) expression in cultured ovaries in control medium (C1, C2, C3) or medium containing VCD (V1, V2, V3). One representative ACTB blot from d2 is shown, however, ACTB expression was measured at each time point. (B) AMH protein intensity was normalized to ACTB protein and expressed as mean  $\pm$  SEM; n = 3; 10 ovaries/pool for cultured ovary samples; different superscript letters indicate a differences between time points (P < 0.05) difference; \*P < 0.05, different from control.



**Figure 4.**

Effect of exogenous AMH on VCD-induced follicle loss. PND4 F344 rat ovaries were cultured in culture medium or medium containing VCD (30  $\mu$ M) with or without recombinant human AMH (100, 200, 400 ng/ml; labeled AMH 100, AMH 200 or AMH 400, respectively) for 8 days. Ovaries were collected and processed for histological evaluation as described in Materials and Methods. Healthy follicles in every 12<sup>th</sup> section were counted. Values indicate the mean number of healthy (A) primordial follicles, (B) small primary follicles, (C) large primary follicles, (D) secondary follicles counted per ovary  $\pm$  SEM; n = 5 ovaries per treatment group (A-D); \*P < 0.05 different from control.