Xenobiotics; Effects on Female Fertility

Alexander Peter Sobinoff

BSc (Biotech) (Hons) Class I

Doctor of Philosophy

April, 2012

Declaration

I hereby certify that this thesis is submitted in the form of a series of published papers of which I am a joint author. I have included as part of the thesis a written statement from each co-author; and endorsed by the Faculty Assistant Dean (Research Training), attesting to my contribution to the joint publications.

(Signed)…….

Acknowledgments

First of all I'd like to thank the University of Newcastle's Reproductive Science group for all of its support and assistance over the past four years. I would also like to express my deepest gratitude to my supervisors: Prof. Eileen Mclaughlin, Dr Shaun Roman and A/Prof. Brett Nixon. Your sponsorship, academic example and independent style of supervision have all been invaluable to me throughout my candidature. A very special thanks goes out to my fellow "lab monkeys" and research assistants. You made the monotonous exciting and the difficult doable with friendly advice, humour, and companionship. In particular, I'd like to acknowledge my good friend Kate Redgrove; thanks for taking the journey with me. I'd also like to thank my "school friends" for all their support and approval. More importantly I'd like to thank them for providing me with a distraction from the lab, without which I'm sure I'd never have reached this point.

Like almost every other aspect of my life, my family has been crucial to my success during my candidature. Thankyou Mum for all your encouragement (99.9!), Pup for your grounding, Andrew for your loyalty and Elizabeth for your laughter; I love you all very much. Finally, I'd like to thank the most important person in the world to me, my gorgeous wife Jacqueline Elizabeth Sobinoff. Your love inspires me to new heights everyday; without you none of this would be possible.

Publications included as part of this thesis

Literature review:

Sobinoff, A. P., Bernstein, I. R., and McLaughlin, E. A. (2012). All Your Eggs in One Basket; Mechanisms of Xenobiotic Induced Female Reproductive Senescence. In Senescence (T. Nagata, ed., pp. 559-584. InTech Press Rijeka, Croatia.

Chapter 1:

Sobinoff, A. P., Pye, V., Nixon, B., Roman, S. D., and McLaughlin, E. A. (2010). Adding Insult to Injury: Effects of Xenobiotic-Induced Preantral Ovotoxicity on Ovarian Development and Oocyte Fusibility. *Toxicological Sciences* **118**, 653-666.

Chapter 2:

Sobinoff, A. P., Mahony, M., Nixon, B., Roman, S. D., and McLaughlin, E. A. (2011). Understanding the Villain: DMBA-Induced Preantral Ovotoxicity Involves Selective Follicular Destruction and Primordial Follicle Activation through PI3K/Akt and mTOR Signaling. *Toxicological Sciences* **123**, 563-575.

Chapter 3:

Sobinoff, A. P., Pye, V., Nixon, B., Roman, S. D., and McLaughlin, E. A. (2012). Jumping the gun: Smoking constituent BaP causes premature primordial follicle activation and impairs oocyte fusibility through oxidative stress. *Toxicology and Applied Pharmacology* **260**, 70-80.

Chapter 4:

Sobinoff, A. P., Mahony, M., Nixon, B., Roman, S. D., and McLaughlin, E. A. (2012). Staying alive: PI3K pathway promotes primordial follicle activation and survival in response to 3-MC induced ovotoxicity. *Toxicological Sciences*, doi:10.1093/toxsci/kfs137.

Statements of Contribution

I attest that the Research Higher Degree candidate Alexander Peter Sobinoff has contributed upwards of 50% towards data collection/analysis and manuscript preparation for all the publications included in this thesis for which I am a co-author.

…….

Prof. Eileen Anne McLaughlin

Date……..

…….

Dr Shaun Daryl Roman

Date……..

…….

A/Prof. Brett Nixon

Date……..

…….

Mrs. Victoria Jane Pye

Date……..

…….

Mrs. Michelle Mahony

Date……..

…….

A/Prof. Jenny Cameron (Assistant Dean Research Training)

Date……..

Additional Publications with Relevance to this Thesis

Chapter in book:

McLaughlin Eileen Anne, Sobinoff Alexander (2010) 'Contraception targets in mammalian ovarian development', *Fertility Control*, Springer, Berlin, 45-66

Conference Publications:

A. P. Sobinoff, V. Pye, B. Nixon, S. D. Roman and E. A. McLaughlin (2009) 'Xenobiotics; Influence on ovarian follicular development', *Reproduction, Fertility and Development*, Adelaide, SA

Pye Victoria Jayne, Sobinoff Alexander, Nixon Brett, Roman Shaun Daryl, McLaughlin Eileen Anne (2010) 'Xenobiotics; influence on long term oocyte viability', *OzBio 2010: The Molecules of Life - from Discovery to Biotechnology. Poster Abstracts*, Melbourne, VIC

Sobinoff Alexander, Pye Victoria Jayne, Nixon Brett, Roman Shaun Daryl, McLaughlin Eileen Anne (2010) 'Consistent mechanism of primordial follicle activation in neonatal mouse ovotoxicity', *OzBio 2010: The Molecules of Life - from Discovery to Biotechnology. Poster Abstracts*, Melbourne, VIC

Sobinoff Alexander, Pye Victoria Jayne, Nixon Brett, Roman Shaun Daryl, McLaughlin Eileen Anne (2010) 'Short Term Xenobiotic Exposure Compromises Long Term Oocyte Viability', *Reproduction, Fertility and Development*, Sydney, NSW

Sobinoff Alexander, Nixon Brett, Roman Shaun Daryl, McLaughlin Eileen Anne (2011) 'Evidence of selective follicular destruction and primordial follicle activation in DMBA induced ovotoxicity' *Reproduction, Fertility and Development*, Cairns, QLD

Sobinoff Alexander, Pye Victoria Jayne, Nixon Brett, Roman Shaun Daryl, McLaughlin Eileen Anne (2011) 'Neonatal xenobiotic exposure compromises adult oocyte viability' *Reproduction, Fertility and Development*, Cairns, QLD

Table of Contents

Abstract

Over the course of the 20th century there has been an increasing trend in western women opting to delay childbirth in the pursuit of social and economic stability. This development has highlighted the need to identify and characterise ovotoxic xenobiotics (foreign chemical compounds) which threaten their fertility. Arguably the most insidious ovotoxic xenobiotics are those which target the irreplaceable primordial follicle pool for destruction, resulting in pre-mature ovarian senescence. Although many of these xenobiotics have been identified, the molecular mechanisms behind their ovotoxicity remain largely unknown. Employing a neonatal mouse model rich in primordial follicles, the studies presented in this thesis were aimed at characterising the mechanisms of ovotoxicity for six xenobiotics known to target immature follicles (4-vinylcyclohexene diepoxide; Methoxychlor; Menadione; Benzo-a-pyrene, 7,12-Dimethylbenz-[a]anthracene; 3-Methylcholanthrene). The effects of short term xenobiotic exposure on long term oocyte viability were also examined to determine whether follicles which survive ovotoxic destruction were still functionally viable.

Microarray analysis and quantitative PCR revealed a unique ovarian response to each xenobiotic involving a number of genes linked to follicular growth/development, cell death and tumorigenesis *in vitro*. Immunohistological and histomorphological analysis confirmed the microarray data, and revealed a consistent mechanism of ovotoxicity involving primordial follicle activation alongside developing follicle atresia *in vitro* and *in vivo*. Immunohistological and pharmacological inhibition studies also revealed an essential role for the PI3K/Akt/mTOR signalling pathways in 3-Methylcholanthrene and 7,12-Dimethylbenz-[a]anthracene induced primordial follicle activation and survival. Studies into the effects of short term neonatal exposure on long term female fertility revealed no difference in the number of healthy oocytes ovulated in neonatally treated adults compared to controls. However, comprehensive oocyte viability analysis revealed a decreased capacity for fertilisation caused by oxidative damage to the oolemma membrane due to mitochondrial electron transport chain leakage.

The studies conducted in this thesis have identified a common mechanism of xenobiotic induced primordial follicle depletion via a homeostatic mechanism of developing follicle recruitment, with some ovotoxic xenobiotics inducing primordial follicle survival as opposed

to atresia. In addition, the contents of this thesis also provide the first documented evidence of short term neonatal exposure causing long term oocyte dysfunction through xenobiotic induced oxidative stress.

Aims and Hypothesises

The overall aim of this thesis was to determine the molecular mechanisms behind xenobiotic induced follicular depletion and to examine the long term effects of ovotoxic xenobiotic exposure after its removal from the follicular environment. Given the discrepancies seen in the current literature, it was hypothesised that xenobiotic induced primordial follicle depletion is not solely due to follicular atresia, and that short term xenobiotic exposure has long term effects on female fertility. The first aim was investigated through a series of in vitro and in vivo culture experiments using histological/immunohistological techniques combined with microarray analysis. The second involved an extensive in vivo experiment where neonatal mice were dosed with varying concentrations of xenobiotic over several days. These neonatal mice were then allowed to reach sexual maturity, and parameters of female fertility were assessed 6 weeks after they were last treated with xenobiotics. Overall six xenobiotics were assayed. These were 4-vinylcyclohexene diepoxide, methoxychlor, menadione, 9:10-dimethyl-1:2-benzanthracene, benzo[a]pyrene, and 3-methylcholanthrene.

Literature review: All Your Eggs in One Basket; Mechanisms of Xenobiotic Induced Female Reproductive Senescence

23

All Your Eggs in One Basket: Mechanisms of Xenobiotic Induced Female Reproductive Senescence

Alexander P. Sobinoff, Ilana R. Bernstein and Eileen A. McLaughlin
Priority Research Centre in Chemical Biology,
University of Newcastle
Australia

1. Introduction

The irreplaceable mammalian primordial follicle represents the basic unit of female fertility, serving as the primary source of all developing oocytes in the ovary. These primordial follicles remain quiescent, often for decades, until recruited into the growing pool throughout a woman's adult reproductive years. Once recruited, <1% will reach ovulation, with the remainder undergoing an apoptotic process known as atresia (Hirshfield, 1991). Menopause, or ovarian senescence, occurs when the pool of primordial follicles becomes exhausted.

Pre-mature ovarian failure (POF; or early menopause) is an ovarian defect characterised by the premature loss of menstrual cyclicity before the age of 40, well below the median age of natural menopause (51 years). Approximately 1-4% of the female population suffers from this condition, making POF a significant contributor of female infertility (Coulam et al., 1986). There is now a growing body of evidence which suggests that foreign synthetic chemicals, also known as xenobiotics, are capable of causing POF by inducing premature follicular depletion. Indeed, exposure to pesticides, workplace chemicals, chemotherapeutic agents and cigarette smoke have all been associated with primordial follicle reduction resulting in premature ovarian senescence (Hoyer and Devine, 2001; Mattison et al., 1983a, 1983b; Sobinoff et al., 2010, 2011).

In addition to infertility, the loss of ovarian hormones which accompanies POF has been connected with an increased risk of early morbidity and mortality (Shuster et al., 2010). With current statistics indicating an increasing trend in western women opting to delay childbirth, xenobiotic exposure could have long lasting repercussions for both the fertility and long term health of these women. In this review we discuss the susceptible nature of primordial follicles and the consequences of xenobiotic induced POF. We then examine the mechanisms of ovotoxicity for environmental toxicants and xenobiotics known to target immature follicles, and discuss the development of novel methods of wildlife fertility control utilizing these ovotoxicants.

2. The primordial follicle: Precious and vulnerable

Oocyte development and maturation occurs within ovarian follicles. These follicles assemble when primary oocytes (arrested at meiosis prophase I) are enveloped by a single layer of flattened granulosa cells, forming the most immature stage of follicular development, the primordial follicle. The timing of this event is species-specific, but generally occurs in the primitive ovary during foetal development (McNatty et al., 2000). Due to the nature of follicular formation, the number of oocytes established around the time of birth is finite, and represents the total number of germ cells available to the mammalian female throughout her entire life (Edson et al., 2009). It is therefore the size and persistence of this primordial follicle pool which determines the female reproductive lifespan (Fig. 1).

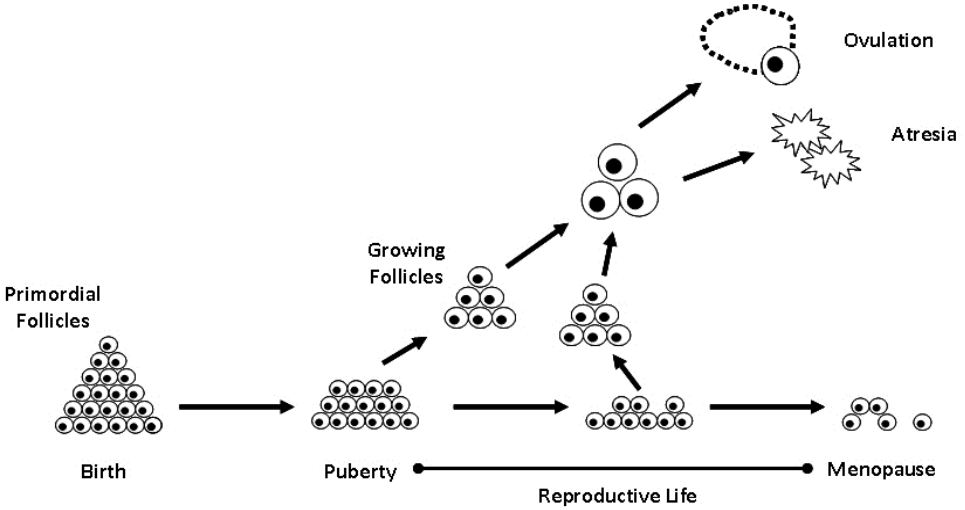


Fig. 1. Simple mechanistic diagram of the human female reproductive lifespan.

The first stage of folliculogenesis involves the recruitment of selected primordial follicles into the growing population. To prolong the length of the female reproductive lifespan, only a few primordial follicles are chosen for recruitment at any one time, with some follicles remaining in a quiescent (non-proliferative) state for months or years (Fig 1). This event occurs in regular waves, and is continuous from birth until ovarian senescence (McGee and Hsueh, 2000). Overall, only a few hundred of all the recruited follicles will complete folliculogenesis and undergo ovulation, with the vast majority being lost to atresia (Hirshfield, 1991). Atresia is thought to be an apoptotic process which selects the healthiest follicles for ovulation, although its mechanism of action is poorly understood. As virtually all follicles are lost during optimal follicular development, it is important that primordial follicles not only survive but also are maintained in a healthy state. Over-stimulation of primordial follicle activation and premature atresia results in the extensive depletion of the primordial follicle pool, resulting in premature ovarian senescence (Reddy et al., 2008).

There is now an increasing volume of studies which link primordial follicle depletion with xenobiotic exposure, suggesting that these irreplaceable follicles are highly sensitive to

cytotoxic insult (Hoyer and Devine, 2001; Mattison et al., 1983a, 1983b; Sobinoff et al., 2010, 2011). It is thought that this sensitivity may stem from the primordial follicles quiescent nature. For example, somatic cells which undergo regular rounds of proliferation constantly renew macromolecules and organelles by virtue of mitosis. However, the oocyte and granulosa cells of the primordial follicle are non-proliferative, and do not benefit from mitotic renewal, perhaps making them excessively vulnerable to xenobiotics which cause sub-lethal damage to mitochondria and other structures over time (Tarin, 1996). Similarly the location of the primordial follicle population within a poorly vascularised region of the ovarian cortex also makes them highly susceptible to toxins which damage ovarian blood vessels, with the resulting cortexual fibrosis destroying primordial follicle rich segments of the ovary (Guraya, 1985; Meirow et al., 2007; van Wezel and Rodgers, 1996).

In addition to direct primordial follicle injury, certain xenobiotics which target developing follicles have been shown to cause excessive primordial follicle activation (Keating, 2009; Sobinoff et al., 2010, 2011). This may be due to a homeostatic mechanism of follicular replacement, in which destroyed developing follicles result in primordial follicle activation to replace the developing pool. If the offending xenobiotic is not removed, this could potentially lead to a vicious cycle of primordial follicle depletion.

3. Consequences of xenobiotic induced primordial follicle depletion

The overall impact of xenobiotic induced follicular depletion on female reproduction depends on the type of follicle targeted for destruction, dose, and duration of exposure (Hoyer and Sipes, 1996). For example, xenobiotics which target large developing follicles have an immediately noticeable effect on female fertility. Antral follicles are the primary producers of ovarian estrogen, and therefore play an important role in the FSH-LH negative feedback loop responsible for ovulation. Xenobiotics which selectively target antral follicles consequently have harmful effects on ovarian cyclicity, effectively acting as endocrine disruptors (Jarrell et al., 1991; Mattison and Schulman, 1980). Fortunately, both prolonged and acute exposure to these ovotoxic agents only causes temporary infertility, as these follicles can be replaced by the primordial follicle pool once the harmful xenobiotic is removed from the immediate environment.

Conversely, xenobiotics which target small pre-antral follicles have more permanent effects on female fertility which could potentially go unrecognised for years. Due to the non-renewing nature of primordial follicles, these xenobiotics are particularly damaging to female fertility, causing permanent infertility and premature ovarian senescence. What makes this type of ovotoxicity concerning is that it has a delayed effect on reproduction which is not made apparent until such a time that follicular recruitment cannot be supported (Hooser et al., 1994). Thus this extended period of time between cause and effect means that the detrimental action of xenobiotic contact often goes unnoticed, and consequently steps are not taken to minimise exposure until it is too late. Thus even a systemic low dose of xenobiotics may produce cumulative effects over time, resulting in the same consequences on female fertility as a large single exposure. With current statistics suggesting an increasing trend in developed countries of women opting to delay childbirth until late in their reproductive life (>30 years), accelerated follicle loss resulting from xenobiotic exposure can deprive these women of the chance to start a family in the conventional manner (Martin et al., 2003).

In addition to permanent infertility, the loss of ovarian hormones which accompanies early menopause has been associated with an increased risk for a variety of health problems. For example, estrogen deficiency (a consequence of menopause) is the most common cause of osteoporosis in humans (Cenci et al., 2003). Bone loss results from the absence of estrogen production by maturing ovarian follicles, which leads to a subsequent increase in FSH production due to the negative feedback of estrogen on pituitary gonadotropin secretion. In terms of bone remodelling, increased FSH production stimulates tumor necrosis factor (TNF) secretion, which in turn increases osteoclast formation and bone reabsorption (Cenci et al., 2003). Menopause induced estrogen withdrawal has also been associated with an increase in many traditional cardiovascular risk factors, including body fat redistribution, insulin resistance and high blood pressure, increased plasma triglyceride levels and high-density lipid cholesterol absorption (Bilianou, 2008; Rosano et al., 2007). Increased risk for Alzheimer's disease is also associated with the menopause induced loss of sex steroid hormones as evidenced by various epidemiological and experimental studies, although some clinical findings refute this evidence (Pike et al., 2009).

Over the course of the 20th century, the average life expectancy for women in the developing world has increased by ~40%, resulting in women now living up to a third of their lives in post menopausal years. Unfortunately, this means that women are now spending a larger proportion of their life with increased health risks brought about by the onset of menopause. In addition, increased risk resulting from xenobiotic induced premature menopause means an enhanced chance for problems. It is therefore important to understand the mechanisms behind xenobiotic induced primordial follicle depletion.

4. Mechanisms of xenobiotic induced primordial follicle depletion

4.1 The Aryl Hydrocarbon Receptor

The Aryl Hydrocarbon Receptor (Ahr) is a ligand activated transcription factor implicated in the regulation of a variety of physiological and developmental effects, including xenobiotic metabolism, cell cycle progression, apoptosis and oxidative stress (Denison and Heath-Pagliuso, 1998; Nebert et al., 2000). In its inactivated state, Ahr is found in the cytoplasm bound to a number of molecular chaperones including hsp90, Xap2, and p23 (Carlson and Perdew, 2002; Petrulis and Perdew, 2002). Ligand binding causes conformational changes which expose a nuclear import signal on the Ahr, resulting in its translocation into the nucleus (Pollenz et al., 1994). Once imported the Ahr-ligand receptor complex disassociates with its chaperones and dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) to form an active transcription factor with high affinity to specific DNA sequences known as xenobiotic-response elements (XRE) within the promoter region of a variety of genes, inducing transcription (Fig.2) (Reyes et al., 1992).

The Ahr-ARNT ligand activated transcription factor is known to regulate the toxicity of various xenobiotic compounds such as polycyclic aromatic hydrocarbons, polychlorinated dibenzofurans and polychlorinated biphenyls which are found ubiquitously in the environment and are highly resistant to metabolic breakdown (Nguyen and Bradfield, 2007; Stapleton and Baker, 2003). In an adaptive response to their accumulation in the cell, Ahr induces the expression of a number of xenobiotic metabolising enzymes, including members of the cytochrome P450 A and B families which oxygenate the intruding xenobiotic as part

of a three tiered enzymatic detoxification mechanism (Conney, 1982). Unfortunately, this oxygenation often results in the bioactivation of the parent xenobiotic into a more reactive and therefore toxic metabolite (Harrigan et al., 2004; Melendez-Colon et al., 1999). Indeed, many of Ahr's known xenobiotic ligands, such as the polycyclic aromatic hydrocarbons benzo[a]pyrene (BaP), 9:10-dimethyl-1:2-benzanthracene (DMBA), and 3-methyl-cholanthrene (3-MC), cause primordial follicle destruction through Ahr initiated cytochrome P450 induced bioactivation (Borman et al., 2000; Mattison and Thorgeirsson, 1979). For example, BaP is initially metabolised by Ahr regulated cyp1A1 and cyp1B1 enzymes resulting in its biotransformation into 7,8-diol, and 9,10-diol macromolecular-adduct forming metabolites within the ovary. Inhibition of Ahr by α-naphthoflavone nullifies its effects on primordial follicle destruction (Bengtsson et al., 1983; Mattison et al., 1983a).

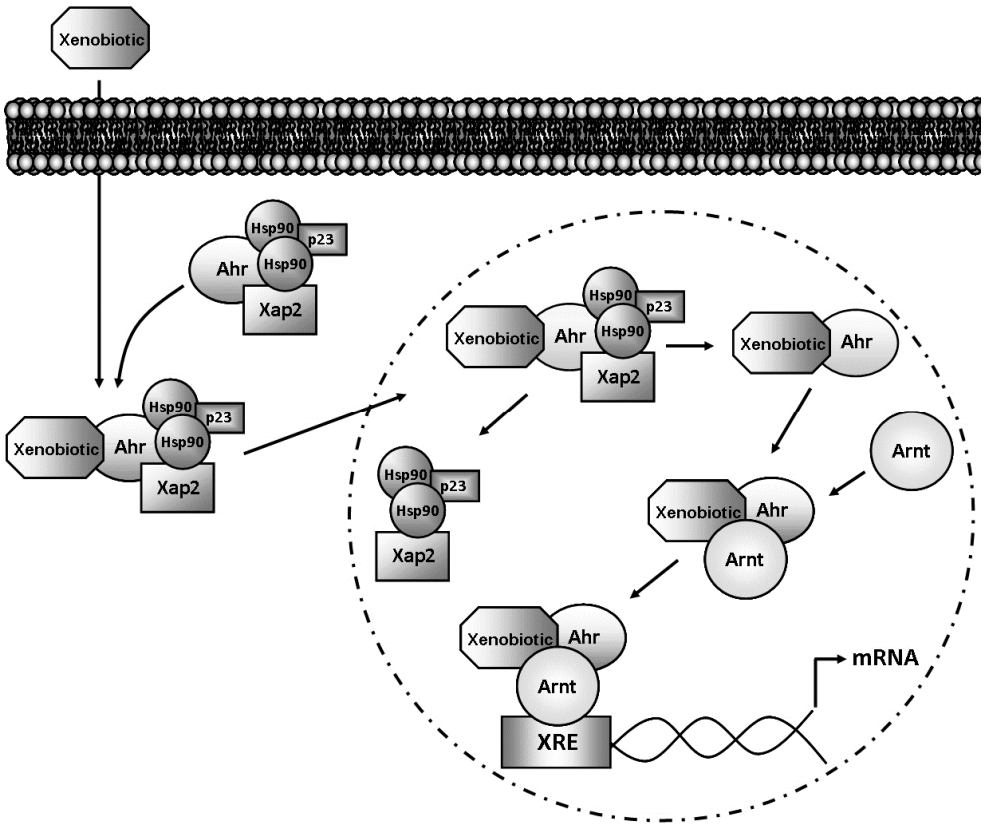


Fig. 2. Molecular mechanism of Arh-Arnt directed gene expression in response to xenobiotic exposure.

In addition to detoxification, the development of Ahr-deficient mice has revealed a physiological role for Ahr in regulating reproduction, growth and development (Benedict et al., 2000, 2003; Nebert et al., 1984; Robles et al., 2000; Schmidt et al., 1996). Benedict et al.

(2000) demonstrated that ovaries from mice deficient for Ahr expression contained significantly more fully formed primordial follicles compared to wild type mice on PND2-3. Robles et al (2000) found similar results, identifying more than a two-fold significant increase in the primordial follicle pool of Ahr deficient PND4 mice compared to wild type mice. These results suggest a developmental role for Ahr in regulating primordial follicle formation and atresia in the mouse. Although the exact details of Ahr role in the regulation of the primordial follicle pool have yet to be determined, given that Ahr xenobiotic ligands cause primordial follicle depletion, we hypothesise that part of these ovotoxic compounds method of ovotoxicity may involve perturbed AhR developmental signalling, inducing premature primordial follicle atresia.

4.2 Bioactivation

Humans come into contact with a variety of xenobiotics over the course of their lifetime, and have evolved a number of physiological mechanisms designed to remove their harmful influence from within the body. Hydrophilic xenobiotics tend to be less toxic, as the body is able to directly excrete them relatively unchanged. However, if the xenobiotic is lipophilic, it will need to be modified by a series of biochemical reactions before it can be eliminated (Pavek and Dvorak, 2008). This series of biochemical reactions is termed biotransformation, and can be divided into two phases. Phase I metabolism involves the introduction or exposure of a reactive polar group on the xenobiotic via oxidation, resulting in a more reactive/water soluble metabolite to facilitate excretion and/or the induction of phase II metabolism. The cytochrome p450 super family of oxidases catalyse the majority of these reactions, although other oxidases, esterases, amidases, and monooxygenases can also be involved (Schroer et al., 2010). Phase II metabolism involves the conjugation of charged species such as glutathione, sulphate, glycine or glucuronic acid to the phase I metabolite to increase its water solubility (Kohalmy and Vrzal, 2011). The addition of these large anionic groups detoxifies reactive electrophiles, resulting in a more polar metabolite which can be actively transported out of the cell. These reactions are carried out by a broad range of transferases, such as glutathione S-transferase, UDP-glucuronosyltransferases, sulfotransferases, N-acetyltransferases, and methyltransferases (Jancovaa et al., 2010).

Unfortunately, phase I metabolism of xenobiotics by the liver and other tissues occasionally results in the production of a more cytotoxic metabolite, a process known as bioactivation (Dekant, 2009). These highly reactive metabolites are electrophilic, and are capable of forming covalent bonds (or adducts) with the nucleophilic centers of cellular macromolecules, such as proteins, DNA, and RNA. Cellular toxicity occurs when these adducts disrupt the normal structure and/or function of these macromolecules, resulting in apoptosis, necrosis or carcinogenesis. The main site of xenobiotic biotransformation within the body is the liver, although the ovary is capable of both phase I and phase II metabolism (Igawa et al., 2009; Rajapaksa et al., 2007a, 2007b; Shimada et al., 2003). Therefore, there is potential for the vulnerable primordial follicle to come into contact with bioactivated ovotoxic metabolites via several routes of exposure. Bioactivated metabolites produced by the liver maybe stable enough to diffuse back into the venous circulatory system, resulting in direct ovarian exposure. Additionally, as the primordial follicle is capable of expressing xenobiotic metabolising enzymes itself, oocytes may be exposed to localised bioactivation. Finally, the xenobiotic may be bioactivated locally into its ovotoxic metabolite by

neighbouring somatic ovarian cells and taken up by the primordial oocyte, contributing to localised bioactivation.

A number of studies performed *in vitro* have revealed that the ovary is capable of the localised bioactivation of a number of xenobiotics into ovotoxic intermediates which target primordial follicles for destruction (Rajapaksa et al., 2007a, 2007b). An example of this localised bioactivation is the reported metabolism of the polycyclic aromatic hydrocarbon DMBA (Fig.3). Ovarian exposure to DMBA disrupts folliculogenesis, resulting in the destruction of all follicle populations leading to POF in rodents, although recent evidence suggests an alternate mechanism of ovotoxicity resulting in primordial follicle depletion in the mouse (Mattison and Schulman, 1980; Sobinoff et al., 2011). This toxicity has been attributed to the bioactivation of DMBA into its ultimate DNA-adduct forming intermediate DMBA-3,4-diol-1,2-epoxide (Shiromizu and Mattison, 1985). DMBA is bioactivated by Cyp1B1 to a 3,4-epoxide which is then converted into a 3,4-diol by the microsomal epoxide hydrolase (MeH) phase II enzyme. This intermediate is then further modified by either Cyp1A1 or Cyp1B1 to form the ultimate ovotoxicant DMBA-3,4-diol-1,2-epoxide (Shimada and Fujii Kuriyama, 2004; Shimada et al., 2001). These three enzymes required for DMBA's biotransformation are all expressed and induced by DMBA exposure in the murine ovary (Igawa et al., 2009; Rajapaksa et al., 2007b; Shimada et al., 2003). In further support of localised DMBA bioactivation, inhibition of MeH in cultured rat ovaries inhibited DMBA induced ovotoxicity, while ovarian culture in the presence of DMBA-3,4-diol induced significantly more primordial follicle depletion than DMBA alone (Igawa et al., 2009; Rajapaksa et al., 2007b).

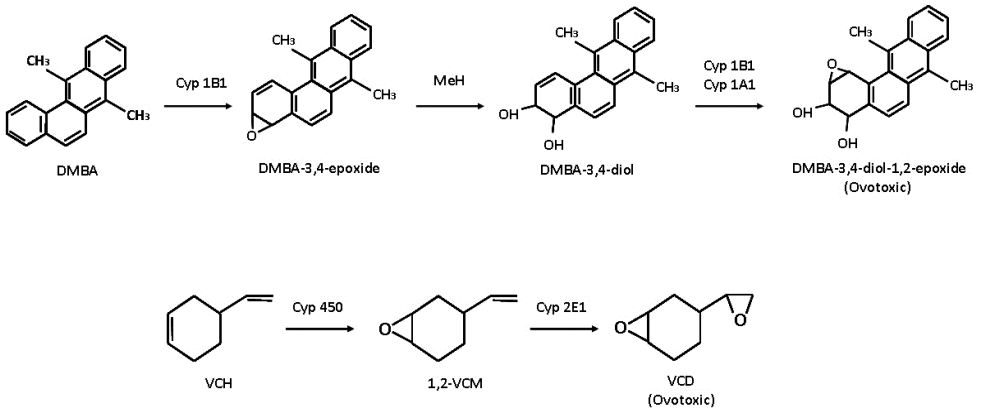


Fig. 3. Metabolism of DMBA and VCD into their ovotoxic metabolites.

Another example of localised bioactivation is the conversion of the industrial chemical 4-Vinylcyclohexene (VCH) into VCH diepoxide (VCD) (Fig. 3). VCH is metabolised by cytochrome P450 phase I enzymes to form VCM-monoepoxide (VCM), which is then converted into VCD. Studies have shown VCD to be the ultimate ovotoxicant, targeting both primordial and primary follicles for depletion (Hu et al., 2001; Smith et al., 1990; Sobinoff et al., 2010). As demonstrated *in vivo* and *in vitro* via knockout studies, VCH/VCM is bioactivated into VCD exclusively by the cyp2e1 isoform in the ovary (Rajapaksa et al.,

2007a). Rajapaksa et al (2007a) cultured neonatal ovaries from both cyp2e1$^{+/+}$ and cyp2e1$^{-/-}$ neonatal mice in VCM and VCD containing media. Both VCH metabolites caused primordial follicle depletion in cyp2e1$^{+/+}$ cultured ovaries. However, unlike VCD, VCM did not produce an ovotoxic affect in cyp2e1$^{-/-}$ cultured ovaries, thus demonstrating its role in VCH induced bioactivation.

4.3 Xenobiotic induced reactive oxygen species generation

Reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide and the highly toxic hydroxyl free radical, are highly reactive oxygen-containing molecules which are produced naturally as a consequence of oxidative energy metabolism (Valko et al., 2007). These short lived ROS play an important role in regulating signal transduction, selectively oxidizing cysteine residues on proteins resulting in a variety of reversible molecular interactions (Janssen-Heininger et al., 2008). However, in excess these highly unstable molecules may lead to perturbed signal transduction and/or oxidative damage to cellular macromolecules, inducing DNA mutations, lipid peroxidation and premature protein degradation. These molecular lesions coupled with perturbed signal transduction can ultimately result in abnormal cellular function, apoptosis and necrosis (Valko et al., 2006, 2007; Wells et al., 2009).

The ovary is a highly redox sensitive organ, with oocytes themselves being particularly vulnerable to excess ROS exposure due to the low rates of oxidative repair in post-mitotic cells (Cadenas and Davies, 2000; Terman et al., 2006). According to the free radical hypothesis of ageing, non-renewing primordial follicles, which can remain quiescent for many years, gradually produce ROS through electron leakage from the mitochondrial electron transport chain (Tarin, 1996). Over time this excess ROS damages the mitochondrial membranes, leading to more electron leakage and further ROS production. Given the redox sensitive nature of primordial follicles, it is reasonable to assume that the generation of xenobiotic induced ROS formed through detoxification may exacerbate this process, contributing to primordial follicle loss (Bondy and Naderi, 1994; Danielson, 2002; Wells et al., 2009).

Xenobiotic enhanced ROS formation may occur via several mechanisms in the primordial follicle (Fig.4). If the ovotoxic xenobiotic contains a quinone-like structure, it may undergo redox cycling with the corresponding semiquinone radical to produce superoxide anions. Further enzymatic and/or spontaneous dismutation of the superoxide anions produces hydrogen peroxide, which can further react with trace amounts of iron or other transition metals to form hydroxyl free radicals (Bolton et al., 2000). Given the futile cyclical nature of redox cycling, this would allow a relatively small concentration of quinone-like xenobiotics to generate an amplified production of ROS in the ovary (Park et al., 2005). For example, menadione (MEN), a synthetic vitamin K with a quinone-like structure, is a potent toxicant which exerts its cytotoxic affect via quinone cycling (Thor et al., 1982). Recently, we examined the effects of MEN on folliculogenesis in neonatal mouse ovaries *in vitro* (Sobinoff et al., 2010). This study found that MEN caused wide spread oxidative stress and DNA damage resulting in primordial and small developing follicle destruction, as evidenced by the detection of increased levels of the hydroxyl radical–induced mutagenic DNA lesion 8-

hydroxyguanine, and Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) analysis (Klaunig and Kamendulis, 2004).

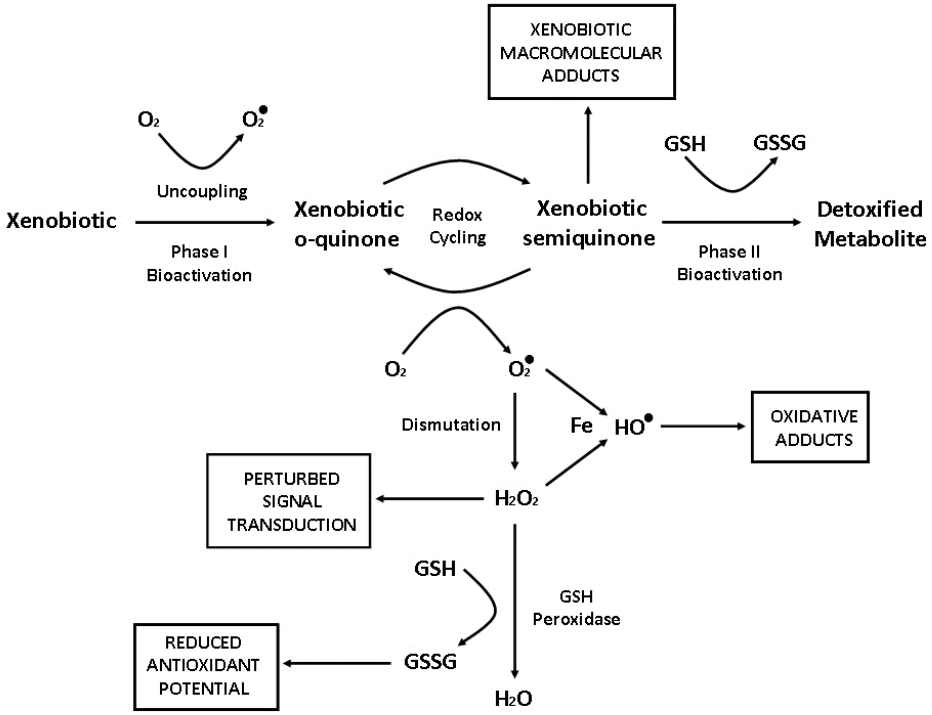


Fig. 4. Biochemical pathways outlining the mechanisms of xenobiotic induced ROS production which may contribute to primordial follicle depletion. Abbreviations: Fe, iron; $O_2^{\bullet}$, superoxide; H_2O_2, hydrogen peroxide; $HO^{\bullet}$, hydroxyl radical.

Another mechanism of xenobiotic induced ROS formation is the phase I bioactivation of the offending xenobiotic into reactive and redox active o-quinone metabolites. As mentioned previously, the PAH BaP is converted into 7,8-diol, and 9,10-diol by Ahr induced cyp1A1 and cyp1B1 enzymes in the ovary (Bengtsson et al., 1983). Studies have also shown that cyp1A1 is also capable of converting BaP into the BaP o-quinones benzo[a]pyrene-3,6-dione and benzo[a]pyrene-6,12-dion (Schwarz et al., 2001). Additionally, cyp 1A1 bioactivated BaP 7,8-diol can be further metabolised via NAD(P)$^+$-dependent oxidation by the phase I dihydrodiol dehydrogenase Akr1c1 enzyme, resulting in the formation of a ketol. This ketol then undergoes tautomerisation to form catechol 7,8-dihydroxybenzo[a]pyrene. Two subsequent 1-electron auto-oxidation events produce a *o*-semiquinone anion, followed by the formation of the o-quinone benzo[a]pyrene-7,8-dione (Trevor et al., 1996). Given the increases observed in cyp 1A1 expression in the ovary in response to BaP exposure, and the relatively high level of dihydrodiol dehydrogenase expression in the ovary compared to the

liver, it is reasonable to assume BaP may be exerting part of its ovotoxic affect through o-quinone formation (Hou et al., 1994). Indeed, both benzo[a]pyrene-3,6-dione and benzo[a]pyrene-6,12-dion were detected in rat ovaries after a single dose exposure of BaP in rodents (Ramesh et al., 2010).

The un-natural "uncoupling" of phase I cytochrome P450 enzymes may also contribute to xenobiotic induced ovotoxicity via ROS production. Cytochrome P450 enzymes use H^+ obtained from NADPH to reduce O_2, which leads to the production of hydrogen peroxide and/or superoxide anion radicals as part of phase I oxygenation. Unfortunately, the P450 catalytic cycle can be uncoupled, resulting in the release of the reactive hydrogen peroxide and/or superoxide anion radical from the enzyme substrate complex (Meunier et al., 2004). Although all cytochrome P450 enzymes experience uncoupling, cyp 2E1 experiences a high rate of the phenomenon (Caro and Cederbaum, 2004). Even in the absence of substrate, cyp 2E1 undergoes un-natural "uncoupling" due to its NADPH oxidase activity independent of phase I metabolism (Ekstrom and Ingelman-Sundberg, 1989). As described previously, VCH is exclusively bioactivated by cyp 2E1 to produce the ovotoxic metabolite VCD. It is therefore possible that VCH may partially cause primordial follicle depletion via excess ROS production. Indeed, studies conducted in our laboratory have demonstrated VCD itself, along with the pesticide methoxychlor (MXC) and MEN, is capable of inducing cyp 2E1 expression and oxidative stress in the form of 8-hydroxyguanine adduct formation in primordial follicles (Sobinoff et al., 2010).

Another mechanism by which ovotoxic xenobiotics may cause oxidative stress is through the depletion of glutathione peroxidase (GSH) via detoxification. GSH is the body's most abundant antioxidant, providing protection against all forms of oxidative stress by scavenging ROS by virtue of its reducing thiol group, forming oxidised glutathione disulfide (GSSG) (Kidd, 1997). The glutathione system (GSH/GSSG ratio) acts as a homoeostatic redox buffer that contributes to maintenance of the cellular redox balance, with a reduction in the GSH/GSSG ratio indicating oxidative stress (Schafer and Buettner, 2001). In addition to its function as a ROS scavenger, GSH is also employed in the phase II metabolism of many ovotoxic xenobiotics (Keating et al., 2010; Tsai-Turton et al., 2007; Wu and Berger, 2008). For example, VCD is conjugated to GSH by the glutathione S-transferase Gst isoform pi (Gstp) as part of phase II detoxification in the ovary (Keating et al., 2010).

The mammalian ovary itself is highly redox sensitive, with maturing oocytes containing the highest concentration of GSH compared to any other cell type in the body (Calvin et al., 1986; Clague et al., 1992; Luderer et al., 2001). It is therefore likely that ovarian somatic and germ cell GSH plays an important role in protecting ovarian follicles from damage by ovotoxic xenobiotics. This is especially evident in primordial follicles, where a natural decrease in the GSH/GSSG ratio with advancing reproductive age increases primordial follicle susceptibility to xenobiotic induced destruction (Mattison et al., 1983b). Therefore, we hypothesise that the detoxification of ovotoxic xenobiotics via GSH conjugation reduces the GSH/GSSG ratio in primordial follicles, leaving them vulnerable to oxidative stress and primordial follicle depletion. Indeed, DMBA detoxification involves GSH conjugation, and its ovotoxic ROS production can be reduced through the addition of GSH, curbing its ovotoxicity (Tsai-Turton et al., 2007). There is controversial evidence for this mechanism of ovotoxicity in VCD induced primordial follicle loss. Rodent exposure to VCD was shown to reduce GSH concentrations by 25% and 55% in rat and mouse ovaries 2 hours after VCD

administration (Bhattacharya and Keating, 2011). Additionally, rodents given the same dose of VCD over a period of several days caused specific primordial follicle depletion only after 15 days of continual dosing (Springer et al., 1996). The significant decrease in GSH concentrations almost immediately after exposure, coupled with the delayed loss of follicles following chronic exposure suggest that GSH reduction over time due to VCD detoxification could leave the susceptible primordial follicle vulnerable to increasing concentrations of xenobiotic induced ROS, resulting in primordial follicle destruction. It is pertinent that a single dose of a higher concentration of VCD (320 mg/kg) causes significant primordial follicle depletion 6 days after exposure, but is not specific to the primordial follicle pool (Devine et al., 2004). Additionally, VCD *in vitro* culture assays have linked an increase in Gstp expression with the first signs of primordial follicle loss after 6 days of exposure in neonatal rat ovaries (Bhattacharya and Keating, 2011; Keating et al., 2010). As Gstp catalyses VCD-GSH conjugation, the increase in enzymatic expression and therefore activity could have contributed to the observed primordial follicle loss due to a reduction in GSH/GSSG oxidative buffer. Conversely, substituting VCD culture media with antioxidant such as GSH does not prevent primordial follicle depletion, suggesting it is not the ultimate cause of depletion (Devine et al., 2004).

4.4 Xenobiotic induced primordial follicle activation

Traditionally, studies attempting to identify the molecular mechanisms behind xenobiotic induced POF have focused on premature follicular atresia as the main source of primordial follicle depletion. However, there is now a growing body of evidence which suggests that xenobiotics cause primordial follicle depletion through accelerated primordial follicle activation (Keating, 2009, 2011; Sobinoff et al., 2010, 2011). A study of VCD and MXC induced primordial follicle depletion has revealed a selective mechanism of pre-antral ovotoxicity involving small developing follicle atresia and primordial follicle activation both *in vitro* and *in vivo* (Sobinoff et al., 2010). Extracted neonatal mouse ovaries cultured in either VCD or MXC were immunopositive for the apoptotic markers caspase 2, caspase 3, and TUNEL in small developing follicles from the primary stage onward, but were absent in primordial follicles (Fig.5). In addition, the primordial follicles in VCD and MXC cultured ovaries expressed proliferating cell nuclear antigen (PCNA), a marker of primordial follicle activation (Picut et al., 2008; Tománek and Chronowska, 2006). VCD and MXC exposure also induces primordial follicle activation and developing follicle atresia *in vivo* as evidenced by increased primordial follicle PCNA expression and histomorphological analysis (Sobinoff et al., 2010). Microarray analysis confirmed via qPCR also showed VCD and MXC up-regulated PI3K/Akt and mTOR signalling, two synergistic pathways intimately associated with primordial follicle activation (Reddy et al., 2010). Further evidence for PI3K/Akt signalling in VCD induced primordial follicle activation comes from a study conducted by Hoyer et al (2009), in which LY294002, an inhibitor of PI3K, prevented primordial follicle depletion in cultured rat ovaries (Vlahos et al., 1994).

The polycyclic aromatic hydrocarbon DMBA, which was previously thought to cause indiscriminate follicular destruction, has also been shown to cause pre-antral ovotoxicity through selective immature follicle destruction and primordial follicle activation (Mattison and Schulman, 1980; Sobinoff et al., 2011). In addition to showing signs of maturing follicle atresia (caspase 2, caspase 3, TUNEL) and primordial follicle activation (PCNA), DMBA

induced Akt1 phosphorylation, mTOR activation, and decreased FOXO3a expression in DMBA cultured primordial oocytes. All of these events occur downstream of the PI3K/Akt and mTOR signalling pathways, providing evidence for these pathways involvement in xenobiotic induced primordial follicle depletion (Reddy et al., 2010). Unlike VCD however, PI3K/Akt inhibitor studies utilising LY294002 in DMBA cultured rat ovaries caused accelerated primordial follicle depletion (Keating, 2009). In addition to its role in primordial follicle activation, PI3k/Akt signalling is also responsible for augmenting cellular survival by inhibiting the activation of proapoptotic proteins and transcription factors (Blume-Jensen et al., 1998; Testa and Bellacosa, 2001). Therefore, in addition to acting synergistically with mTOR signalling to cause primordial follicle activation, PI3k/Akt signalling may help preserve the primordial follicle pool in times of cytotoxic stress. Interestingly however, mTOR signalling does not require PI3k/Akt signalling to induce primordial follicle activation, and in fact may be the sole driver of DMBA induced primordial follicle activation (Adhikari et al., 2010).

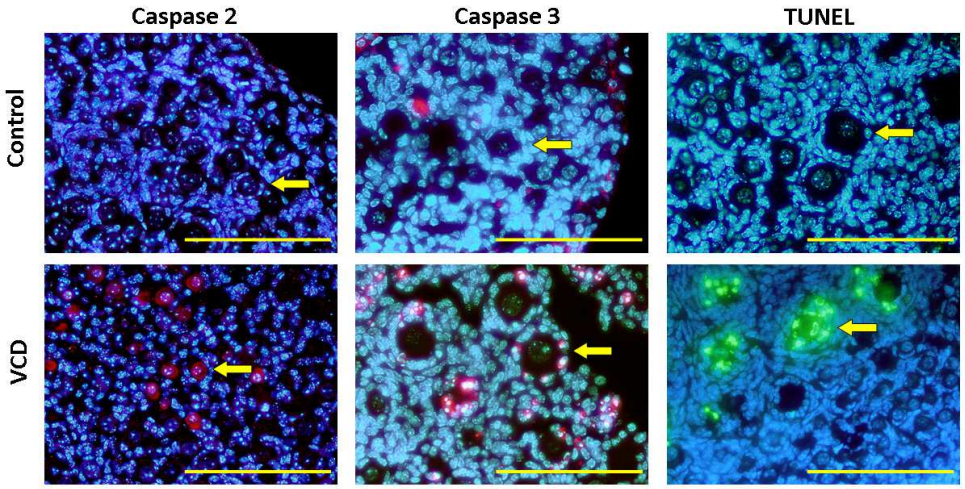


Fig. 5. Immunohistological staining of apoptotic markers in VCD exposed neonatal mouse ovaries. Blue staining (DAPI) represents nuclear staining; red staining (Cy-5) represents specific staining for the protein of interest; green staining (Fluorescein) represents specific staining for degraded DNA (TUNEL). Thin arrow=developing follicle; scale bar is equal to 50μm.

As xenobiotic induced primordial follicle activation is reportedly accompanied by small pre-antral follicular destruction, it has been hypothesised that xenobiotic induced primordial follicle depletion is the result of a homeostatic mechanism of follicular replacement (Keating, 2009; Sobinoff et al., 2010). In this hypothesis, the ovotoxic xenobiotic targets and destroys developing follicles, leading to increased primordial follicle recruitment to maintain the developing pool (Fig. 6). Although the developing pool may be maintained for some time, eventually the rate of developing follicle destruction will exceed the dwindling primordial follicle pools rate of replacement, resulting in POF. Indeed, it is well known that

rapidly dividing cells, such as the granulosa cells of developing follicles, are highly susceptible to the action of cytotoxic xenobiotics (Blumenfeld and Haim, 1997; Hirshfield, 1991). Therefore, if the xenobiotic targeted these proliferating granulosa cells for destruction, the entire follicular structure would demise (Hughes and Gorospe, 1991). Even given the vulnerable nature of the primordial follicle explained earlier in this review, the primordial follicles quiescent nature may reduce their susceptibility to certain xenobiotics, and are only destroyed once a commitment to activation/recruitment has been made.

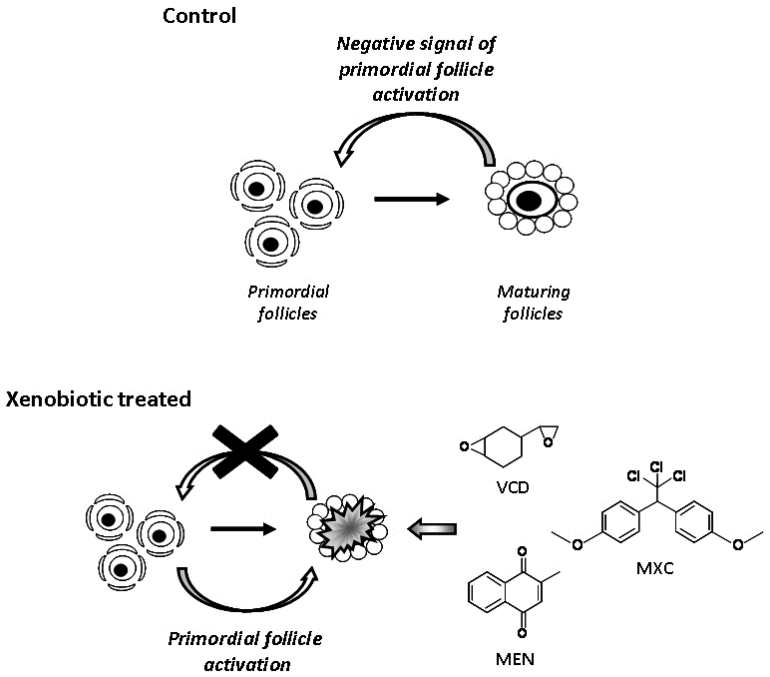


Fig. 6. Homeostatic mechanism of follicular replacement hypothesis. Under control conditions, premature primordial follicle activation is prevented by negative cytokine signals excreted from the developing pool of follicles. Xenobiotic exposure results in the destruction of this developing pool, removing these negative signals causing primordial follicle activation.

Another proposed mechanism of xenobiotic induced primordial follicle activation may involve perturbed signal transduction caused by oxidative stress. As described previously, ROS play a physiological role in regulating signal transduction by selectively oxidising cysteine residues on proteins resulting in a variety of reversible molecular interactions (Wells et al., 2009). It is therefore conceivable that increased levels of xenobiotic induced ROS could lead to abnormal cysteine oxidation and consequently dysregulated signal transduction. For example, the PI3K/Akt signalling pathway has been shown to be up-regulated by increased levels of ROS through the H_2O_2 oxidation of phosphatases which

negatively regulate the pathway (Kim et al., 2005; Naughton et al., 2009). Given the PI3K/Akt pathway's role in the regulation of primordial follicle recruitment, increased ROS production could potentially cause primordial follicle activation in xenobiotic treated ovaries. Indeed, all three xenobiotics which have thus far been reported to induce primordial follicle activation also cause oxidative stress and induce the expression/activation of members of the PI3K/Akt signalling pathway in the ovary (Sobinoff et al., 2010, 2011; Tsai-Turton et al., 2007).

Xenobiotic induced primordial follicle activation may also be the result of abnormal cross-talk between signalling pathways. For example, DMBA exposure was shown to induce *Dnajb6* expression, a heat shock protein whose expression is normally induced by Nrf2 and Hsf1 in response to oxidative stress (Sobinoff et al., 2011; Thimmulappa et al., 2002; Wang, K. et al., 2009). Dnajb6 responds 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). In turn NFAT positively regulates PTEN expression, a known inhibitor of Akt1 phosphorylation. Therefore DMBA induced Dnajb6 expression may inhibit NFAT transcriptional activity, reducing PTEN expression and stimulating Akt1 phosphorylation, resulting in primordial follicle activation (Baksh et al., 2002; Reddy et al., 2010; Wang, Q. et al.).

4.5 Xenobiotic induced cell death

Ovarian follicles undergo physiological cell death via the apoptotic process of atresia, which is thought to select dysfunctional follicles and thus reserving the healthiest follicles for ovulation (Tilly et al., 1991). A number of studies have concluded that ovotoxic xenobiotics which target primordial follicles for destruction do so by inducing premature follicular atresia (Hu et al., 2001; Matikainen et al., 2001; Tilly and Robles, 1999). In this review we have already discussed the mechanisms by which ovotoxic xenobiotics may induce follicular atresia in primordial follicles (Ahr activation, Bioactivation, and ROS generation). However, other forms of cell death have been reportedly induced by xenobiotic exposure. Cell death by necrosis usually occurs in response to tissue injury, and elicits an inflammatory response in the surrounding tissue. Necrosis can be distinguished from apoptosis via histomorphological and ultrastructural analysis (Gobe and Harmon, 2001). In a study by Mattison (1980), the three PAHs BaP, 3-MC, and DMBA were shown to cause morphological changes in mouse primordial follicle oocytes which were consistent with necrosis (Mattison, 1980). The alkylating chemotherapeutic agent cyclophosphamide was also shown to cause necrotic damage in mouse primordial follicle oocytes three days after a single i.p injection (Plowchalk and Mattison, 1992). However, lower doses of cyclophosphamide produced atretic changes in primordial follicle oocytes, suggesting the type of cell death (apoptosis/necrosis) caused by xenobiotic exposure depends upon the dose given, and the duration of exposure. Therefore, concentrations of xenobiotic which cause mild cellular damage may result in active cell death, or apoptosis, while concentration which result in severe damage will result in passive cell death, or necrosis (Raffray and Gerald, 1997).

Autophagy or "self eating" is another possible non-apoptotic mechanism of cell death which may result in primordial follicle depletion. This conserved catabolic process involves the lysosomal-dependant turnover of cytoplasmic organelles and proteins during times of

starvation or nutrient deficiency, allowing the regeneration of metabolic precursor molecules to ensure survival (Levine and Klionsky, 2004). Increased incidences of autophagy have also been observed in response to other environmental stresses, including hypoxia, oxidative stress, and xenobiotic exposure (Kiffin et al., 2006; Kondo et al., 2005). Under these conditions autophagy may renew damaged or dysfunctional organelles, thereby maintaining a healthy cell population. Although the activation of autophagy in response to cell stress may be a cellular adaptation to promote survival, excessive activation beyond a key threshold may result in cellular collapse and atrophy, a process known as autophagic cell death (Galluzzi et al., 2008). While debatable whether autophagic cell death is independent from apoptosis, it has been almost universally accepted that excess autophagy can induce apoptosis (Levine and Yuan, 2005; Maiuri et al., 2007). Recent studies have suggested autophagy as an alternate form of programmed cell death in the ovary, with evidence indicating it is the main mechanism by which oogonia are lost prior to primordial follicle formation (Duerrschmidt et al., 2006; Lobascio et al., 2007; Rodrigues et al., 2009). Thus prolonged xenobiotic exposure resulting in organelle damage may induce autophagic cell death in primordial follicles, resulting in depletion. Indeed, proteins responsible for regulating apoptosis, such as members of the Bcl2 family, have also been found to regulate autophagy (Maiuri et al., 2007; Shimizu et al., 2004). Therefore, gene expression studies in which these pathways have been thought to induce xenobiotic atresia could be inducing primordial follicle destruction by apoptotic independent or dependent autophagy (Flaws et al., 2006).

5. Ovotoxic xenobiotics as agents for wildlife fertility control

Population control of native and exotic pest species is necessary to prevent environmental degradation, competition and predation of native wildlife, the spread of pathogenic diseases, and conflicts with humans over food production. Traditionally, population control has involved the elimination of the target species through poisoning, trapping and shooting (McAlpine et al., 2007). Although effective immediately, these methods are seen as inhuman, unsustainable, and ineffective over the long term. Manipulating the reproductive rate, particularly in females, instead of increasing the mortality rate is potentially more humane, species specific, and effective at curtailing populations (Kirkpatrick, 2007). The use of ovotoxic xenobiotics as agents of contraception/sterilisation represents a novel approach to fertility control. Of particular interest are xenobiotics which have been shown to cause POF by specifically targeting the primordial follicle population for degradation (Hoyer and Devine, 2001; Sobinoff et al., 2010), thus causing permanent sterility.

To achieve widespread efficacy ovotoxic xenobiotics in fertility control must be delivered via single or minimal oral administration. To be successful an oral agent must also have permanent or very long lasting effects, be specific for the target pest species and be humane/environmentally safe (Castle and Dean, 1996). Rodents such as the rice-field rat represent a serious pest in cereal agriculture, accounting for an average annual loss of between 5-10% of rice crops in Asia, 17% of rice crops in Indonesia, between 15-100% of maize in Africa, and between 5-90% of total crop production in South America (Geddes, 1992; Mwanjabe and Leirs, 1997; Rodríguez and Jaime, 1993; Singleton, 2003; Taylor, 1968).

VCD represents an ideal fertility control agent due to its ability to induce rapid small follicle depletion resulting in POF in rodents at concentrations which do not cause widespread

cytotoxicity (Springer et al., 1996). Additionally, VCD metabolism in the liver and hepatic tissue of rodents results in the production and excretion of the inert compound from the body, potentially reducing its effects on predators and its bioaccumulation in the environment (Flaws et al., 1994; Keating et al., 2010; Rajapaksa et al., 2007a). However, VCD does have disadvantages which make it fall short of the ideal fertility control agent. As described previously, VCD requires multiple doses to cause complete infertility in the rodent model (Springer et al., 1996). In addition, a VCD containing bait would need to be both attractive and palatable to the pest species, but not palatable or accessible to non-pest species. Currently, VCD is being trialled as an oral fertility control agent in the rice-field rat *Rattus argentiventer*. Registered by SenesTech Inc. as ContraPest®, the company website suggests the formulated bait is palatable, causes complete sterility within one month's ingestion, and does not adversely affect the animal's health and well being (http://www.senestech.com/). The use of other ovotoxicants as oral fertility control agents has been less successful. In a study by Sanders et al (2011) ERL-4221, a less toxic diepoxide, cycloaliphatic epoxide resin, which recently replaced VCD in industry, was investigated as a possible fertility control agent for pigs. A 20 day treatment period using palatable bait containing 16.0 mg ERL-4221 kg–1 bodyweight failed to produce any difference in follicular composition compared to control treated animals (Sanders et al., 2011). In summary, ovotoxicants represent potential fertility control agents, provided the xenobiotic delivers significant follicle depletion without side effects, and does not adversely affect the environment or food chain.

6. Conclusions

Ovotoxic xenobiotics cause primordial follicle depletion via several mechanisms which ultimately lead to their destruction or activation. These chemicals are rarely ovotoxic by themselves, and require hepatic or ovarian metabolism to exert their destructive effects on reproduction. This type of ovotoxicity is insidious in its nature, and is not usually detected until the primordial follicle pool has become severely depleted, resulting in premature reproductive senescence. Besides a loss in fertility, reproductive senescence is also associated with an increased incidence of a variety of health problems. Despite the negatives associated with ovotoxic xenobiotics, there is potential to use their destructive nature for wildlife control and agricultural gain. It is a form of poetic justice that ovotoxic xenobiotics which prevent women from conceiving may be used to combat one of the biggest causes of death in the third world, starvation. Future research should be aimed at further elaborating the specific mechanisms of primordial follicle ovotoxicity, improving our ability to predict/detect human risk from environmental exposure, and investigating the possibility of using these ovotoxicants for the environmental control of pest species.

7. Acknowledgments

The authors gratefully acknowledge the financial assistance to EAM by the Australian Research Council, Hunter Medical Research Institute and the Newcastle Permanent Building Society Charitable Trust. APS is the recipient of an Australian Postgraduate Award PhD scholarship. This work was supported by National Health and Medical Research Council (Project grant #510735) to EAM.

8. References

Adhikari, D., W. Zheng, Y. Shen, N. Gorre, T. Hämäläinen, A. J. Cooney, I. Huhtaniemi, Z. J. Lan and K. Liu (2010). "Tsc/mTORC1 signaling in oocytes governs the quiescence and activation of primordial follicles." *Human molecular genetics* 19(3): pp. 397-410, ISSN 0964-6906.

Baksh, S., H. R. Widlund, A. A. Frazer-Abel, J. Du, S. Fosmire, D. E. Fisher, J. A. DeCaprio, J. F. Modiano and S. J. Burakoff (2002). "NFATc2-mediated repression of cyclin-dependent kinase 4 expression." *Molecular cell* 10(5): pp. 1071-1081, 1097-2765.

Benedict, J. C., T. M. Lin, I. K. Loeffler, R. E. Peterson and J. A. Flaws (2000). "Physiological role of the aryl hydrocarbon receptor in mouse ovary development." *Toxicological Sciences* 56(2): pp. 382-388, ISSN 1096-6080.

Benedict, J. C., K. P. Miller, T. M. Lin, C. Greenfeld, J. K. Babus, R. E. Peterson and J. A. Flaws (2003). "Aryl hydrocarbon receptor regulates growth, but not atresia, of mouse preantral and antral follicles." *Biology of reproduction* 68(5): pp. 1511-1517, ISSN 0006-3363.

Bengtsson, M., J. Montelius, L. Mankowitz and J. Rydström (1983). "Metabolism of polycyclic aromatic hydrocarbons in the rat ovary comparison with metabolism in adrenal and liver tissues." *Biochemical Pharmacology* 32(1): pp. 129-136, ISSN 0006-2952.

Bhattacharya, P. and A. F. Keating (2011). "Ovarian metabolism of xenobiotics." *Experimental Biology and Medicine* 236(7): pp. 765-771, ISSN 1535-3702.

Blume-Jensen, P., R. Janknecht and T. Hunter (1998). "The kit receptor promotes cell survival via activation of PI 3-kinase and subsequent Akt-mediated phosphorylation of Bad on Ser136." *Current biology* 8(13): pp. 779-785, ISSN 0960-9822.

Blumenfeld, Z. and N. Haim (1997). "Prevention of gonadal damage during cytotoxic therapy." *Annals of medicine* 29(3): pp. 199-206, ISSN 0785-3890.

Bolton, J. L., M. A. Trush, M. Trevor, G. Dryhurst and T. J. Monks (2000). "Role of quinones in toxicology." *Chemical Research in Toxicology* 13(3): pp. 135-160, ISSN 0893-228X.

Bondy, S. C. and S. Naderi (1994). "Contribution of hepatic cytochrome P450 systems to the generation of reactive oxygen species." *Biochemical pharmacology* 48(1): pp. 155-159, ISSN 0006-2952.

Borman, S. M., P. J. Christian, I. G. Sipes and P. B. Hoyer (2000). "Ovotoxicity in female Fischer rats and B6 mice induced by low-dose exposure to three polycyclic aromatic hydrocarbons: comparison through calculation of an ovotoxic index." *Toxicology and applied pharmacology* 167(3): pp. 191-198, ISSN 0041-008X.

Cadenas, E. and K. J. A. Davies (2000). "Mitochondrial free radical generation, oxidative stress, and aging." *Free Radical Biology and Medicine* 29(3-4): pp. 222-230, ISSN 0891-5849.

Calvin, H. I., K. Grosshans and E. J. Blake (1986). "Estimation and manipulation of glutathione levels in prepuberal mouse ovaries and ova: relevance to sperm nucleus transformation in the fertilized egg." *Gamete research* 14(3): pp. 265-275, ISSN 1554-3919.

Carlson, D. B. and G. H. Perdew (2002). "A dynamic role for the Ah receptor in cell signaling? Insights from a diverse group of Ah receptor interacting proteins." *Journal of biochemical and molecular toxicology* 16(6): pp. 317-325, ISSN 1099-0461.

Caro, A. A. and A. I. Cederbaum (2004). "Oxidative Stress, Toxicology, and Pharmacology of CYP2E1*." *Annu. Rev. Pharmacol. Toxicol.* 44: pp. 27-42, ISSN 0066-4170.

Castle, P. E. and J. Dean (1996). "Molecular genetics of the zona pellucida: implications for immunocontraceptive strategies." *Journal of reproduction and fertility. Supplement* 50: pp. 1-8, ISSN 0449-3087.

Cenci, S., G. Toraldo, M. N. Weitzmann, C. Roggia, Y. Gao, W. P. Qian, O. Sierra and R. Pacifici (2003). "Estrogen deficiency induces bone loss by increasing T cell proliferation and lifespan through IFN- -induced class II transactivator." *Proceedings of the National Academy of Sciences of the United States of America* 100(18): pp. 10405-10410, ISSN 0027-8424.

Clague, N., M. Sevcik, G. Stuart, M. Brännström, P. O. Janson and J. F. Jarrell (1992). "The effect of estrous cycle and buthionine sulfoximine on glutathione release from the in vitro perfused rat ovary." *Reproductive Toxicology* 6(6): pp. 533-539, ISSN 0890-6238.

Conney, A. H. (1982). "Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: GHA Clowes Memorial Lecture." *Cancer research* 42(12): pp. 4875-4917, ISSN 0008-5472.

Coulam, C. B., S. C. Adamson and J. F. Annegers (1986). "Incidence of premature ovarian failure." *Obstetrics & Gynecology* 67(4): pp. 604-606, ISSN 0029-7844.

Dai, Y. S., J. Xu and J. D. Molkentin (2005). "The DnaJ-related factor Mrj interacts with nuclear factor of activated T cells c3 and mediates transcriptional repression through class II histone deacetylase recruitment." *Molecular and cellular biology* 25(22): pp. 9936, 0270-7306.

Danielson, P. B. (2002). "The cytochrome P450 superfamily: biochemistry, evolution and drug metabolism in humans." *Current Drug Metabolism* 3(6): pp. 561-597, ISSN 1389-2002.

Dekant, W. (2009). "The role of biotransformation and bioactivation in toxicity." *Molecular, Clinical and Environmental Toxicology* 99: pp. 57-86, ISSN 1023-294X.

Denison, M. S. and S. Heath-Pagliuso (1998). "The Ah receptor: a regulator of the biochemical and toxicological actions of structurally diverse chemicals." *Bulletin of environmental contamination and toxicology* 61(5): pp. 557-568, ISSN 0007-4861.

Devine, P. J., I. G. Sipes and P. B. Hoyer (2004). "Initiation of delayed ovotoxicity by in vitro and in vivo exposures of rat ovaries to 4-vinylcyclohexene diepoxide." *Reproductive Toxicology* 19(1): pp. 71-77, ISSN 0890-6238.

Duerrschmidt, N., O. Zabirnyk, M. Nowicki, A. Ricken, F. A. Hmeidan, V. Blumenauer, J. Borlak and K. Spanel-Borowski (2006). "Lectin-like oxidized low-density lipoprotein receptor-1-mediated autophagy in human granulosa cells as an alternative of programmed cell death." *Endocrinology* 147(8): pp. 3851-3860, ISSN 0013-7227.

Edson, M. A., A. K. Nagaraja and M. M. Matzuk (2009). "The mammalian ovary from genesis to revelation." *Endocrine reviews* 30(6): pp. 624-712, ISSN 1945-7189.

Ekstrom, G. and M. Ingelman-Sundberg (1989). "Rat liver microsomal NADPH-supported oxidase activity and lipid peroxidation dependent on ethanol-inducible cytochrome P-450 (P-450IIE1)." *Biochemical pharmacology* 38(8): pp. 1313-1319, ISSN 0006-2952.

Flaws, J. A., S. L. Marion, K. P. Miller, P. J. Christian, J. K. Babus and P. B. Hoyer (2006). "Effect of bcl-2 overexpression in mice on ovotoxicity caused by 4-vinylcyclohexene." *Toxicology and applied pharmacology* 215(1): pp. 51-56, ISSN 0041-008X.

Flaws, J. A., K. L. Salyers, I. G. Sipes and P. B. Hoyer (1994). "Reduced ability of rat preantral ovarian follicles to metabolize 4-vinyl-1-cyclohexene diepoxide in vitro." *Toxicology and applied pharmacology* 126(2): pp. 286-294, ISSN 0041-008X.

Galluzzi, L., J. M. Vicencio, O. Kepp, E. Tasdemir, M. C. Maiuri and G. Kroemer (2008). "To die or not to die: that is the autophagic question." *Curr Mol Med* 8(2): pp. 78-91, ISSN 1566-5240.

Geddes, A. M. W. (1992). The relative importance of pre-harvest crop pests in Indonesia, *Natural Resources Institute Bulletin (United Kingdom)*, A. M. W. Geddes, pp. 1-70, Natural Resources Institute, ISBN 0-85954-295-5. Chatham, UK.

Gobe, G. and B. Harmon (2001). Apoptosis: Morphological Criteria and Other Assays, *Encyclopedia of Life Sciences*, Y. Zheng, <http://dx.doi.org/10.1002/9780470015902.a0002569.pub3 >

Guraya, S. S. (1985). Primordial follicle, *Biology of Ovarian Follicles in Mammals*, pp. 3-14, Springer-Verlag, ISBN 9783540150220. Berlin, Germany.

Harrigan, J. A., C. M. Vezina, B. P. McGarrigle, N. Ersing, H. C. Box, A. E. Maccubbin and J. R. Olson (2004). "DNA adduct formation in precision-cut rat liver and lung slices exposed to benzo [a] pyrene." *Toxicological Sciences* 77(2): pp. 307-314, ISSN 1096-6080.

Hirshfield, A. N. (1991). "Development of follicles in the mammalian ovary." *International review of cytology* 124: pp. 43-101, ISSN 0074-7696.

Hooser, S. B., D. P. Douds, D. G. DeMerell, P. B. Hoyer and I. G. Sipes (1994). "Long-term ovarian and gonadotropin changes in mice exposed to 4-vinylcyclohexene." *Reproductive Toxicology* 8(4): pp. 315-323, ISSN 0890-6238.

Hou, Y. T., W. Xia, J. E. Pawlowski and T. M. Penning (1994). "Rat dihydrodiol dehydrogenase: complexity of gene structure and tissue-specific and sexually dimorphic gene expression." *Cancer research* 54(1): pp. 247-255, ISSN 0008-5472.

Hoyer, P. B. and P. J. Devine (2001). "Ovarian toxicity of 4-vinylcyclohexene diepoxide: a mechanistic model." *Toxicologic pathology* 29(1): pp. 91-99, ISSN 0192-6233.

Hoyer, P. B. and I. G. Sipes (1996). "Assessment of follicle destruction in chemical-induced ovarian toxicity." *Annual review of pharmacology and toxicology* 36(1): pp. 307-331, ISSN 0362-1642.

Hu, X., P. J. Christian, K. E. Thompson, I. Glenn Sipes and P. B. Hoyer (2001). "Apoptosis induced in rats by 4-vinylcyclohexene diepoxide is associated with activation of the caspase cascades." *Biology of reproduction* 65(1): pp. 87-93, ISSN 0006-3363.

Hughes, F. M. and W. C. Gorospe (1991). "Biochemical identification of apoptosis (programmed cell death) in granulosa cells: evidence for a potential mechanism underlying follicular atresia." *Endocrinology* 129(5): pp. 2415-2422, ISSN 0013-7227.

Igawa, Y., A. F. Keating, K. S. Rajapaksa, I. G. Sipes and P. B. Hoyer (2009). "Evaluation of ovotoxicity induced by 7, 12-dimethylbenz [a] anthracene and its 3, 4-diol metabolite utilizing a rat in vitro ovarian culture system." *Toxicology and applied pharmacology* 234(3): pp. 361-369, ISSN 0041-008X.

Jancovaa, P., P. Anzenbacherb and E. Anzenbacherovaa (2010). "Phase II drug metabolizing enzymes." *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* 154(2): pp. 103-116, ISSN 1213-8118.

Janssen-Heininger, Y. M. W., B. T. Mossman, N. H. Heintz, H. J. Forman, B. Kalyanaraman, T. Finkel, J. S. Stamler, S. G. Rhee and A. van der Vliet (2008). "Redox-based regulation of signal transduction: principles, pitfalls, and promises." *Free Radical Biology and Medicine* 45(1): pp. 1-17, ISSN 0891-5849.

Jarrell, J. F., L. Bodo, E. V. Younglai, R. D. Barr and G. J. O'Connell (1991). "The short-term reproductive toxicity of cyclophosphamide in the female rat." *Reproductive Toxicology* 5(6): pp. 481-485, ISSN 0890-6238.

Keating, A. F. (2009). "Effect of phosphatidylinositol-3 kinase inhibition on ovotoxicity caused by 4-vinylcyclohexene diepoxide and 7, 12-dimethylbenz [a] anthracene in neonatal rat ovaries." *Toxicology and applied pharmacology* 241(2): pp. 127-134, ISSN 0041-008X.

Keating, A. F., S. M. Fernandez, C. J. Mark-Kappeler, N. Sen, I. G. Sipes and P. B. Hoyer (2011). "Inhibition of PIK3 Signaling Pathway Members by the Ovotoxicant 4-Vinylcyclohexene Diepoxide in Rats." *Biology of reproduction* 84(4): pp. 743-751, ISSN 0006-3363.

Keating, A. F., N. Sen, I. G. Sipes and P. B. Hoyer (2010). "Dual protective role for Glutathione S-transferase class pi against VCD-induced ovotoxicity in the rat ovary." *Toxicology and applied pharmacology* 247(2): pp. 71-75, ISSN 0041-008X.

Kidd, P. M. (1997). "Glutathione: systemic protectant against oxidative and free radical damage." *Alternative Medicine Review* 2(3): pp. 155-176, ISSN 1089-5159.

Kiffin, R., U. Bandyopadhyay and A. M. Cuervo (2006). "Oxidative stress and autophagy." *Antioxidants & redox signaling* 8(1-2): pp. 152-162, ISSN 1523-0864.

Kirkpatrick, J. F. (2007). "Measuring the effects of wildlife contraception: the argument for comparing apples with oranges." *Reproduction, Fertility and Development* 19(4): pp. 548-552, ISSN 1448-5990.

Klaunig, J. E. and L. M. Kamendulis (2004). "The role of oxidative stress in carcinogenesis." *Annu. Rev. Pharmacol. Toxicol.* 44: pp. 239-267, ISSN 0066-4170.

Kohalmy, K. and R. Vrzal (2011). "Regulation of Phase II Biotransformation Enzymes by Steroid Hormones." *Current Drug Metabolism* 12(2): pp. 104-123, ISSN 1389-2002.

Kondo, Y., T. Kanzawa, R. Sawaya and S. Kondo (2005). "Role of autophagy in cancer development and response to therapy." *Nature Reviews Cancer* 5(9): pp. 726-734, ISSN 1474-175X.

Levine, B. and D. J. Klionsky (2004). "Development by Self-Digestion:: Molecular Mechanisms and Biological Functions of Autophagy." *Developmental cell* 6(4): pp. 463-477, ISSN 1534-5807.

Levine, B. and J. Yuan (2005). "Autophagy in cell death: an innocent convict?" *Journal of Clinical Investigation* 115(10): pp. 2679-2688, ISSN 0021-9738.

Lobascio, A. M., F. G. Klinger, M. L. Scaldaferri, D. Farini and M. De Felici (2007). "Analysis of programmed cell death in mouse fetal oocytes." *Reproduction* 134(2): pp. 241-252, ISSN 1470-1626.

Luderer, U., T. J. Kavanagh, C. C. White and E. M. Faustman (2001). "Gonadotropin regulation of glutathione synthesis in the rat ovary." *Reproductive Toxicology* 15(5): pp. 495-504, ISSN 0890-6238.

Maiuri, M. C., E. Zalckvar, A. Kimchi and G. Kroemer (2007). "Self-eating and self-killing: crosstalk between autophagy and apoptosis." *Nature Reviews Molecular Cell Biology* 8(9): pp. 741-752, ISSN 1471-0072.

Martin, J. A., B. E. Hamilton, P. D. Sutton, S. J. Ventura, F. Menacker and M. L. Munson (2003). "Births: final data for 2002." *National vital statistics reports* 52(10): pp. 1-113, ISSN 1551-8930.

Matikainen, T., G. I. Perez, A. Jurisicova, J. K. Pru, J. J. Schlezinger, H. Y. Ryu, J. Laine, T. Sakai, S. J. Korsmeyer and R. F. Casper (2001). "Aromatic hydrocarbon receptor-driven Bax gene expression is required for premature ovarian failure caused by biohazardous environmental chemicals." *Nature Genetics* 28(4): pp. 355-360, ISSN 1061-4036.

Mattison, D. R. (1980). "Morphology of oocyte and follicle destruction by polycyclic aromatic hydrocarbons in mice." *Toxicology and Applied Pharmacology* 53(2): pp. 249-259, ISSN 0041-008X.

Mattison, D. R. and J. D. Schulman (1980). "How xenobiotic chemicals can destroy oocytes." *Contemp Obstet Gynecol* 15: pp. 157-169, ISSN 1471-8359.

Mattison, D. R., K. Shiromizu and M. S. Nightingale (1983a). "Oocyte destruction by polycyclic aromatic hydrocarbons." *American journal of industrial medicine* 4(1-2): pp. 191-202, ISSN 0271-3586.

Mattison, D. R., K. Shiromizu, J. A. Pendergrass and S. S. Thorgeirsson (1983b). "Ontogeny of ovarian glutathione and sensitivity to primordial oocyte destruction by cyclophosphamide." *Pediatric pharmacology (New York, NY)* 3(1): pp. 49-55, ISSN 0270-322X.

Mattison, D. R. and S. S. Thorgeirsson (1979). "Ovarian aryl hydrocarbon hydroxylase activity and primordial oocyte toxicity of polycyclic aromatic hydrocarbons in mice." *Cancer Research* 39(9): pp. 3471-3475, ISSN 0008-5472.

McAlpine, C. A., S. Heyenga, B. Taylor, A. N. N. Peterson and G. McDonald (2007). "Regional planning in Queensland's rangelands: challenges and prospects for biodiversity conservation." *Geographical Research* 45(1): pp. 27-42, ISSN 1745-5871.

McGee, E. A. and A. J. W. Hsueh (2000). "Initial and cyclic recruitment of ovarian follicles." *Endocrine reviews* 21(2): pp. 200-214, ISSN 1945-7189.

McNatty, K. P., A. E. Fidler, J. L. Juengel, L. D. Quirke, P. R. Smith, D. A. Heath, T. Lundy, A. O'Connell and D. J. Tisdall (2000). "Growth and paracrine factors regulating follicular formation and cellular function." *Molecular and Cellular Endocrinology* 163(1-2): pp. 11-20, ISSN 0303-7207.

Meirow, D., J. Dor, B. Kaufman, A. Shrim, J. Rabinovici, E. Schiff, H. Raanani, J. Levron and E. Fridman (2007). "Cortical fibrosis and blood-vessels damage in human ovaries exposed to chemotherapy. Potential mechanisms of ovarian injury." *Human reproduction* 22(6): pp. 1626-1633, ISSN 0268-1161.

Melendez-Colon, V. J., A. Luch, A. Seidel and W. M. Baird (1999). "Cancer initiation by polycyclic aromatic hydrocarbons results from formation of stable DNA adducts rather than apurinic sites." *Carcinogenesis* 20(10): pp. 1885-1891, ISSN 0143-3334.

Meunier, B., S. P. De Visser and S. Shaik (2004). "Mechanism of oxidation reactions catalyzed by cytochrome P450 enzymes." *Chemical reviews* 104(9): pp. 3947-3980, ISSN 0009-2665.

Mwanjabe, P. S. and H. Leirs (1997). "An early warning system for IPM-based rodent control in smallholder farming systems in Tanzania." *Belgian Journal of Zoology* 127: pp. 49-58, ISSN 0777-6276.

Nebert, D. W., D. D. Brown, D. W. Towne and H. J. Eisen (1984). "Association of fertility, fitness and longevity with the murine Ah locus among (C57BL/6N)(C3H/HeN) recombinant inbred lines." *Biology of reproduction* 30(2): pp. 363-373, ISSN 0006-3363.

Nebert, D. W., A. L. Roe, M. Z. Dieter, W. A. Solis, Y. I. Yang and T. P. Dalton (2000). "Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis." *Biochemical pharmacology* 59(1): pp. 65-85, ISSN 0006-2952.

Nguyen, L. P. and C. A. Bradfield (2007). "The search for endogenous activators of the aryl hydrocarbon receptor." *Chemical research in toxicology* 21(1): pp. 102-116, ISSN 0893-228X.

Park, J. H., S. Gopishetty, L. M. Szewczuk, A. B. Troxel, R. G. Harvey and M. Trevor (2005). "Formation of 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxo-dGuo) by PAH o-quinones: involvement of reactive oxygen species and copper (II)/copper (I) redox cycling." *Chemical research in toxicology* 18(6): pp. 1026-1037, ISSN 0893-228X.

Pavek, P. and Z. Dvorak (2008). "Xenobiotic-induced transcriptional regulation of xenobiotic metabolizing enzymes of the cytochrome P450 superfamily in human extrahepatic tissues." *Current drug metabolism* 9(2): pp. 129-143, ISSN 1389-2002.

Petrulis, J. R. and G. H. Perdew (2002). "The role of chaperone proteins in the aryl hydrocarbon receptor core complex." *Chemico-biological interactions* 141(1-2): pp. 25-40, ISSN 0009-2797.

Picut, C. A., C. L. Swanson, K. L. Scully, V. C. Roseman, R. F. Parker and A. K. Remick (2008). "Ovarian follicle counts using proliferating cell nuclear antigen (PCNA) and semi-automated image analysis in rats." *Toxicologic pathology* 36(5): pp. 674-679, ISSN 0192-6233.

Plowchalk, D. R. and D. R. Mattison (1992). "Reproductive toxicity of cyclophosphamide in the C57BL/6N mouse: 1. Effects on ovarian structure and function." *Reproductive Toxicology* 6(5): pp. 411-421, ISSN 0890-6238.

Pollenz, R. S., C. A. Sattler and A. Poland (1994). "The aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein show distinct subcellular localizations in Hepa 1c1c7 cells by immunofluorescence microscopy." *Molecular pharmacology* 45(3): pp. 428-438, ISSN 0026-895X.

Raffray, M. and M. C. Gerald (1997). "Apoptosis and necrosis in toxicology: a continuum or distinct modes of cell death?" *Pharmacology & therapeutics* 75(3): pp. 153-177, ISSN 0163-7258.

Rajapaksa, K. S., E. A. Cannady, I. G. Sipes and P. B. Hoyer (2007a). "Involvement of CYP 2E1 enzyme in ovotoxicity caused by 4-vinylcyclohexene and its metabolites." *Toxicology and applied pharmacology* 221(2): pp. 215-221, ISSN 0041-008X.

Rajapaksa, K. S., I. G. Sipes and P. B. Hoyer (2007b). "Involvement of microsomal epoxide hydrolase enzyme in ovotoxicity caused by 7, 12-dimethylbenz [a] anthracene." *Toxicological sciences* 96(2): pp. 327-334, ISSN 1096-6080.

Ramesh, A., A. E. Archibong and M. S. Niaz (2010). "Ovarian Susceptibility to Benzo [a] Pyrene: Tissue Burden of Metabolites and DNA Adducts in F-344 Rats." *Journal of toxicology and environmental health. Part A* 73(23): pp. 1611-1625, ISSN 1528-7394.

Reddy, P., L. Liu, D. Adhikari, K. Jagarlamudi, S. Rajareddy, Y. Shen, C. Du, W. Tang, T. Hämäläinen and S. L. Peng (2008). "Oocyte-specific deletion of Pten causes premature activation of the primordial follicle pool." *Science* 319(5863): pp. 611-613, ISSN 0036-8075.

Reddy, P., W. Zheng and K. Liu (2010). "Mechanisms maintaining the dormancy and survival of mammalian primordial follicles." *Trends in Endocrinology & Metabolism* 21(2): pp. 96-103, ISSN 1043-2760.

Reyes, H., S. Reisz-Porszasz and O. Hankinson (1992). "Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor." *Science* 256(5060): pp. 1193-1195, ISSN 0036-8075.

Robles, R., Y. Morita, K. K. Mann, G. I. Perez, S. Yang, T. Matikainen, D. H. Sherr and J. L. Tilly (2000). "The aryl hydrocarbon receptor, a basic helix-loop-helix transcription factor of the PAS gene family, is required for normal ovarian germ cell dynamics in the mouse." *Endocrinology* 141(1): pp. 450-453, ISSN 0013-7227.

Rodrigues, P., D. Limback, L. K. McGinnis, C. E. Plancha and D. F. Albertini (2009). "Multiple mechanisms of germ cell loss in the perinatal mouse ovary." *Reproduction* 137(4): pp. 709-720, ISSN 1470-1626.

Rodríguez, M. and A. Jaime (1993). Rodent plague: a continuing problem in Latin America and the Caribbean. *Latin American Conference on Rodent Biology and Control of Plague*, ISBN 9718709053, Santa Cruz, Bolivia, October 1991.

Sanders, D. L., F. Xie, R. E. Mauldin, J. C. Hurley, L. A. Miller, M. R. Garcia, R. W. DeYoung, D. B. Long and T. A. Campbell (2011). "Efficacy of ERL-4221 as an ovotoxin for feral pigs (Sus scrofa)." *Wildlife Research* 38(2): pp. 168-172, ISSN 1448-5494.

Schafer, F. Q. and G. R. Buettner (2001). "Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple." *Free Radical Biology and Medicine* 30(11): pp. 1191-1212, ISSN 0891-5849.

Schmidt, J. V., G. H. Su, J. K. Reddy, M. C. Simon and C. A. Bradfield (1996). "Characterization of a murine Ahr null allele: involvement of the Ah receptor in hepatic growth and development." *Proceedings of the National Academy of Sciences* 93(13): pp. 6731-6736, ISSN 0027-8424.

Schroer, K., M. Kittelmann and S. Lütz (2010). "Recombinant human cytochrome P450 monooxygenases for drug metabolite synthesis." *Biotechnology and bioengineering* 106(5): pp. 699-706, ISSN 1097-0290.

Schwarz, D., P. Kisselev, I. Cascorbi, W. H. Schunck and I. Roots (2001). "Differential metabolism of benzo [a] pyrene and benzo [a] pyrene-7, 8-dihydrodiol by human CYP1A1 variants." *Carcinogenesis* 22(3): pp. 453-459, ISSN 0143-3334.

Shimada, T. and Y. Fujii Kuriyama (2004). "Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and1B1." *Cancer science* 95(1): pp. 1-6, ISSN 1349-7006.

Shimada, T., Y. Oda, E. M. J. Gillam, F. P. Guengerich and K. Inoue (2001). "Metabolic activation of polycyclic aromatic hydrocarbons and other procarcinogens by cytochromes P450 1A1 and P450 1B1 allelic variants and other human cytochromes P450 in Salmonella typhimurium NM2009." *Drug metabolism and disposition* 29(9): pp. 1176-1182, ISSN 0090-9556.

Shimada, T., A. Sugie, M. Shindo, T. Nakajima, E. Azuma, M. Hashimoto and K. Inoue (2003). "Tissue-specific induction of cytochromes P450 1A1 and 1B1 by polycyclic aromatic hydrocarbons and polychlorinated biphenyls in engineered C57BL/6J mice of arylhydrocarbon receptor gene." *Toxicology and applied pharmacology* 187(1): pp. 1-10, ISSN 0041-008X.

Shimizu, S., T. Kanaseki, N. Mizushima, T. Mizuta, S. Arakawa-Kobayashi, C. B. Thompson and Y. Tsujimoto (2004). "Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes." *Nature cell biology* 6(12): pp. 1221-1228, ISSN 1465-7392.

Shiromizu, K. and D. R. Mattison (1985). "Murine oocyte destruction following intraovarian treatment with 3 methylcholanthrene or 7, 12 dimethylbenz (a) anthracene: Protection by alpha naphthoflavone." *Teratogenesis, carcinogenesis, and mutagenesis* 5(6): pp. 463-472, ISSN 1520-6866.

Shuster, L. T., D. J. Rhodes, B. S. Gostout, B. R. Grossardt and W. A. Rocca (2010). "Premature menopause or early menopause: long-term health consequences." *Maturitas* 65(2): pp. 161-166, ISSN 0378-5122.

Singleton, G. R. (2003). "Impacts of rodents on rice production in Asia." *IRRI Discuss. Pap. Ser* 45: pp. 1-30, ISSN 0115-3862.

Smith, B. J., D. R. Mattison and I. G. Sipes (1990). "The role of epoxidation in 4-vinylcyclohexene-induced ovarian toxicity." *Toxicology and applied pharmacology* 105(3): pp. 372-381, ISSN 0041-008X.

Sobinoff, A. P., M. Mahony, B. Nixon, S. D. Roman and E. A. McLaughlin (2011). "Understanding the villain: DMBA induced pre-antral ovotoxicity involves selective follicular destruction and primordial follicle activation through PI3K/Akt and mTOR signalling." *Toxicological Sciences*: pp. kfr195v1-kfr195, ISSN 1096-6080.

Sobinoff, A. P., V. Pye, B. Nixon, S. D. Roman and E. A. McLaughlin (2010). "Adding Insult to Injury: Effects of Xenobiotic-Induced Preantral Ovotoxicity on Ovarian Development and Oocyte Fusibility." *Toxicological Sciences* 118(2): pp. 653-666, ISSN 1096-6080.

Springer, L. N., M. E. McAsey, J. A. Flaws, J. L. Tilly, I. G. Sipes and P. B. Hoyer (1996). "Involvement of apoptosis in 4-vinylcyclohexene diepoxide-induced ovotoxicity in rats." *Toxicology and applied pharmacology* 139(2): pp. 394-401, ISSN 0041-008X.

Stapleton, H. M. and J. E. Baker (2003). "Comparing polybrominated diphenyl ether and polychlorinated biphenyl bioaccumulation in a food web in Grand Traverse Bay, Lake Michigan." *Archives of environmental contamination and toxicology* 45(2): pp. 227-234, ISSN 0090-4341.

Tarin, J. J. (1996). "Potential effects of age-associated oxidative stress on mammalian oocytes/embryos." *Molecular human reproduction* 2(10): pp. 717-724, ISSN 1360-9947.

Taylor, K. D. (1968). "An outbreak of rats in agricultural areas of Kenya in 1962." *East African Agricultural and Forestry Journal* 34(1): pp. 66-77, ISSN 0012-8325.

Terman, A., B. Gustafsson and U. T. Brunk (2006). "The lysosomal-mitochondrial axis theory of postmitotic aging and cell death." *Chemico-biological interactions* 163(1-2): pp. 29-37, ISSN 0009-2797.

Testa, J. R. and A. Bellacosa (2001). "AKT plays a central role in tumorigenesis." *Proceedings of the National Academy of Sciences* 98(20): pp. 10983-10985, ISSN 0027-8424.

Thimmulappa, R. K., K. H. Mai, S. Srisuma, T. W. Kensler, M. Yamamoto and S. Biswal (2002). "Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray." *Cancer research* 62(18): pp. 5196-5203, ISSN 0008-5472.

Thor, H., M. T. Smith, P. Hartzell, G. Bellomo, S. A. Jewell and S. Orrenius (1982). "The metabolism of menadione (2-methyl-1, 4-naphthoquinone) by isolated hepatocytes. A study of the implications of oxidative stress in intact cells." *Journal of Biological Chemistry* 257(20): pp. 12419-12425, ISSN 0021-9258.

Tilly, J. L., K. I. Kowalski, A. L. Johnson and A. J. W. Hsueh (1991). "Involvement of apoptosis in ovarian follicular atresia and postovulatory regression." *Endocrinology* 129(5): pp. 2799-2801, ISSN 0013-7227.

Tilly, J. L. and R. Robles (1999). Apoptosis and its impact in clinical reproductive medicine, *Molecular biology in reproductive medicine*, B. C. J. M. Fauser, A. J. Rutherford, J. F. Strauss and A. Van Steirteghem, pp. 79–101, ISBN 1850709947. New York, USA.

Tománek, M. and E. Chronowska (2006). "Immunohistochemical localization of proliferating cell nuclear antigen (PCNA) in the pig ovary." *Folia histochemica et Cytobiologica* 44(4): pp. 269-274, ISSN 0239-8508.

Trevor, M., S. T. Ohnishi, T. Ohnishi and R. G. Harvey (1996). "Generation of reactive oxygen species during the enzymatic oxidation of polycyclic aromatic hydrocarbon trans-dihydrodiols catalyzed by dihydrodiol dehydrogenase." *Chemical research in toxicology* 9(1): pp. 84-92, ISSN 0893-228X.

Tsai-Turton, M., B. N. Nakamura and U. Luderer (2007). "Induction of apoptosis by 9, 10-dimethyl-1, 2-benzanthracene in cultured preovulatory rat follicles is preceded by a rise in reactive oxygen species and is prevented by glutathione." *Biology of Reproduction* 77(3): pp. 442-451, ISSN 0006-3363.

Valko, M., D. Leibfritz, J. Moncol, M. T. D. Cronin, M. Mazur and J. Telser (2007). "Free radicals and antioxidants in normal physiological functions and human disease." *The international journal of biochemistry & cell biology* 39(1): pp. 44-84, ISSN 1357-2725.

Valko, M., C. J. Rhodes, J. Moncol, M. Izakovic and M. Mazur (2006). "Free radicals, metals and antioxidants in oxidative stress-induced cancer." *Chemico-biological interactions* 160(1): pp. 1-40, ISSN 0009-2797.

van Wezel, I. L. and R. J. Rodgers (1996). "Morphological characterization of bovine primordial follicles and their environment in vivo." *Biology of reproduction* 55(5): pp. 1003-1011, ISSN 0006-3363.

Vlahos, C. J., W. F. Matter, K. Y. Hui and R. F. Brown (1994). "A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002)." *Journal of Biological Chemistry* 269(7): pp. 5241-5248, ISSN 0021-9258.

Wang, K., H. Fang, D. Xiao, X. Zhu, M. He, X. Pan, J. Shi, H. Zhang, X. Jia and Y. Du (2009). "Converting redox signaling to apoptotic activities by stress-responsive regulators

HSF1 and NRF2 in fenretinide treated cancer cells." *PLoS One* 4(10): pp. e7538, ISSN 1932-6203.

Wang, Q., Y. Zhou, L. N. Jackson, S. M. Johnson, C. W. Chow and B. M. Evers "Nuclear factor of activated T cells (NFAT) signaling regulates PTEN expression and intestinal cell differentiation." *Molecular Biology of the Cell* 22(3): pp. 412,

Wells, P. G., G. P. McCallum, C. S. Chen, J. T. Henderson, C. J. J. Lee, J. Perstin, T. J. Preston, M. J. Wiley and A. W. Wong (2009). "Oxidative stress in developmental origins of disease: teratogenesis, neurodevelopmental deficits, and cancer." *Toxicological sciences* 108(1): pp. 4-18, ISSN 1096-6080.

Wu, K. L. and T. Berger (2008). "Reduction in rat oocyte fertilizability mediated by S-(1, 2-dichlorovinyl)-L-cysteine: a trichloroethylene metabolite produced by the glutathione conjugation pathway." *Bulletin of environmental contamination and toxicology* 81(5): pp. 490-493, ISSN 0007-4861.

Chapter 1: Adding Insult to Injury: Effects of Xenobiotic-Induced Preantral Ovotoxicity on Ovarian Development and Oocyte Fusibility

TOXICOLOGICAL SCIENCES **118(2)**, 653–666 (2010)
doi:10.1093/toxsci/kfq272
Advance Access publication September 9, 2010

Adding Insult to Injury: Effects of Xenobiotic-Induced Preantral Ovotoxicity on Ovarian Development and Oocyte Fusibility

Alexander P. Sobinoff,* Victoria Pye,* Brett Nixon,*,† Shaun D. Roman,*,† and Eileen A. McLaughlin*,†,[1]

*Reproductive Science Group; and †Australian Research Council Center of Excellence in Biotechnology and Development, School of Environmental and Life Sciences, University of Newcastle, Callaghan, New South Wales 2308, Australia

[1]To whom correspondence should be addressed. Fax: +612-4921-6308. E-mail: eileen.mclaughlin@newcastle.edu.au.

Received July 7, 2010; accepted September 3, 2010

Mammalian females are born with a finite number of nonrenewing primordial follicles, the majority of which remain in a quiescent state for many years. Because of their nonrenewing nature, these "resting" oocytes are particularly vulnerable to xenobiotic insult, resulting in premature ovarian senescence and the formation of dysfunctional oocytes. In this study, we characterized the mechanisms of ovotoxicity for three ovotoxic agents, 4-vinylcyclohexene diepoxide (VCD), methoxychlor (MXC), and menadione (MEN), all of which target immature follicles. Microarray analysis of neonatal mouse ovaries exposed to these xenobiotics *in vitro* revealed a more than twofold significant difference in transcript expression ($p < 0.05$) for a number of genes associated with apoptotic cell death and primordial follicle activation. Histomorphological and immunohistological analysis supported the microarray data, showing signs of primordial follicle activation and preantral follicle atresia both *in vitro* and *in vivo*. Sperm-oocyte fusion assays on oocytes obtained from adult Swiss mice treated neonatally revealed severely reduced sperm-egg binding and fusion in a dose-dependent manner for all the xenobiotic treatments. Additionally, lipid peroxidation analysis on xenobiotic-cultured oocytes indicated a dose-dependent increase in oocyte lipid peroxidation for all three xenobiotics *in vitro*. Our results reveal a novel mechanism of preantral ovotoxicity involving the homeostatic recruitment of primordial follicles to maintain the pool of developing follicles destroyed by xenobiotic exposure and to our knowledge provide the first documented evidence of short-term, low- and high-dose (VCD 40–80 mg/kg/day, MXC 50–100 mg/kg/day, MEN 7.5–15 mg/kg/day) neonatal exposure to xenobiotics causing long-term reactive oxygen species–induced oocyte dysfunction.

Key Words: **xenobiotic; fertility; ovary; primordial follicle; oocyte dysfunction.**

The mammalian female reproductive life span is largely defined by a finite pool of primordial follicles established around the time of birth. These follicles serve as the primary source of all developing oocytes in the ovary and cannot be regenerated after fetal development (Hirshfield, 1991). Only a few primordial follicles are recruited into the growing pool of follicles at any one time, with some follicles remaining in a quiescent state for many years. This event occurs in regular waves and is continuous from birth until the primordial follicle pool is depleted, resulting in menopause (McGee and Hsueh, 2000). Overall, < 1% of all follicles recruited into the growing pool are destined for ovulation, with the vast majority being lost during development in an apoptotic process called atresia (Hirshfield, 1991). Although atresia is a normal physiological process, it is now known that it can be abnormally triggered through exposure to synthetic chemical compounds (or xenobiotics) with disastrous effects on female fertility (Hoyer and Sipes, 1996). Because of their nonrenewing nature, xenobiotics that destroy primordial follicles are particularly damaging to female fertility, causing permanent infertility and premature ovarian failure (Borgeest *et al.*, 2004; Borman *et al.*, 2000; Neal *et al.*, 2007).

Although the ovotoxic effects of many xenobiotics are well documented, the exact molecular mechanisms behind their action are only just being elucidated. One of the most extensively characterized mechanisms underpinning primordial follicle ovotoxicity has been explored using the xenobiotic 4-vinylcyclohexene diepoxide (VCD), an ovotoxic metabolite of 4-vinylcyclohexene used as a solvent for epoxides in industry (Hoyer and Sipes, 1996). Repeated dosing of VCD in rodents induces preantral follicle loss, specifically primordial and primary follicle destruction (Kao *et al.*, 1999). Molecular mechanistic studies demonstrate that VCD enhances follicular atresia in preantral follicles via the activation of Bcl-2 family of proto-oncogenes and proapoptotic members of the mitogen-activated protein kinase (MAPK) family (Hu *et al.*, 2001, 2002). VCD-induced primordial follicle ovotoxicity has also been shown to be nullified by phosphatidylinositol 3-kinase (PI3 kinase) inhibition *in vitro* (Keating *et al.*, 2009). As PI3 kinase is believed to be required for primordial follicle activation, growth, and survival, this suggests that VCD may induce primordial follicle loss through increased follicular activation (McLaughlin and McIver, 2009). Whereas increased follicle activation and atresia contributes to VCD-induced follicular atresia *in vitro*, the

entire *in vivo* mechanism underpinning VCD and other xenobiotic ovotoxicity remains unclear.

In addition to the molecular mechanisms behind xenobiotic ovotoxicity, the effects of xenobiotic exposure on long-term oocyte function have not been studied. One potential impact of xenobiotic exposure on egg and embryo quality is the induction of the formation of reactive oxygen species (ROS). Ovarian somatic and germ cells normally remove the harmful influence of xenobiotics through a three-tier enzymatic defence mechanism. The initial detoxification step involves the bioactivation of xenobiotic compounds into free radical intermediates by the cytochrome p450 family of oxidases (Danielson, 2002). Xenobiotic-enhanced ROS formation can occur in the oocyte via the unnatural uncoupling of the oxidative reaction or, if the hydroxylated metabolite forms a quinone, through redox cycling (Wells *et al*., 2009). In the mammalian ovary, cytochrome P450 enzyme mRNA levels rise in response to xenobiotic exposure (Cannady *et al*., 2003), potentially leading to disturbed oocyte redox potential, resulting in oxidative damage to cellular macromolecules and/or perturbed signal transduction (Tarin, 1996). In terms of oocyte functional competence, peroxidative damage to the oocyte plasma membrane lipids may also alter membrane fluidity and elasticity, inhibiting sperm/oocyte fusion and fertilization.

To better understand the mechanisms underpinning xenobiotic-induced ovotoxicity, we examined the effects of three ovotoxic environmental chemicals on ovarian follicle signaling pathways and oocyte dysfunction. In addition to VCD, the ovarian xenobiotics, methoxychlor (MXC), and menadione (MEN) were used in this study. MXC is a synthetic organochlorine insecticide that has been shown to directly induce follicular atresia *in vivo*, specifically targeting antral follicles in adult cycling female mice (Borgeest *et al*., 2004, 2002). Additionally, preliminary studies in our laboratory have also shown that MXC is capable of inducing primordial follicle loss in neonatal ovaries *in vitro*, possibly because of an independent mechanism of prepubertal ovotoxicity. The effects of the synthetic vitamin K quinone MEN on ovarian folliculogenesis have not been reported; however, pilot studies in our laboratory demonstrated that it is a potent ovotoxicant capable of inducing follicular atresia in neonatal ovaries cultured *in vitro*. Microarray, follicle counts, and immunohistological analysis revealed a consistent mechanism of primordial follicle activation in VCD and MXC ovotoxicity *in vitro* and all three xenobiotics *in vivo*. Sperm-oocyte fusion assays and lipid peroxidation analysis demonstrated that short-term xenobiotic exposure causes long-term oocyte dysfunction possibly because of xenobiotic ROS–induced oxidative stress.

MATERIALS AND METHODS

Reagents. Unless otherwise stated, chemicals, xenobiotics (> 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), mouse monoclonal anti-human p63 (anti-p63, ab3239), and rabbit polyclonal anti-cytochrome p450 2E1 (anti-Cyp2E1, ab73878) were obtained from Abcam (Cambridge, MA). Mouse monoclonal anti-8-oxoguanine (anti-8ox, MAB3560) was obtained from Chemicon (Billerica, MA). Alexa Fluor 594 goat anti-rabbit immunoglobulin G (IgG) (A11012), Alexa Fluor 594 goat anti-mouse IgG (A11005), 4,4-difluoro-5-(4-phenyl-1,3-buttadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid 581/591 C11 (BODIPY; D3861), fetal bovine serum, L-glutamine, and Insulin-Transferrin-Selenium (ITS) were purchased from the Invitrogen Co. (Carlsbad, CA). L-Ascorbic Acid was obtained from MP Biomedicals (Solon, OH) and 0.4-μm Culture Plate Inserts were purchased from Millipore (Billerica, MA). All culture dishes and cell culture plates were obtained from Greiner Bio-One (Monroe, NC). Oligo(dT)15 primer, RNasin, dNTPs, M-MLV-Reverse Transcriptase, RQ1 DNAse, GoTaq Felxi, $MgCl_2$, GoTaq quantitative PCR (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 21–22°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 (< 0.5 ml/kg/day dimethyl sulfoxide [DMSO]) or sesame oil containing a low and high dose of VCD (40 and 80 mg/kg/day), MXC (50 and 100 mg/kg/day), or MEN (7.5 and 15 mg/kg/day). The dosage, routes of administration, and dosing time courses were based on previous studies and were chosen with the intention of inducing partial ovotoxicity with minimal cytotoxicity (Borgeest *et al*., 2002; Cannady *et al*., 2003; Gupta *et al*., 2006; Radjendirane *et al*., 1998). Animals were observed daily for symptoms of toxicity and mortality. Half of the treated animals were culled by CO_2 asphyxiation 24 h after the last injection. The remaining animals were weaned and then superovulated at 6 weeks of age via ip injection of 10 IU of Folligon (equine chorionic gonadotropin; Intervet, Sydney, Australia) followed by ip administration of 10 IU of Chorulon (human chorionic gonadotrophin [hCG]; Intervet) 48 h later.

Ovarian culture. Ovaries from days 3–4 Swiss neonatal mice were cultured as described previously (Holt *et al*., 2006). Briefly, Swiss neonates were sacrificed by CO_2 inhalation followed by decapitation. Ovaries were excised, trimmed of excess tissue, and placed on culture plate inserts in six-well tissue culture plate wells floating atop 1.5 ml Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 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. Using fine forceps, 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% CO_2 in air, with media changes every 2 days. Ovaries were treated with vehicle control medium (0.1% DMSO), VCD (25μM), MXC (25μM), or MEN (5μM). Xenobiotic culture concentrations were determined by pilot studies performed in our laboratory with the intention of inducing overt toxicity.

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 fourth slide counterstained with hematoxylin and eosin.

Healthy oocyte–containing follicles were then counted in every hematoxylin- and eosin-stained section. Follicles with eosinophilic oocytes were not counted as they could not be definitively identified as follicles. Primordial follicles were classified as those with a single layer of squamous granulosa cells. Activating follicles were identified as those that contained one or more cuboidal granulosa cells in a single layer. Primary follicles were classified as those that 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 and active Casp3 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/tris-buffered saline (TBS) for 1.5 h at room temperature. The following solutions were diluted in TBS containing 1% BSA. Sections were incubated with anti-PCNA (1:80), anti-Casp2 (1:200), or anti-Casp3 (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 IgG and 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).

TUNEL 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, New South Wales, Australia), according to the manufacturer's instructions. Slides were then counterstained with DAPI for 5 min, mounted in Mowiol, and observed on an Axio Imager A1 fluorescent microscope (Carl Zeiss) under fluorescent optics and pictures taken using an Olympus DP70 microscope camera (Olympus).

Protein extraction and immunoblotting. Ovaries were solubilized with SDS lysis buffer and quantified aliquots separated by electrophoresis and transferred onto a nitrocellulose Hybond C-Extra membrane (Amersham) prior to blocking for 2 h in 5% skim milk powder in TBST (0.1% Tween-20) and then incubated in a 1:1000 dilution of anti-Cyp2E1 in 1% BSA/TBST overnight at 4°C. Following washing and incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz, sc-2004) at a 1:5000 dilution for 1 h at room temperature, proteins were visualized using an ECL Detection Kit (Amersham) according to manufacturer's instructions. The membrane was then stripped in 100mM β-mercaptoethanol, 2% SDS, and 62.5mM Tris (pH 6.7) at 60°C for 1 h and reprobed using a mouse monoclonal anti-α-tubulin (Sigma, T5168) as a loading control.

RNA extraction. Total RNA was isolated from ovaries using two rounds of a modified acid guanidinium thiocyanate-phenol-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 $2^{-\Delta\Delta C(t)}$, 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 5 μg) was isolated from xenobiotic-cultured neonatal ovaries and prepared for microarray analysis at the Australian Genome Research Facility (AGRF) using an Affymetrix Mouse Genome 430 2.0 Array platform. Labeling, hybridizing, washing, and array scanning were performed by the AGRF using the Affymetrix protocol on a GeneChip scanner 3000 (Affymetrix, Santa Clara, CA). All experiments were performed in triplicate with independently extracted RNAs. Data analysis and normalization were also performed by AGRF using the Robust Multichip Average method. Briefly, control (DMSO)-treated neonatal ovaries were used as a background to generate expression signal log ratios with basis 2 to determine fold changes (*n*-fold) between control- and xenobiotic-treated ovaries. Statistically significant genes with more than a twofold difference in gene expression determined through the use of a "volcano plot" were then analyzed using Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, Redwood City, CA) software to identify canonical signaling pathways influenced by xenobiotic exposure. The data discussed in this publication have been deposited in National Center for Biotechnology Information's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE23725 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23725).

Sperm-oocyte fusion assay. Adult mice (6 weeks) treated with xenobiotics over a 7-day period after birth were superovulated as described previously under animal dosing. Cumulus-intact oocytes were recovered 12–15 h after the final hCG injection by rupturing the oviductal ampullae of superovulated animals in M2 medium. Adherent cumulus cells were then dispersed by treating the collected oocytes with 300 IU/ml hyaluronidase solution and washing twice in M2 medium under oil. The zona pellucida was then removed from these oocytes by brief treatment with low-pH (2.5) acid Tyrode's solution and allowed to recover for at least 1 h at 37°C in an atmosphere of 5% CO_2 in air. Sperm were collected from mature male mice by dissecting the cauda epididymides and squeezing out the dense sperm mass along the tube. The sperm were then allowed to disperse into 800 μl M2 medium, diluted to a final concentration of 2×10^5 sperm per milliliter in M2 medium, and allowed to capacitate for 3 h at 37°C in 5% CO_2 in air. Following capacitation, zona-free oocytes were preloaded with DAPI for 15 min; 12–25 oocytes were then added to the sperm suspensions and coincubated for 15 min at 37°C in 5% CO_2 in air. Using serial aspiration through a finely drawn pipette, unbound and loosely adhered spermatozoa were removed from oocytes. Oocytes were then mounted on slides and the number of sperm bound to the oocyte membrane counted using phase contrast microscopy. Sperm-oocyte fusion was then assayed by counting the number of DAPI-stained sperm heads attached to the oocyte membrane using fluorescent microscopy.

Oocyte lipid peroxidation assay. Adult female mice (6–8 weeks) were superovulated, oocytes recovered, and adherent cumulus cells removed as described previously. Oocytes were then treated with vehicle control medium (0.1% DMSO) or xenobiotics (5, 15, and 25μM) for 2 h under oil at 37°C and 5% CO_2 in air. An oxidative stress–positive control was also carried out by exposing oocytes to 80mM H_2O_2 for 30 min under oil at 37°C and 5% CO_2 in air. Oocytes were then washed twice in M2 medium under oil and incubated in 10mM BODIPY stain for 30 min at 37°C in 5% CO_2 in air. The dye-loaded oocytes were then washed twice in M2 medium and mounted on slides. Green and red

fluorescence of BODIPY were determined using an LSM510 laser-scanning microscope (Carl Zeiss MicroImaging, Inc.) equipped with argon and helium/neon lasers at excitation wavelengths of 488 and 543 nm and emission spectra of 500–530 nm (green) and greater than 560 nm (red). Histogram analysis was then used to determine the ratio of green to red fluorescence.

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

Experimental design. A summary of the experimental design can be found in supplementary data (Supplementary fig. 1).

RESULTS

Influence of Xenobiotic Exposure on the Neonatal Ovary Transcriptome

All three xenobiotic treatments led to significant differences in ovarian gene expression (Fig. 1). Similarities between the neonatal ovarian responses to all three xenobiotics were assessed using Ingenuity Pathways comparison analysis software (IPA) (Fig. 1D; Supplementary table 1). Overall, only six genes were commonly regulated between the three xenobiotic-treated groups. However, 70 common genes were regulated in response to VCD and MXC exposure, making up 6% of the total number of genes affected by VCD and 16% of the total number of genes affected by MXC. This suggested an overlapping mechanism between VCD- and MXC-induced ovotoxicity.

Significantly altered genes were then categorized according to biological function (Table 1). In agreement with the current literature, xenobiotic exposure influenced a large number of genes involved in cell death. Interestingly, all three xenobiotics also influenced the expression of genes implicated in the cell cycle, cellular assembly and organization, development, and growth/proliferation. This indicated that the observed ovarian follicular response to these xenobiotics was not limited to follicular atresia but also involved a number of other biological processes.

VCD and MXC Exposure Upregulate Signaling Pathways Implicated in Follicular Development and Atresia

In order to confirm a multilayered mechanism of xenobiotic-induced preantral ovotoxicity involving various biological functions in neonatal ovaries, differentially expressed genes were analyzed for signaling pathways and molecular functions using IPA (Fig. 2; Supplementary Table 2). VCD exposure caused the significant upregulation of a large number of canonical signaling pathways associated with follicular development (vascular endothelial growth factor [VEGF], peroxisome proliferator-activated receptor alpha/retinoid X receptor alpha, Integrin, and insulin-like growth factor 1 signaling) and primordial follicle activation (Phosphatidylinositol 3-kinase/serine-threonine protein kinase [PI3K/Akt], mammalian target of rapamycin, and extracellular signal-regulated kinase/MAPK signaling) in contrast to a small number of pathways involved in follicular atresia (PTEN, protein 53 [p53], and myelocytomatosis oncogene (myc)-mediated apoptosis signaling). MXC exposure also resulted in

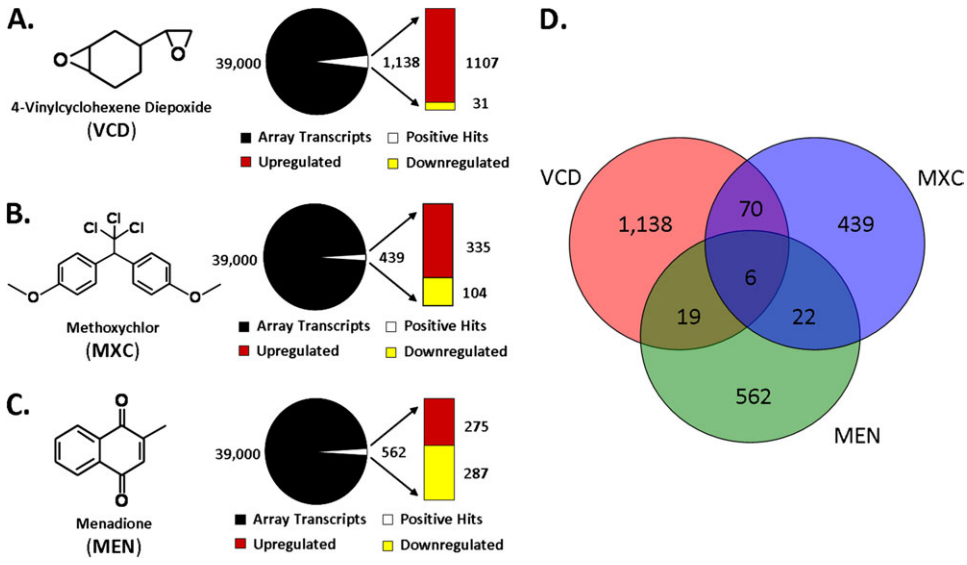


FIG. 1. Microarray analysis of DMSO-cultured ovaries versus xenobiotic-cultured ovaries. Ovaries were excised from neonatal mice (4 days old, $n = 15$) and cultured in xenobiotic-treated medium for 96 h, RNA extracted, and prepared for microarray analysis as described in the Materials and Methods section. (A–C) Summary of microarray results for each xenobiotic treatment. Total number of genes found on an Affymetrix Mouse Genome 430 2.0 Array platform are presented as nonregulated (black) and regulated (white) genes with a significant change in expression (> twofold change, $p < 0.05$). The top bar represents the number of positively regulated genes, and the bottom bar represents the number of negatively regulated genes in xenobiotic-cultured ovaries. (D) Venn diagrams of genes with significantly altered gene expression in xenobiotic-cultured ovaries. Between the VCD- and MXC-cultured ovaries, 70 common genes were regulated in response to xenobiotic exposure.

TABLE 1

Functional Classification of Genes that Were Upregulated or Downregulated by Xenobiotic Exposure in Cultured Neonatal Ovaries. Genes Were Analyzed Using IPA (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

	VCD		MXC		MEN	
Molecular and cellular function	Upregulated	Downregulated	Upregulated	Downregulated	Upregulated	Downregulated
Cell cycle	50	1	21	2	2	4
Cell death	129	4	33	12	20	19
Cell morphology	65	2	31	5	14	14
Cell signaling	5	1	11	5	12	5
Cell-to-cell signaling and interaction	40	—	18	11	27	15
Cellular assembly and organization	64	1	38	4	17	13
Cellular development	151	4	50	16	18	33
Cellular function and maintenance	43	3	28	6	19	14
Cellular growth and proliferation	129	4	13	18	12	25
DNA replication and repair	24	1	12	6	3	13
Gene expression	102	2	38	4	3	21

the significant upregulation of canonical signaling pathways associated with follicular development (retinoic acid receptor activation, VEGF, granulocyte-macrophage colony stimulating factor, Gα12/13, and aryl hydrocarbon receptor signaling), primordial follicle activation (PI3K/Akt signaling), and follicular atresia (PTEN, p53, myc-mediated apoptosis, and apoptosis signaling). However, unlike VCD and MXC, MEN only influenced two pathways implicated in follicular atresia (ataxia telangiectasia–mutated gene and β-alanine signaling) and a number of other pathways involved in the immune response and xenobiotic metabolism.

qPCR Validation of Microarray Results: VCD and MXC Upregulate Common Genes Involved in Folliculogenesis

Validation of the microarray results using qPCR confirmed the upregulation of all 10 selected genes in both VCD- and MXC-cultured ovaries (Table 2). Of these genes, two (*MAPK8* and *Bcl2l1*) had been associated with either VCD- or MXC-induced atresia, *Ccnd2* has been implicated in MXC ovotoxicity, and two were linked with the PTEN/PI3K/PDK1/Akt signaling pathway, which has been coupled with VCD-induced primordial follicle activation (*Akt1* and *Akt2*). The remaining five genes were novel and two have been implicated in apoptosis (*Med1* and *Cdkn2a*) and the other three in later follicular development (*Sox4*, *Dlg4* and *Ccnd2*). These results suggest that MXC and VCD have additional ovotoxicity mechanisms.

Effects of Xenobiotic Exposure on Primordial Follicle Activation and Follicular Atresia In Vitro

Neonatal ovaries cocultured with xenobiotics were probed for markers of primordial follicle activation and cell death. PCNA staining was detected in the granulosa cells and oocyte nuclei of primary and secondary follicles in vehicle control (DMSO)–cultured ovaries and was absent in primordial follicles, indicating that they were in their quiescent state (Fig. 3). However, PCNA staining was detected in both the granulosa cells and the oocytes of primordial follicles in all three xenobiotic-cultured ovaries, indicating a commitment to follicular development. In contrast to the vehicle control, both active Casp2 and Casp3 were detected in the majority of primary and secondary follicles in all three xenobiotic-cultured ovaries, with active Casp2 being localized to developing oocytes and active Casp3 to developing granulosa cells (Fig. 3). Interestingly, both activated Casp2 and Casp3 were not detected in any primordial follicles in the xenobiotic-cultured ovaries. This suggests that both the granulosa cells and the oocytes of developing preantral follicles had committed to apoptosis, but not primordial follicles. TUNEL staining was also detected in primary and secondary stage follicles in both VCD- and MXC-cultured ovaries but was detected in all follicle types in MEN-treated ovaries, indicating widespread DNA damage. These results indicate selective apoptosis in developing preantral follicles in both VCD- and MXC-cultured ovaries and widespread cell death in MEN-cultured ovaries.

Effects of Xenobiotic Exposure on Primordial Follicle Activation and Follicular Atresia In Vivo

Female Swiss neonatal mice were administered daily injections of either high- or low-dose xenobiotic for 7 days. PCNA was detected in large clusters of primordial follicles in all three high-dose xenobiotic treatments (Fig. 4), with a staining pattern similar to that observed *in vitro*. The follicular composition of ovaries from all three low-dose xenobiotic treatments revealed an observable reduction in the number of preantral follicles (Fig. 5A). The low-dose VCD treatment also induced a slight but significant reduction in the

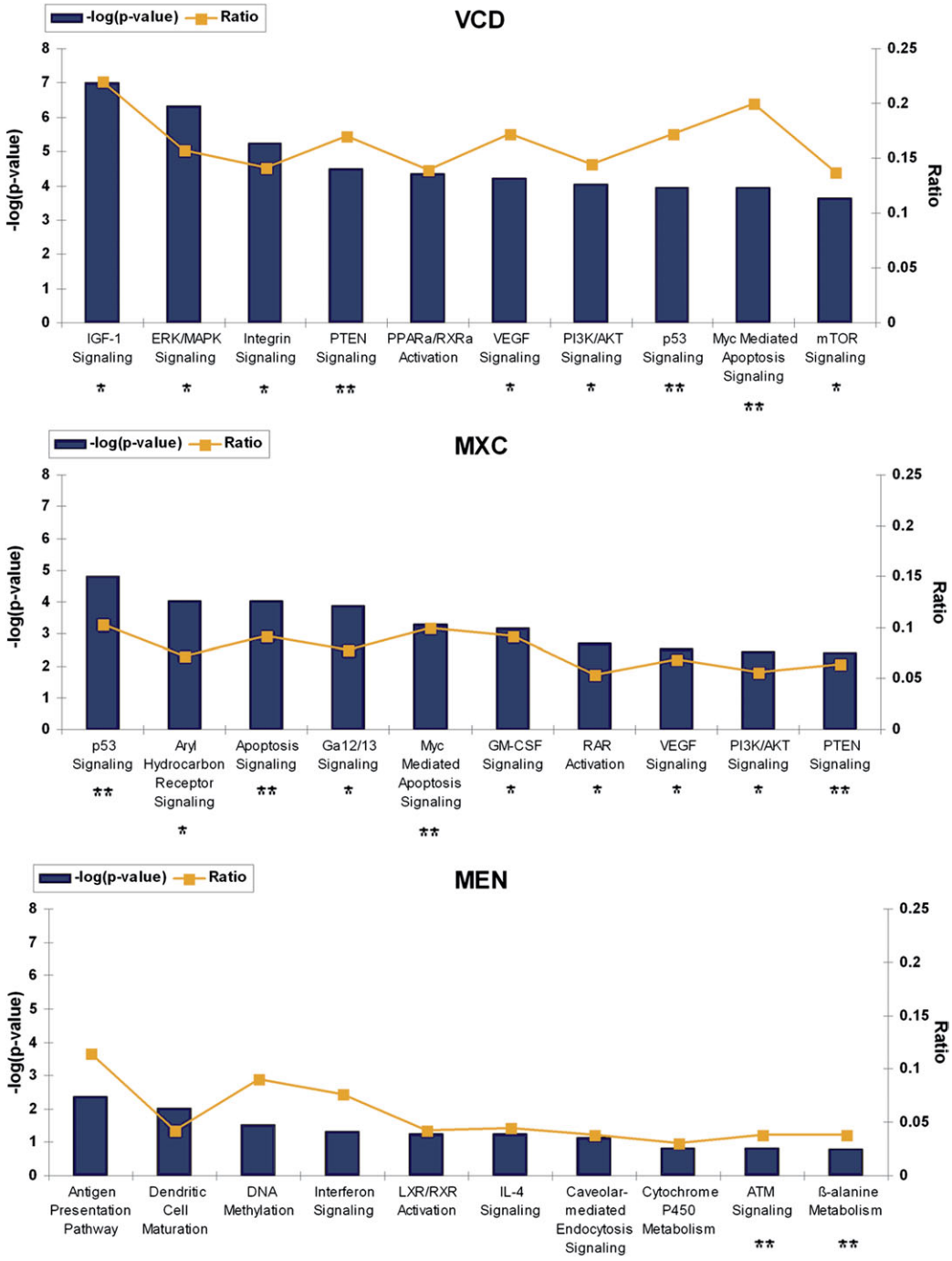


FIG. 2. Top canonical pathways that were significantly upregulated by xenobiotic-cultured neonatal ovaries as identified by IPA. The significance of the association between upregulated genes and the canonical pathway was evaluated using a right-tailed Fisher's exact test to calculate a *p* value determining the probability that the association is explained by chance alone (y-axis). Ratios referring to the proportion of upregulated genes from a pathway related to the total number of molecules that make up that particular pathway are also displayed (line graph, z-axis). Asterisks indicate pathways associated with follicular development, and double asterisks indicate pathways associated with follicular atresia.

number of primordial follicles, mirrored by a comparable increase in activating primordial follicles. Both the low-dose MXC and MEN treated ovaries also revealed a slight reduction in the number of secondary follicles, although this was not significant. Analysis indicated that the low-dose MEN treatment induced a significant decrease in follicular number (54% of the control). In agreement with the PCNA results, the follicular composition of ovaries from all three high-dose

TABLE 2

qPCR Validation of Microarray Results for Select Transcripts Upregulated by VCD- and MXC-Cultured Neonatal Ovaries. 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. Fold Change (Mean ± SE) and Summary of Function Relating to Folliculogenesis Are Included. All Fold Changes Were Statistically Significant ($p < 0.05$)

			Fold change	
Gene symbol	Gene name	Summary of function	VCD	MXC
Sox4	SRY-box–containing gene 4	Transcription factor; positive regulator of the Wnt receptor pathway implicated in folliculogenesis (Sinner *et al*., 2007)	5.34 ± 0.9	6.34 ± 0.5
Akt1	Thymoma viral proto-oncogene 1	Member of the PTEN/PI3K/PDK1/Akt signaling pathway implicated in follicle activation and granulosa cell proliferation (Brown *et al*., 2010; Reddy *et al*., 2009)	4.58 ± 0.4	4.54 ± 0.4
Akt2	Thymoma viral proto-oncogene 2	Implicated in primordial germ cell growth, cell death, and proliferation (Brown *et al*., 2010; Datta *et al*., 1999)	2.72 ± 0.2	1.73 ± 0.1
Dlg4	Discs, large homolog 4 (Drosophila)	Plasma membrane kinase; expressed in activated primordial follicle granulosa cells with a suspected role in regulating folliculogenesis (Huang *et al*., 2003)	2.85 ± 0.5	2.17 ± 0.3
Ccnd2	Cyclin D2	Essential for granulosa cell proliferation, decreased expression associated with MXC antral follicle ovotoxicity (Gupta *et al*., 2006; Muñiz *et al*., 2006)	4.25 ± 0.7	4.26 ± 0.5
Mapk8	Mitogen-activated protein kinase 8	Increased expression is associated with VCD-induced atresia (Hu *et al*., 2002)	2.02 ± 0.3	4.81 ± 1.3
Bcl2l1	Bcl-2-like 1	Associated with granulosa cell proliferation and survival; overexpression reduces MXC ovotoxic effect in antral follicles (Borgeest *et al*., 2004; Brown *et al*., 2010; Rucker *et al*., 2000)	3.22 ± 0.3	2.98 ± 0.7
Rarg	Retinoic acid receptor, gamma	Expressed in primary follicle granulosa cells; shown to transcriptionally inhibit FSH receptor transcription (Kascheike and Walther, 1997; Xing and Sairam, 2002)	2.40 ± 0.1	1.71 ± 0.1
Cdkn2a	Cyclin-dependent kinase inhibitor 2A	Increased expression associated with follicular atresia; decreased expression associated with primordial follicle activation (Bayrak and Oktay, 2003)	4.92 ± 0.6	2.89 ± 0.2
Med1	Mediator complex subunit 1	DNA repair gene involved in p53-dependent apoptosis (Frade *et al*., 2002)	2.99 ± 0.3	2.80 ± 0.2

Note. SRY, sex-determining region Y; Wnt, wingless; FSH, follicle-stimulating hormone.

xenobiotic treatments indicated a large significant reduction in primordial follicles, with a comparably large increase in the number of activating follicles (Fig. 5B). Additionally, the high-dose xenobiotic treatments also caused a similar decrease in the number of preantral follicles to that observed in the low-dose treatments. Individually, VCD caused no further changes in follicular composition, MXC caused a significant increase in the composition of primary (~fourfold increase) and secondary (~twofold increase) follicles, and MEN caused an observable increase in the composition of primary follicles (~twofold increase). Analysis of the average number of follicles per section demonstrated a significant decrease in follicular number for VCD (43% of the control), MXC (59% of the control), and MEN (53% of the control).

Short-Term Xenobiotic Exposure Reduces Long-Term Oocyte Fusibility In Vivo

Adult Swiss mice treated with either a low or a high dose of xenobiotics for a 7-day period after birth were superovulated and their oocytes tested in sperm-oocyte fusion assays. Oocytes from both low- and high-dose xenobiotic treatments exhibited severely reduced sperm-egg binding compared with the control, with both MXC and MEN oocytes exhibiting a dose-dependent response (Fig. 6B). Similarly, sperm-egg fusion was

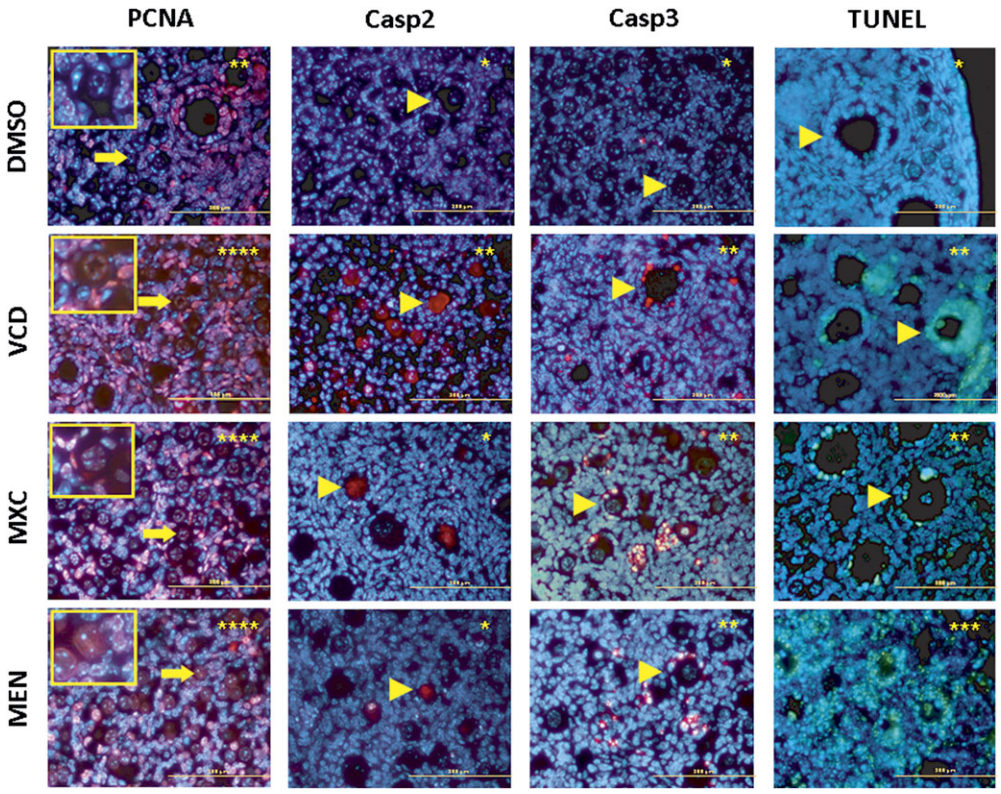


FIG. 3. Fluorescent immunohistological and TUNEL staining as visualized via fluorescent microscopy. Ovaries excised from neonatal mice (4 days old) were cultured in xenobiotic-treated medium for 96 h and processed for immunohistochemistry and TUNEL analysis as described in the Materials and Methods section. Ovarian sections were incubated with antibodies against PCNA, active caspase 2, and active caspase 3 or subjected to TUNEL analysis. 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%, and **** = 76–100%. Thin arrow, primordial follicle; arrow head, primary follicle; scale bar is equal to 100 μm.

reduced to ~20% of the control for all three low-dose xenobiotic treatments, with negligible levels of sperm-egg fusion being detected in the oocytes from high-dose exposure (Fig. 6C). These results are intriguing, as they indicate that neonatal exposure to low doses of xenobiotic, which do not cause drastic changes in follicular composition (see Fig. 5A), will significantly reduce oocyte function.

Xenobiotic Exposure Induces Oocyte Lipid Peroxidation In Vitro

To investigate the possibility of xenobiotic-induced oocyte dysfunction resulting from peroxidative damage to the oocyte plasma membrane, lipid peroxidation studies using the fluorescent dye BODIPY were performed on ovulated MII oocytes exposed to xenobiotics *in vitro*. BODIPY is a fluorescent fatty acid analog, which shifts its emission peak from 560 (red) to 500–530 nm (green) upon the oxidation of fatty acids. In these experiments, MXC was substituted for its more ovotoxic metabolite 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane, as MXC itself had no effect on lipid peroxidation (data not shown). All three xenobiotic treatments were capable of significantly inducing varying levels of lipid peroxidation *in vitro* (Fig. 7), and these results confirm the possibility of decreased sperm-egg binding/fusion observed *in vivo* being partially because of peroxidative damage to the oocyte plasma membrane.

DISCUSSION

Microarray analysis indicated a multilayered mechanism of xenobiotic-induced preantral ovotoxicity for both VCD and MEN involving follicular growth/development and atresia. Previous studies have also implicated increased follicular growth/development in VCD-induced ovotoxicity through PI3 kinase–dependent primordial follicle depletion in rodents (Hu *et al.*, 2006; Keating *et al.*, 2009). Indeed, in our study, VCD also upregulated the expression of genes involved in the PI3K/Akt signaling pathway, providing further support for this pathway in VCD-induced ovotoxicity. However, MXC has only been implicated in antral follicle atresia in adult rodents (Borgeest *et al.*, 2002, 2004) and the inhibition of antral follicle growth (Gupta *et al.*, 2009). Those experiments using adult cycling rodents are at odds with our unique findings of MXC upregulating pathways associated with follicular growth/development in neonatal ovaries. This difference may be because

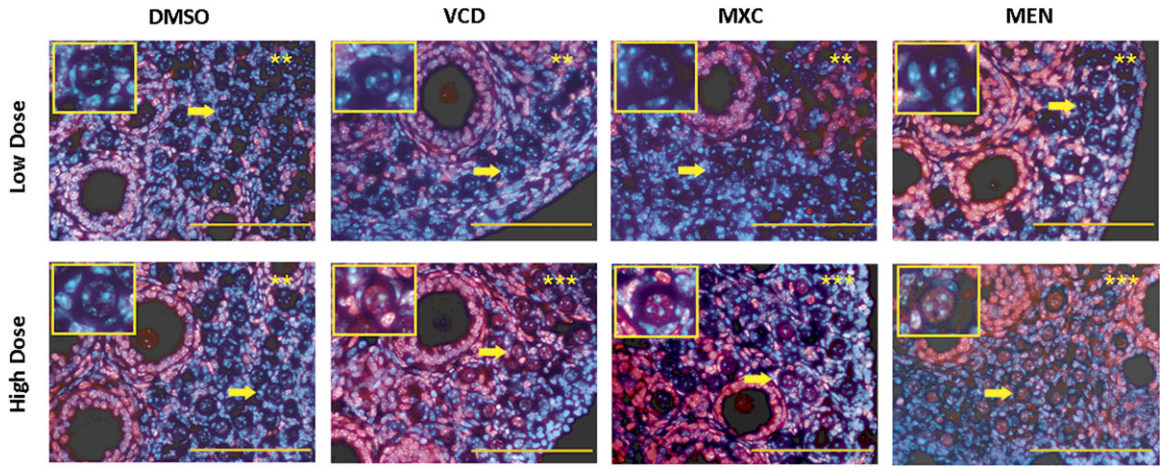


FIG. 4. Fluorescent immunolocalization of PCNA protein in low and high dose xenobiotic–treated ovaries *in vivo*. Neonatal mice (4 days old) were treated with either a low or a high dose of xenobiotics over a 7-day period, culled, and their ovaries extracted and processed for immunohistochemistry as outlined in the Materials and Methods section. The results presented here are representative of $n = 3$ experiments. The percentage of labeled follicles per section is represented by the following scale: * = < 10%, ** = 10–50%, *** = 51–75%, and **** = 76–100%. Thin arrow, primordial follicle; scale bar is equal to 100 μm.

of an independent mechanism of MXC-induced prepubertal ovotoxicity, as evidenced by age-dependent differences between the disposition and metabolism of xenobiotics.

In addition to the global similarities between VCD and MXC upregulated signaling pathways implicated in follicular growth/development and atresia, both xenobiotics influenced a number of common signaling pathways, suggesting an overlapping mechanism of ovotoxicity (Fig. 2). In further support of this hypothesis, qPCR analysis confirmed the upregulation of 10 genes in VCD- and MXC-cultured ovaries, five of which have not been previously associated with preantral ovotoxicity (Table 2). Two isoforms belonging to the Akt family of serine/threonine-directed kinases, *Akt1* and *Akt2*, were found to be upregulated by VCD and MXC. Both kinases have been

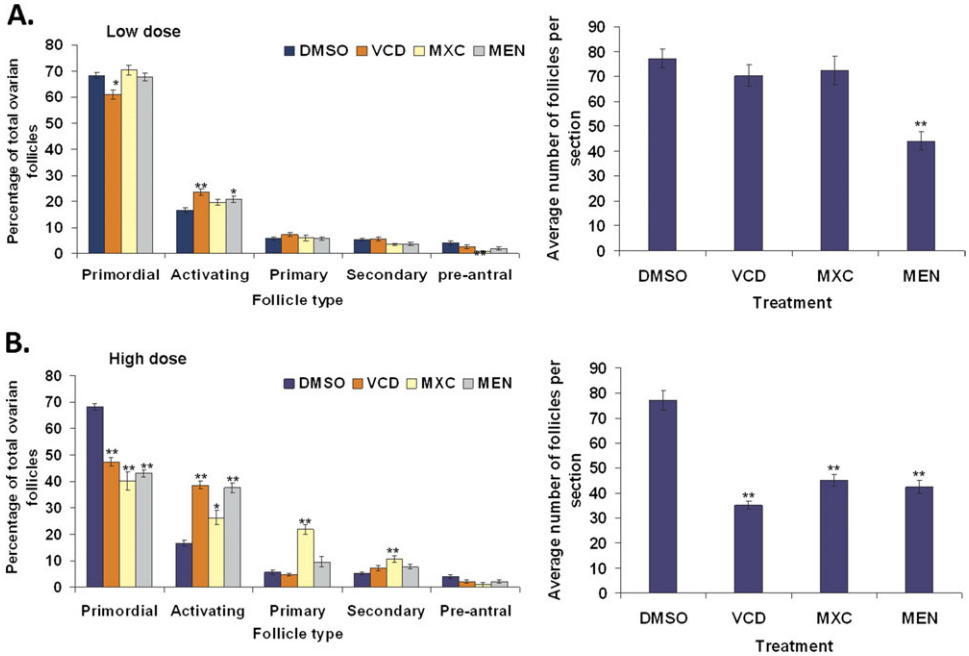


FIG. 5. Effect of xenobiotic exposure on ovarian follicle composition and number *in vivo*. Neonatal mice (4 days old) were treated with either a low or a high dose of xenobiotics over a 7-day period as described in the Materials and Methods section. Ovarian sections were stained with hematoxylin and eosin, and healthy oocyte–containing follicles were classified and counted under a microscope. (A) Low-dose ovarian follicle composition (left panel) and average number of follicles per counted section (right panel). (B) High-dose ovarian follicle composition (left panel) and average number of follicles per counted section (right panel). Values are mean ± SEM, $n = 3$–5 ovaries from three to five mice. The symbols * and ** represent $p < 0.05$ and $p < 0.01$, respectively, in comparison with DMSO control values.

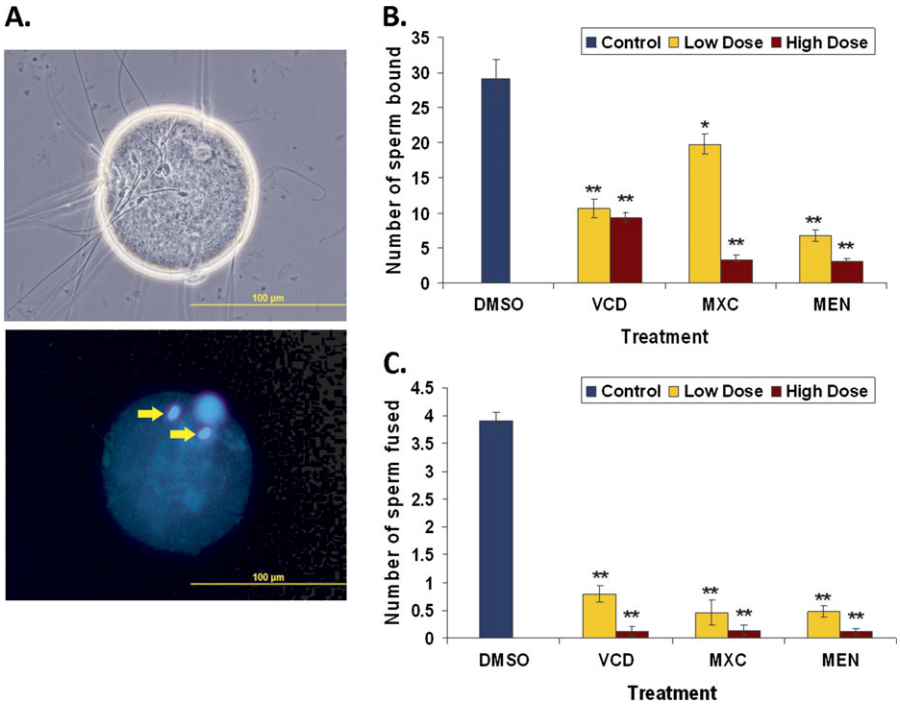


FIG. 6. Effect of neonatal xenobiotic exposure on long-term oocyte quality *in vivo*. Neonatal mice (4 days old) were treated with either a low or a high dose of xenobiotics over a 7-day period, weaned, and superovulated at 6 weeks of age. Collected zona-free oocytes were then coincubated with sperm and assessed for impaired sperm-egg binding and fusion as outlined in the Materials and Methods section. (A) Corresponding phase contrast and fluorescent microscopy images of control zona-free oocyte after sperm-egg binding assay. Arrows, fused sperm nuclei. Scale bar is equal to 100 µm. (B) Number of sperm heads bound to zona-free oocytes after coincubation. (C) Number of fused sperm observed after coincubation. Values are mean ± SE, $n = 12–25$ oocytes from three mice. The symbols * and ** represent $p < 0.05$ and $p < 0.01$, respectively, in comparison with DMSO control values.

implicated in the regulation of follicular development, with *Akt1* knockout mice displaying reduced fertility characterized by abnormal folliculogenesis and *Akt2* overexpression being associated with increased cell survival in the ovary (Brown *et al.*, 2010). *Akt1* has been shown to regulate folliculogenesis via the PI3K/Akt signaling pathway, which itself has been implicated in primordial follicle survival and activation (Reddy *et al.*, 2009).

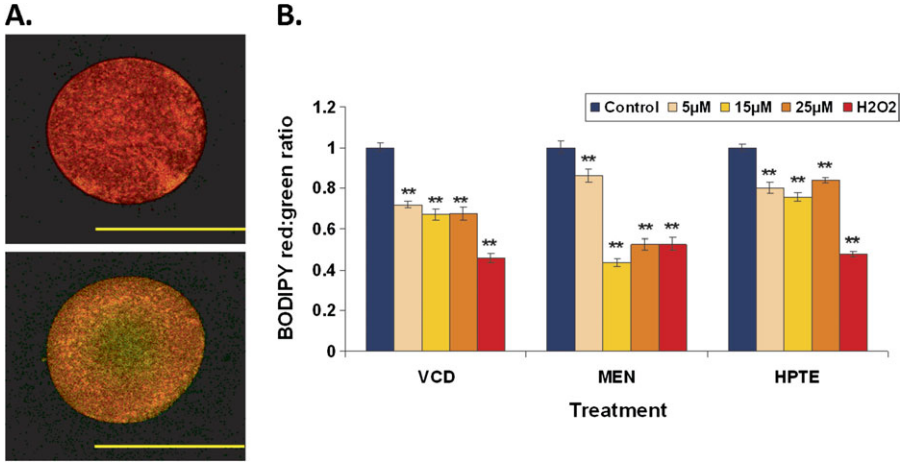


FIG. 7. Xenobiotic-induced oocyte membrane lipid peroxidation *in vitro*. Oocytes obtained from superovulated mice were cultured in a xenobiotic-treated medium for 2 h and labeled with BODIPY fluorescent dye as outlined in the Materials and Methods section. (A) Representative images of BODIPY-labeled DMSO-treated (top) and H_2O_2-treated (bottom) oocytes. Scale bar is equal to 100 µm. (B) Levels of lipid peroxidation as measured by the ratio of red:green fluorescence in varying concentrations of xenobiotics. Results were normalized to DMSO values for each experiment. H_2O_2 (8mM) was used as a representative of free radical ROS–induced lipid peroxidation. Values are mean ± SE, $n = 18–27$ oocytes from four mice. The symbols * and ** represent $p < 0.05$ and $p < 0.01$, respectively, in comparison with DMSO control values.

Another gene upregulated was *Sox4*, an important transcription factor of progenitor cell development and a regulator of the *Wnt* signaling pathway associated with follicular development (Boyer *et al*., 2009; Sinner *et al*., 2007). Interestingly, *Sox4* has also recently been found to transcriptionally activate growth factor receptors associated with PI3K/Akt signaling, making it a possible regulator of primordial follicle activation (Scharer *et al*., 2009). The identification of two members of the Akt family of protein kinases and a possible upstream regulator of the PI3K/Akt signaling pathway indicates an important mechanism of early preantral follicle ovotoxicity. In addition to genes associated with follicular development, VCD and MXC also upregulated the expression of *Mapk8*, and MAPK associated with induced atresia, and *Med1*, a DNA repair gene involved in p53-induced apoptosis (Frade *et al*., 2002; Hu *et al*., 2002).

Evidence of increased primordial follicle activation was found in VCD- and MXC-cultured ovaries by increased staining for PCNA in the granulosa cells and oocytes of primordial follicles (Chapman and Wolgemuth, 1994; Hutt *et al*., 2006). The markers of apoptosis, Casp2, Casp3, and TUNEL were all detected in small developing follicles from the primary stage onward. Studies involving mice deficient in Casp2 have identified the cysteine protease as a marker for cell death in the oocyte, whereas Casp3 is critical for granulosa cell apoptosis (Matikainen *et al*., 2001; Morita *et al*., 2001). Unfortunately, these results do not provide any insight into what cell type (granulosa cell or the oocyte) is targeted directly by VCD and MXC, as Casp2 and Casp3 were observed simultaneously in both primary and secondary follicles. The observed patterns of Casp2, Casp3, and TUNEL staining in VCD-treated ovaries have also been reported in similar studies using mice and rats, but the observed PCNA staining remains unique (Devine *et al*., 2002). Interestingly, these markers were not detected in the primordial follicles of VCD- and MXC-treated ovaries, indicating that these follicles were spared destruction.

Collectively, our results suggest a mechanism of VCD- and MXC-induced ovotoxicity involving small preantral follicular destruction and primordial follicle activation. The observed increase in primordial follicle activation may be because of a homeostatic mechanism of follicular replacement, maintaining the pool of developing follicles destroyed by xenobiotic exposure. Indeed, immunocontraception studies targeting zona proteins in developing follicles in the rabbit and marmoset primates are proposed to cause small preantral follicular destruction and primordial follicle activation through a similar mechanism (Paterson *et al*., 1992; Skinner *et al*., 1984). In further support for this hypothesis, *in vivo* experiments on xenobiotic-dosed neonatal mice also detected increased follicular activation and follicular depletion. This follicular replacement hypothesis has been previously suggested as a mode of VCD-induced primordial follicle depletion *in vitro*, but to our knowledge, this study is the first time that this hypothesis has been supported by microarray analysis and *in vivo* observations (Keating *et al*., 2009).

In addition to the follicular replacement hypothesis, follicle activation may be exacerbated by xenobiotic-induced oxidative stress. We have shown that xenobiotic exposure *in vitro* can cause oxidative stress and DNA damage, as evidenced by the detection of increased levels of 8-hydroxyguanine, a hydroxyl radical–induced DNA molecular lesion (Supplementary fig. 3) (Klaunig and Kamendulis, 2004). As recently reviewed by Wells *et al*. (2009), ROS play an important role in regulating signal transduction. Physiologically, ROS-mediated signal transduction occurs through H_2O_2, which selectively oxidizes cysteine residues on proteins resulting in a variety of reversible molecular interactions. We propose that increased levels of H_2O_2 caused by xenobiotic detoxification could lead to perturbed cysteine oxidation and upregulation of PI3K/Akt signaling through the inactivation of phosphatises, which negatively regulate the pathway via H_2O_2 oxidation (Kim *et al*., 2005; Naughton *et al*., 2009).

Unlike VCD and MXC, MEN upregulated pathways implicated in follicular atresia, the immune response, and xenobiotic metabolism. Although upregulation of apoptotic and xenobiotic-metabolizing pathways was expected, upregulation of immune response pathways such as the dendritic cell maturation pathway, the antigen presentation pathway, and interleukin-4 signaling was not. We propose that these pathways become activated because of oxidative stress following MEN quinone redox cycling (Cadenas and Davies, 2000; Kantengwa *et al*., 2003; Powis *et al*., 1981). MEN-induced ovotoxicity chiefly induces primordial and primary follicular atresia *in vitro* as indicated by increased Casp2 and Casp3 staining in developing follicles and widespread DNA damage in MEN-cultured neonatal ovaries (Fig. 3). Our *in vivo* follicular composition analysis of low-dose MEN supports these results; however, high-dose MEN *in vivo* analysis revealed increased follicular activation alongside increased small preantral follicular depletion. These results are intriguing and could be the result of differences between MEN metabolism in ovarian culture and *in vivo*. In the rodent liver, MEN is metabolized through a two-stage electron reduction into menadiol, which is then enzymatically conjugated to glucuronide and excreted from the body (Jarabak and Jarabak, 1995; Thompson *et al*., 1972). The resulting menadiol glucuronide conjugate is less reactive than MEN and therefore less toxic. The ovary is capable of limited detoxification, as xenobiotic detoxifying enzymes are increased (Supplementary fig. 2) (Cannady *et al*., 2003). However, as evidenced by the effect on follicular destruction in the low-dose treatments, it was still more potent than VCD and MXC.

Gamete interaction assays revealed severely reduced sperm-egg binding and fusion in a dose-dependent manner for all the xenobiotic treatments (Fig. 6). To our knowledge, this is the first evidence of short-term neonatal exposure to xenobiotics reducing fertilization at sexual maturity. Additionally, these results demonstrate that low-dose xenobiotic exposures, which do not profoundly affect the follicular population, can cause

oocyte dysfunction (Figs. 5 and 6). As sperm-egg fusion assays are an indicator of oocyte membrane fluidity, it was suggested that the reduced levels of sperm-egg binding and fusion were because of peroxidative damage to the oocyte plasma membrane. Oocyte lipid peroxidation is a physiological process that is thought to occur after the penetration of the membrane by an oxidizing spermatozoon, with the resulting change in plasma membrane fluidity acting as a block to polyspermy (Perry and Epel, 1985). We propose that xenobiotic-induced ROS initiate this process prematurely in the ovary, causing the observed impaired sperm-egg binding/fusion. To confirm xenobiotic exposure could lead to oocyte lipid peroxidation, levels of lipid peroxidation were measured in xenobiotic-cultured MII oocytes. All xenobiotics or their metabolites (Miller *et al*., 2006) induced lipid peroxidation (Diaz *et al*., 2007; Swain and Smith, 2007). These results support the view that xenobiotic exposure can cause oocyte dysfunction by altering the fluidity of the oocyte plasma membrane, thus impairing fertilization.

These findings challenge the established dogma of preantral ovotoxicity, which suggests that a xenobiotic is only harmful at concentrations where it induces follicular loss and that once it is removed from the immediate environment female fertility can revert to normal. Our data indicate that even a short-term, low-dose xenobiotic exposure that does not induce follicular atresia can cause lasting effects on oocyte quality in the form of xenobiotic ROS–induced oocyte dysfunction, even after it is removed from the environment. Additionally, these results also raise the possibility of using low-dose xenobiotic exposures to study the effects of mitochondrial loss in the ageing oocyte, a postulated cause of poor fertility in older women (Tarin, 1996)

In summary, our results support a mechanism of preantral ovotoxicity involving the homeostatic recruitment of primordial follicles to maintain the pool of developing follicles destroyed by xenobiotic exposure. These results also provide evidence of an overlapping mechanism of preantral ovotoxicity between VCD and MXC demonstrated by similarities in gene expression and affect on folliculogenesis *in vitro* and *in vivo*. To our knowledge, these studies also demonstrate for the first time that short-term xenobiotic exposure can cause long-term oocyte dysfunction, even at levels which do not have an adverse affect on follicular composition.

SUPPLEMENTARY DATA

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

FUNDING

Australian Research Council; Hunter Medical Research Institute; Newcastle Permanent Building Society Charitable Trust to E.A.M.; National Health and Medical Research Council (Project grant #510735) to E.A.M., S.D.R., B.N.

ACKNOWLEDGMENTS

A.P.S. is the recipient of a Australian Postgraduate Award PhD scholarship. The authors would also like to thank Skye Courtney McIver for her technical assistance.

REFERENCES

Bayrak, A., and Oktay, K. (2003). The expression of cyclin-dependent kinase inhibitors p 15, p 16, p 21, and p 27 during ovarian follicle growth initiation in the mouse. *Reprod. Biol. Endocrinol.* **1,** 41.

Borgeest, C., Miller, K. P., Gupta, R., Greenfeld, C., Hruska, K. S., Hoyer, P., and Flaws, J. A. (2004). Methoxychlor-induced atresia in the mouse involves Bcl-2 family members, but not gonadotropins or estradiol. *Biol. Reprod.* **70,** 1828–1835.

Borgeest, C., Symonds, D., Mayer, L. P., Hoyer, P. B., and Flaws, J. A. (2002). Methoxychlor may cause ovarian follicular atresia and proliferation of the ovarian epithelium in the mouse. *Toxicol. Sci.* **68,** 473–478.

Borman, S. M., Christian, P. J., Sipes, I. G., and Hoyer, P. B. (2000). Ovotoxicity in female Fischer rats and B6 mice induced by low-dose exposure to three polycyclic aromatic hydrocarbons: comparison through calculation of an ovotoxic index. *Toxicol. Appl. Pharmacol.* **167,** 191–198.

Boyer, A., Goff, A. K., and Boerboom, D. (2009). WNT signaling in ovarian follicle biology and tumorigenesis. *Trends Endocrinol. Metab.* **21,** 25–32.

Brown, C., LaRocca, J., Pietruska, J., Ota, M., Anderson, L., Duncan Smith, S., Weston, P., Rasoulpour, T., and Hixon, M. L. (2010). Subfertility caused by altered follicular development and oocyte growth in female mice lacking PKBalpha/Akt1. *Biol. Reprod.* **82,** 246–256.

Cadenas, E., and Davies, K. J. A. (2000). Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic. Biol. Med.* **29,** 222–230.

Cannady, E. A., Dyer, C. A., Christian, P. J., Sipes, I. G., and Hoyer, P. B. (2003). Expression and activity of cytochromes P450 2E1, 2A, and 2B in the mouse ovary: the effect of 4-vinylcyclohexene and its diepoxide metabolite. *Toxicol. Sci.* **73,** 423–430.

Chapman, D. L., and Wolgemuth, D. J. (1994). Expression of proliferating cell nuclear antigen in the mouse germ line and surrounding somatic cells suggests both proliferation-dependent and -independent modes of function. *Int. J. Dev. Biol.* **38,** 491–497.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform. *Anal. Biochem.* **162,** 156–159.

Danielson, P. B. (2002). The cytochrome P450 superfamily: biochemistry, evolution and drug metabolism in humans. *Curr. Drug Metab.* **3,** 561–597.

Datta, S. R., Brunet, A., and Greenberg, M. E. (1999). Cellular survival: a play in three Akts. *Genes Dev.* **13,** 2905–2927.

Devine, P. J., Sipes, I. G., Skinner, M. K., and Hoyer, P. B. (2002). Characterization of a rat in vitro ovarian culture system to study the ovarian toxicant 4-vinylcyclohexene diepoxide. *Toxicol. Appl. Pharmacol.* **184,** 107–115.

Diaz, F. J., Wigglesworth, K., and Eppig, J. J. (2007). Oocytes are required for the preantral granulosa cell to cumulus cell transition in mice. *Dev. Biol.* **305,** 300–311.

Frade, R., Balbo, M., and Barel, M. (2002). RB18A regulates p53-dependent apoptosis. *Oncogene* **21,** 861–866.

Gupta, R. K., Meachum, S., Hernández-Ochoa, I., Peretz, J., Yao, H. H., and Flaws, J. A. (2009). Methoxychlor inhibits growth of antral follicles by altering cell cycle regulators. *Toxicol. Appl. Pharmacol.* **240,** 1–7.

Gupta, R. K., Schuh, R. A., Fiskum, G., and Flaws, J. A. (2006). Methoxychlor causes mitochondrial dysfunction and oxidative damage in the mouse ovary. *Toxicol. Appl. Pharmacol.* **216,** 436–445.

Hirshfield, A. N. (1991). Development of follicles in the mammalian ovary. *Int. Rev. Cytol. Surv. Cell Biol.* **124,** 43–101.

Holt, J. E., Jackson, A., Roman, S. D., Aitken, R. J., Koopman, P., and McLaughlin, E. A. (2006). CXCR4/SDF1 interaction inhibits the primordial to primary follicle transition in the neonatal mouse ovary. *Dev. Biol.* **293,** 449–460.

Hoyer, P. B., and Sipes, I. G. (1996). Assessment of follicle destruction in chemical-induced ovarian toxicity. *Annu. Rev. Pharmacol. Toxicol.* **36,** 307–331.

Hu, X., Christian, P., Sipes, I. G., and Hoyer, P. B. (2001). Expression and redistribution of cellular Bad, Bax, and Bcl-xL protein is associated with VCD-induced ovotoxicity in rats. *Biol. Reprod.* **65,** 1489–1495.

Hu, X., Flaws, J. A., Sipes, I. G., and Hoyer, P. B. (2002). Activation of mitogen-activated protein kinases and AP-1 transcription factor in ovotoxicity induced by 4-vinylcyclohexene diepoxide in rats. *Biol. Reprod.* **67,** 718–724.

Hu, X., Roberts, J. R., Apopa, P. L., Kan, Y. W., and Ma, Q. (2006). Accelerated ovarian failure induced by 4-vinyl cyclohexene diepoxide in Nrf2 null mice. *Mol. Cell. Biol.* **26,** 940–954.

Huang, J. H. Y., Rajkovic, A., Szafranski, P., Ochsner, S., Richards, J. A., and Goode, S. (2003). Expression of Drosophila neoplastic tumor suppressor genes discslarge, scribble, and lethal giant larvae in the mammalian ovary. *Gene Expr. Patterns.* **3,** 3–11.

Hutt, K. J., McLaughlin, E. A., and Holland, M. K. (2006). Primordial follicle activation and follicular development in the juvenile rabbit ovary. *Cell Tissue Res.* **326,** 809–822.

Jarabak, R., and Jarabak, J. (1995). Effect of ascorbate on the DT-diaphorase-mediated redox cycling of 2-methyl-1, 4-naphthoquinone. *Arch. Biochem. Biophys.* **318,** 418–423.

Kantengwa, S., Jornot, L., Devenoges, C., and Nicod, L. P. (2003). Superoxide anions induce the maturation of human dendritic cells. *Am. J. Respir. Critic. Care Med.* **167,** 431–437.

Kao, S. W., Sipes, I. G., and Hoyer, P. B. (1999). Early effects of ovotoxicity induced by 4-vinylcyclohexene diepoxide in rats and mice. *Reprod. Toxicol.* **13,** 67–75.

Kascheike, B., and Walther, N. (1997). Alterations in the chromatin structure of the distal promoter region of the bovine oxytocin gene correlate with ovarian expression. *DNA Cell Biol.* **16,** 1237–1248.

Keating, A. F., Mark, C. J., Sen, N., Sipes, I. G., and Hoyer, P. B. (2009). Effect of phosphatidylinositol-3 kinase inhibition on ovotoxicity caused by 4-vinylcyclohexene diepoxide and 7, 12-dimethylbenz [a] anthracene in neonatal rat ovaries. *Toxicol. Appl. Pharmacol.* **241,** 127–134.

Kim, J. H., Chu, S. C., Gramlich, J. L., Pride, Y. B., Babendreier, E., Chauhan, D., Salgia, R., Podar, K., Griffin, J. D., and Sattler, M. (2005). Activation of the PI3K/mTOR pathway by BCR-ABL contributes to increased production of reactive oxygen species. *Blood* **105,** 1717–1723.

Klaunig, J. E., and Kamendulis, L. M. (2004). The role of oxidative stress in carcinogenesis. *Annu. Rev. Pharmacol. Toxicol.* **44,** 239–267.

Matikainen, T., Perez, G. I., Zheng, T. S., Kluzak, T. R., Rueda, B. R., Flavell, R. A., and Tilly, J. L. (2001). Caspase-3 gene knockout defines cell lineage specificity for programmed cell death signaling in the ovary. *Endocrinology* **142,** 2468–2480.

McGee, E. A., and Hsueh, A. J. W. (2000). Initial and cyclic recruitment of ovarian follicles. *Endocr. Rev.* **21,** 200–214.

McLaughlin, E. A., and McIver, S. C. (2009). Awakening the oocyte: controlling primordial follicle development. *Reproduction* **137,** 1–11.

Miller, K. P., Gupta, R. K., and Flaws, J. A. (2006). Methoxychlor metabolites may cause ovarian toxicity through estrogen-regulated pathways. *Toxicol. Sci.* **93,** 180–188.

Morita, Y., Maravei, D. V., Bergeron, L., Wang, S., Perez, G. I., Tsutsumi, O., Taketani, Y., Asano, M., Horai, R., and Korsmeyer, S. J. (2001). Caspase-2 deficiency rescues female germ cells from death due to cytokine insufficiency but not meiotic defects caused by ataxia telangiectasia-mutated (Atm) gene inactivation. *Cell Death Differ.* **8,** 614–620.

Muñiz, L. C., Yehia, G., Mémin, E., Ratnakar, P., and Molina, C. A. (2006). Transcriptional regulation of cyclin D2 by the PKA pathway and inducible cAMP early repressor in granulosa cells. *Biol. Reprod.* **75,** 279–288.

Naughton, R., Quiney, C., Turner, S. D., and Cotter, T. G. (2009). Bcr-Abl-mediated redox regulation of the PI3K/AKT pathway. *Leukemia* **23,** 1432–1440.

Neal, M. S., Zhu, J., Holloway, A. C., and Foster, W. G. (2007). Follicle growth is inhibited by benzo-[a]-pyrene, at concentrations representative of human exposure, in an isolated rat follicle culture assay. *Hum. Reprod.* **22,** 961–967.

Paterson, M., Koothan, P. T., Morris, K. D., O'Byrne, K. T., Braude, P., Williams, A., and Aitken, R. J. (1992). Analysis of the contraceptive potential of antibodies against native and deglycosylated porcine ZP3 in vivo and in vitro. *Biol. Reprod.* **46,** 523–534.

Perry, G., and Epel, D. (1985). Fertilization stimulates lipid peroxidation in the sea urchin egg* 1. *Dev. Biol.* **107,** 58–65.

Powis, G., Svingen, B. A., and Appel, P. (1981). Quinone-stimulated superoxide formation by subcellular fractions, isolated hepatocytes, and other cells. *Mol. Pharmacol.* **20,** 387–394.

Radjendirane, V., Joseph, P., Lee, Y. H., Kimura, S., Klein-Szanto, A. J. P., Gonzalez, F. J., and Jaiswal, A. K. (1998). Disruption of the DT diaphorase (NQO1) gene in mice leads to increased menadione toxicity. *J. Biol. Chem.* **273,** 7382–7389.

Reddy, P., Adhikari, D., Zheng, W., Liang, S., Hamalainen, T., Tohonen, V., Ogawa, W., Noda, T., Volarevic, S., and Huhtaniemi, I. (2009). PDK1 signaling in oocytes controls reproductive aging and lifespan by manipulating the survival of primordial follicles. *Hum. Mol. Genet.* **18,** 2813–2824.

Rucker, E. B., III., Dierisseau, P., Wagner, K. U., Garrett, L., Wynshaw-Boris, A., Flaws, J. A., and Hennighausen, L. (2000). Bcl-x and Bax regulate mouse primordial germ cell survival and apoptosis during embryogenesis. *Mol. Endocrinol.* **14,** 1038–1052.

Scharer, C. D., McCabe, C. D., Ali-Seyed, M., Berger, M. F., Bulyk, M. L., and Moreno, C. S. (2009). Genome-wide promoter analysis of the SOX4 transcriptional network in prostate cancer cells. *Cancer Res.* **69,** 709–717.

Sinner, D., Kordich, J. J., Spence, J. R., Opoka, R., Rankin, S., Lin, S. C. J., Jonatan, D., Zorn, A. M., and Wells, J. M. (2007). Sox17 and Sox4 differentially regulate {beta}-catenin/T-cell factor activity and proliferation of colon carcinoma cells. *Mol. Cell. Biol.* **27,** 7802–7815.

Skinner, S. M., Mills, T., Kirchick, H. J., and Dunbar, B. S. (1984). Immunization with zona pellucida proteins results in abnormal ovarian follicular differentiation and inhibition of gonadotropin-induced steroid secretion. *Endocrinology* **115,** 2418–2432.

Swain, J. E., and Smith, G. D. (2007). Reversible phosphorylation and regulation of mammalian oocyte meiotic chromatin remodeling and segregation. *Soc. Reprod. Fertil. Suppl.* **63,** 343–358.

Tarin, J. J. (1996). Potential effects of age-associated oxidative stress on mammalian oocytes/embryos. *Mol. Hum. Reprod.* **2,** 717–724.

Thompson, R. M., Gerber, N., Seibert, R. A., and Desiderio, D. M. (1972). Identification of 2-methyl-1, 4-naphthohydroguinone monoglucuronide as a metabolite of 2-methyl-1, 4-naphthoquinone (menadione) in rat bile. *Res. Commun. Chem. Pathol. Pharmacol.* **4,** 543–552.

Wells, P. G., McCallum, G. P., Chen, C. S., Henderson, J. T., Lee, C. J. J., Perstin, J., Preston, T. J., Wiley, M. J., and Wong, A. W. (2009). Oxidative stress in developmental origins of disease: teratogenesis, neurodevelopmental deficits, and cancer. *Toxicol. Sci.* **108,** 4–18.

Xing, W., and Sairam, M. R. (2002). Retinoic acid mediates transcriptional repression of ovine follicle-stimulating hormone receptor gene via a pleiotropic nuclear receptor response element. *Biol. Reprod.* **67,** 204–211.

Chapter 2: Understanding the Villain: DMBA-Induced Preantral Ovotoxicity Involves Selective Follicular Destruction and Primordial Follicle Activation through PI3K/Akt and mTOR Signaling

TOXICOLOGICAL SCIENCES **123(2)**, 563–575 (2011)
doi:10.1093/toxsci/kfr195
Advance Access publication July 23, 2011

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

Alexander P. Sobinoff,* Michelle Mahony,* Brett Nixon,*,† Shaun D. Roman,*,† and Eileen A. McLaughlin,*,†,[1]

*Reproductive Science Group, School of Environmental and Life Sciences and †Australian Research Council Centre of Excellence in Biotechnology and Development, University of Newcastle, Callaghan, New South Wales 2308, Australia

[1]To whom correspondence should be addressed at Discipline of Biological Sciences, Australian Research Council Centre of Excellence in Biotechnology and Development, School of Environmental and Life Sciences, University of Newcastle, Callaghan, New South Wales 2308, Australia. Fax: +61-2-4921-6308. E-mail: eileen.mclaughlin@newcastle.edu.au.

Received May 31, 2011; accepted July 13, 2011

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 xenobiotic perturbed developmental process not directly associated with follicular destruction is intriguing and has led to new approaches in studying xenobiotic-induced 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 automotive exhaust (Gelboin, 1980). In addition to its carcinogenic nature, DMBA has also been shown to disrupt folliculogenesis, causing the depletion of all follicle populations leading to POF (Mattison and Schulman,

1980). 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 Akt1 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 Anti-mullerian hormone (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, L-glutamine, and insulin-transferrin-selenium (ITS) were purchased from the Invitrogen Co. (Carlsbad, CA). L-ascorbic acid was obtained from MP Biomedicals (Solon, OH). Rabbit polyclonal anti-phospho-mTOR (phosphor S2448) (anti-pmTOR (S2448), 09-213SP), Rabbit polyclonal anti-phospho-mTOR (phosphor T2446) (anti-pmTOR (T2446), 09-345SP), and 0.4 μm culture plate inserts were purchased from Millipore (Billerica, MA). All culture dishes and cell culture plates were obtained from Greiner Bio-One (Monroe, NC). Oligo(dT)15 primer, RNasin, dNTPs, M-MLV-Reverse Transcriptase, RQ1 DNase, GoTaq Felxi, $MgCl_2$, GoTaq 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 CO_2 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 CO_2 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% CO_2 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/Tris 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

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 a 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-phenol-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 $2^{-\Delta\Delta C(t)}$, 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 ($p < 0.5$) 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%BC%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.

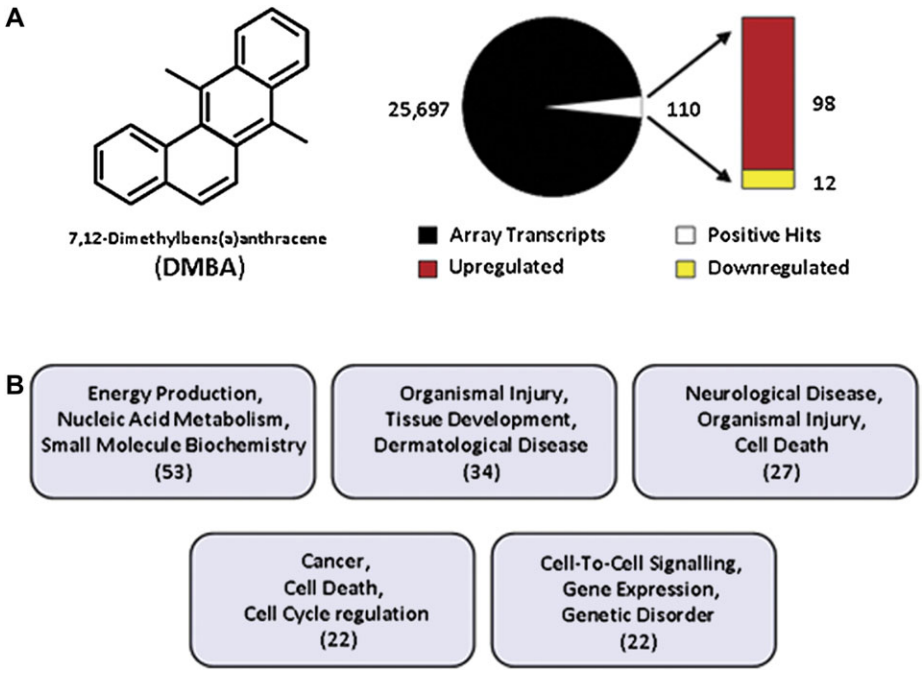


FIG. 1. Microarray analysis of control-cultured ovaries verses DMBA-cultured ovaries. Ovaries were excised from neonatal mice (4 days old, $n = 15$) and cultured in xenobiotic-treated medium for 96 h RNA extracted and subjected to microarray analysis as described in the ''Materials and Methods'' section. (A) Summary of microarray results. Total number of genes found on an Illumina Sentrix Mouse ref8v2 Beadchip are presented as nonregulated (black) and regulated (white) genes with a significant change in expression (> twofold change, $p < 0.05$). The red bar represents the number of positively regulated genes and the yellow bar represents the number of negatively regulated genes in xenobiotic-cultured ovaries. (B) Molecular networks of significantly altered genes influenced by DMBA exposure. Significantly altered genes were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base (Ingenuity Systems). Networks of significantly altered genes were then algorithmically generated based on their connectivity. The networks are ranked according to their scores, and the five highest-ranking networks of genes are displayed. The numbers of genes in each network are shown in brackets. *Note*. See online version for color version.

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 1
Functional Classification of Genes That Were Upregulated or Downregulated by DMBA Exposure in Cultured Neonatal Ovaries

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

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

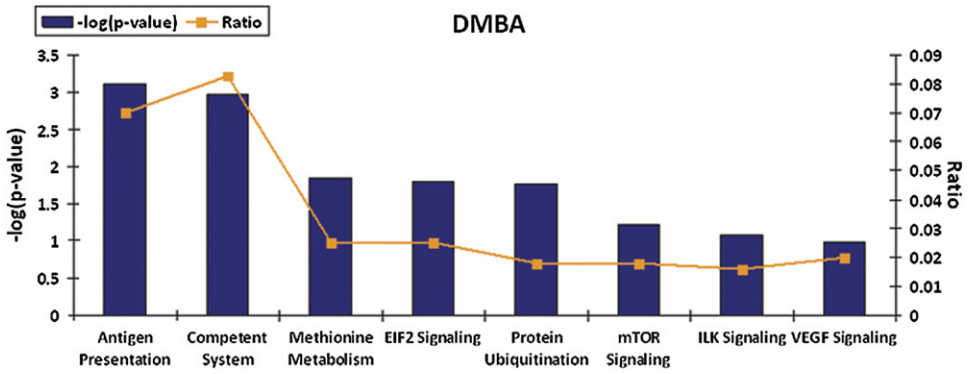


FIG. 2. Top canonical pathways that were significantly upregulated by DMBA-cultured neonatal ovaries as identified by IPA. The significance of the association between upregulated genes and the canonical pathway was evaluated using a right-tailed Fisher's exact test to calculate a *p* value determining the probability that the association is explained by chance alone (blue bars, y-axis). Ratios referring to the proportion of upregulated genes from a pathway related to the total number of molecules that make up that particular pathway are also displayed (line graph, z-axis). *Note*. See online version for color version.

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

TABLE 2
qPCR Validation of Microarray Results for Select Transcripts Upregulated by DMBA-Cultured Neonatal Ovaries

Gene symbol	Gene name	Summary of function	Fold change
Cdkn1a	Cyclin-dependent kinase inhibitor 1A	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)	3.77 ± 0.3
Anapc5	Anaphase-promoting complex subunit 5	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; Koloteva-Levine *et al*., 2004)	2.03 ± 0.2
Hspa8	Heat shock protein 8	Stress-related chaperone; regulates cyclin D1 and cyclin D1-dependent protein kinase accumulation (Diehl *et al*., 2003)	1.93 ± 0.2
Dnajb6	DnaJ (Hsp40) homolog, subfamily B, member 6	Stress-related chaperone; regulates gene expression in response to stress through Class II Histone Deacetylase Recruitment (Dai *et al*., 2005)	2.85 ± 0.5
Ddx5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	RNA helicase; stimulates cell proliferation, coactivator of p53 signaling, overexpression associated with estrogen receptor alpha tumorigenesis (Nicol and Fuller-Pace, 2010)	1.71 ± 0.1
Srsf5	Serine/arginine-rich splicing factor 5	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)	1.85 ± 0.3
Foxn3	Forkhead box N3	Transcription factor; regulator of cell cycle progression at G2 phase, implicated in preventing tumorigenesis (Scott and Plon, 2005)	1.48 ± 0.2
Calr	Calreticulin	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)	1.41 ± 0.1
Gbas	Glioblastoma amplified sequence	Mitochondrial protein; involved in mitochondrial oxidative phosphorylation, expressed in 40% of glioblastomas (Martherus and Sluiter, 2010; Wang *et al*., 1998)	1.68 ± 0.2
Ccnd1	Cyclin D1	Promotes cell cycle progression from G1 to S phase; overexpression associated with breast/ovarian cancer (Hashimoto *et al*., 2011; Robker and Richards, 1998)	5.82 ± 0.5

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.

(Fig. 1; Table 1). 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.

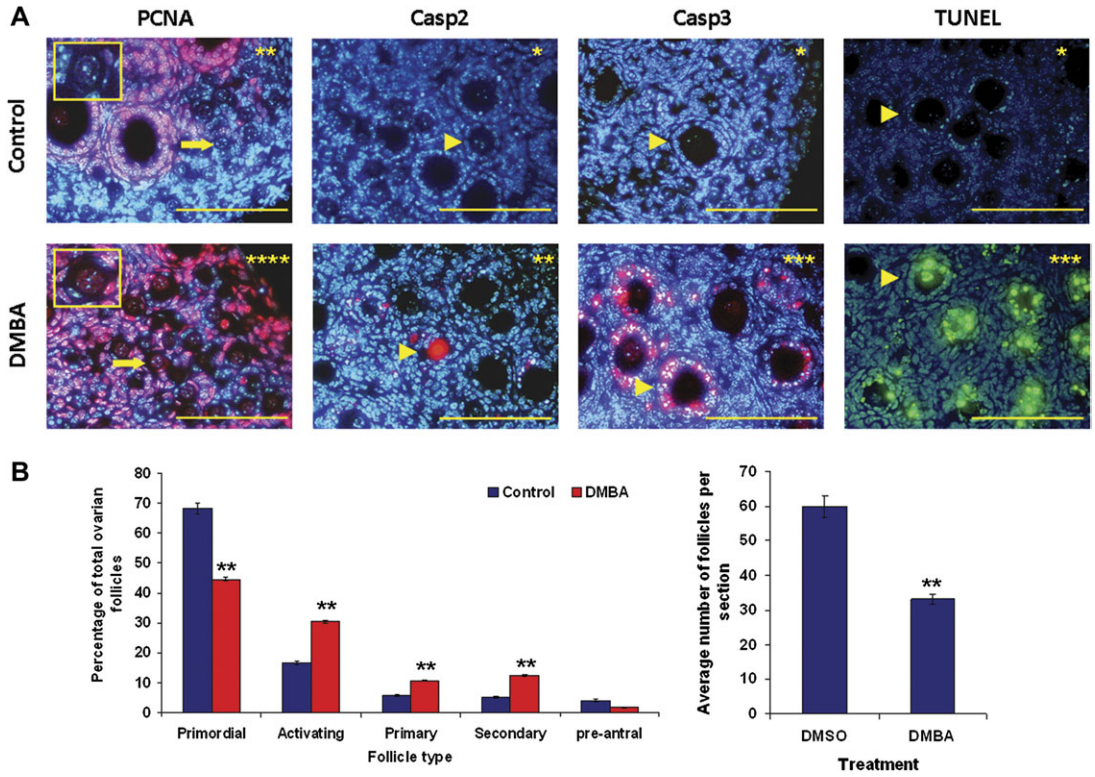


FIG. 3. DMBA exposure causes immature follicular destruction and primordial follicle activation *in vitro* and *in vivo*. (A) Fluorescent immunohistological and TUNEL staining as visualized via fluorescent microscopy. Ovaries excised from neonatal mice (4 days old) were cultured in DMBA-treated medium 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.

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

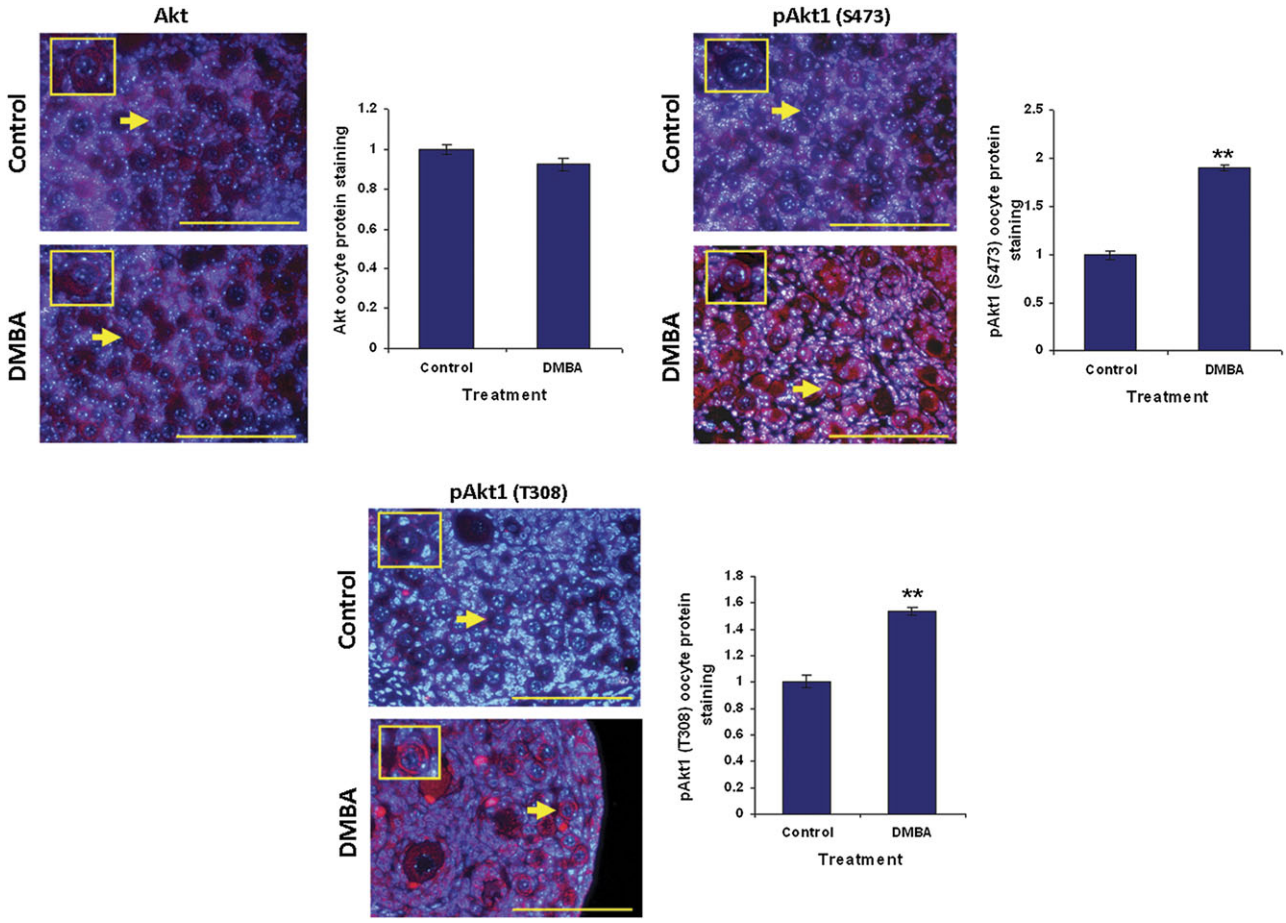


FIG. 4. Fluorescent immunolocalization of Akt, pAkt1 (S473), and pAkt1 (T308) in DMBA-cultured ovaries. Ovaries excised from neonatal mice (4 days old) were cultured in DMBA-treated medium for 96 h and processed for immunohistochemistry as outlined in the ''Materials and Methods'' section. Representative image of both control- and DMBA-treated ovaries (left panel) and quantification of oocyte nuclear staining (right panel). The results presented here are representative of $n = 3$ experiments. Blue staining (DAPI) represents nuclear staining in all cells; red staining (Cy-5) represents specific staining for the described protein. Thin arrow = primordial follicle; scale bar is equal to 100 μm. 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

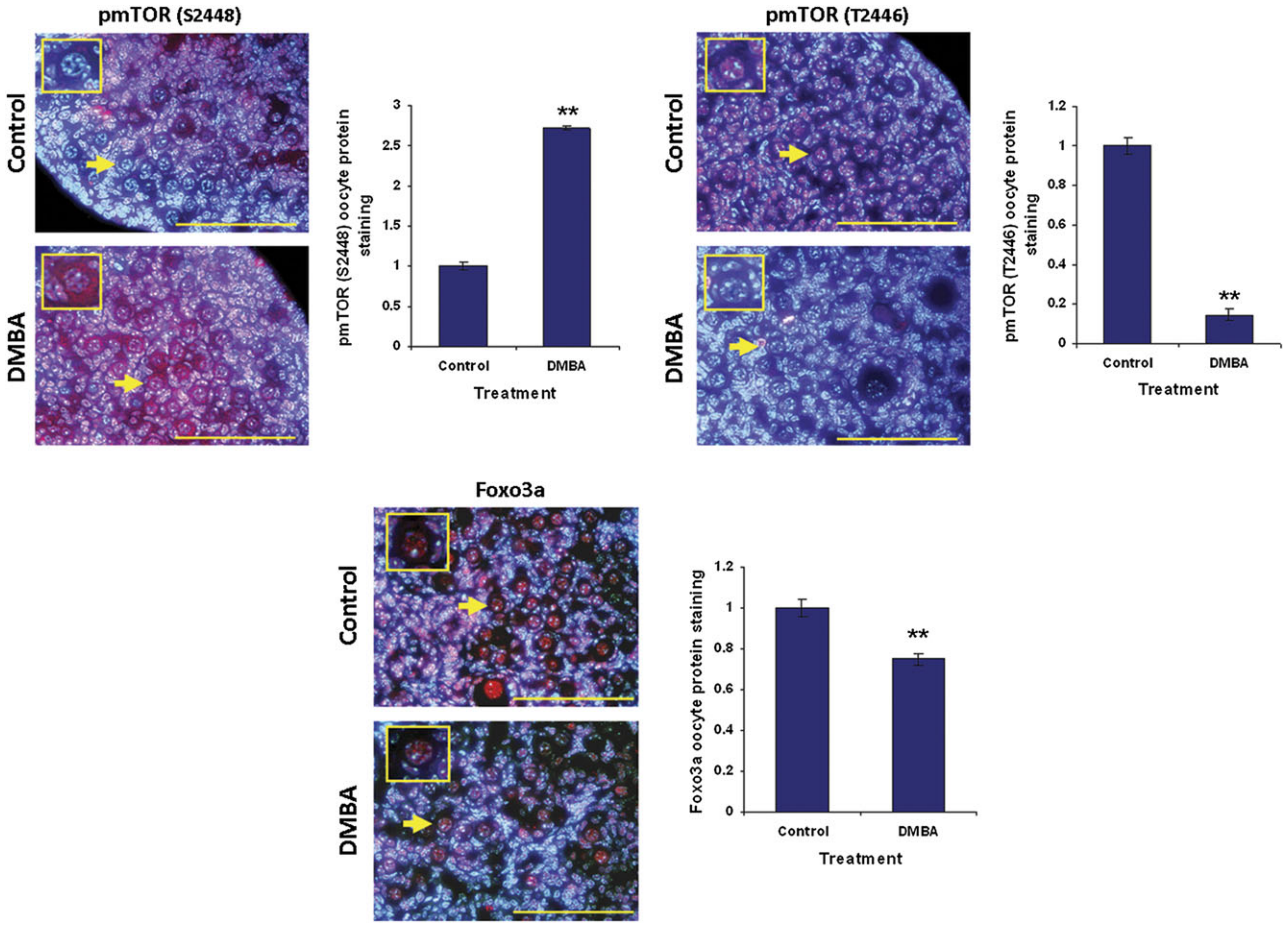


FIG. 5. Fluorescent immunolocalization of pmTOR (S2448), pmTOR (T2446), and Foxo3a in DMBA-cultured ovaries. Ovaries excised from neonatal mice (4 days old) were cultured in DMBA-treated medium for 96 h and processed for immunohistochemistry as outlined in materials and methods. Representative image of both control- and DMBA-treated ovaries (left panel) and quantification of oocyte nuclear staining (right panel). The results presented here are representative of $n = 3$ experiments. Blue staining (DAPI) represents nuclear staining in all cells; red staining (Cy-5) represents specific staining for the described protein. Thin arrow = primordial follicle; scale bar is equal to 100 μm. The symbol ** represents $p < 0.01$ in comparison with control values. *Note*. See online version for color version.

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, tumorigenesis, 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 (IRESs), 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 pre-mRNAs linked with cell cycle progression and increased tumorigenesis (Diamond *et al*., 1993; Huang *et al*., 2007). Calr is a Ca^{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).

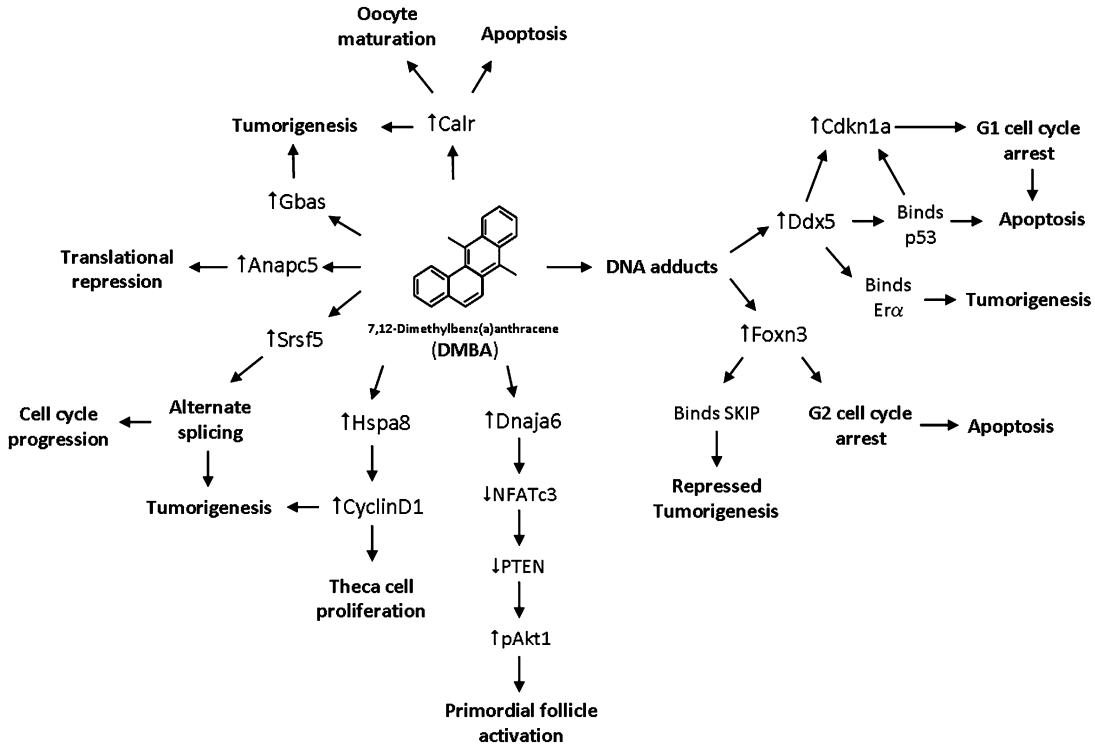


FIG. 6. Summary of qPCR results generating a preliminary model of the ovotoxic effects of DMBA on ovarian folliculogenesis. DMBA influenced the expression of a number of genes which either directly or nondirectly associated with apoptosis, tumorigenesis, and follicular growth/development. ↑ represents upregulation, ↓ represents downregulation.

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 lead 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 the overactivation of primordial follicles in *Tsc1*$^{-/-}$ mutant mice (Adhikari *et al*., 2010). mTOR itself is regulated via a phosphorylation-dependent molecular switch, whereby mTOR Ser^{2448} phosphorylation results in activation and mTOR Thr^{2446} phosphorylation results in inhibition (Cheng *et al*., 2004). We observed an increase in mTOR Ser^{2448} phosphorylation and a decrease in mTOR Thr^{2446} 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 Ser^{2448} phosphorylation was primarily cytoplasmic, whereas mTOR Thr^{2446} 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 ovotoxicity 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/.

FUNDING

National Health and Medical Research Council (Project grant #510735 to E.A.M., S.D.R., and B.N).

ACKNOWLEDGMENTS

The authors gratefully acknowledge the financial assistance to E.A.M. by the Australian Research Council, Hunter Medical Research Institute, and the Newcastle Permanent Building Society Charitable Trust. A.P.S. is the recipient of a Australian Postgraduate Award PhD scholarship.

REFERENCES

Adhikari, D., Zheng, W., Shen, Y., Gorre, N., Hamalainen, T., Cooney, A. J., Huhtaniemi, I., Lan, Z. J., and Liu, K. (2010). Tsc/mTORC 1 signaling in oocytes governs the quiescence and activation of primordial follicles. *Hum. Mol. Genet.* **19,** 397–410.

Agashe, V. R., and Hartl, F. U. (2000). Roles of molecular chaperones in cytoplasmic protein folding. *Semin. Cell Dev. Biol.* **11,** 15–25.

Arnaud, E., Touriol, C., Boutonnet, C., Gensac, M. C., Vagner, S., Prats, H., and Prats, A. C. (1999). A new 34-kilodalton isoform of human fibroblast growth factor 2 is cap dependently synthesized by using a non-AUG start codon and behaves as a survival factor. *Mol. Cell. Biol.* **19,** 505–514.

Baker, D. J., Dawlaty, M. M., Galardy, P., and Van Deursen, J. M. (2007). Mitotic regulation of the anaphase-promoting complex. *Cell. Mol. Life Sci.* **64,** 589–600.

Baksh, S., Widlund, H. R., Frazer-Abel, A. A., Du, J., Fosmire, S., Fisher, D. E., DeCaprio, J. A., Modiano, J. F., and Burakoff, S. J. (2002). NFATc2-mediated repression of cyclin-dependent kinase 4 expression. *Mol. Cell* **10,** 1071–1081.

Barua, A., and Yoshimura, Y. (1999). Immunolocalization of MHC-II+ cells in the ovary of immature, young laying and old laying hens Gallus domesticus. *J. Reprod. Fertil.* **116,** 385–389.

Blachère, N. E., Darnell, R. B., and Albert, M. L. (2005). Apoptotic cells deliver processed antigen to dendritic cells for cross-presentation. *PLoS Biol.* **3,** 1070.

Borman, S. M., Christian, P. J., Sipes, I. G., and Hoyer, P. B. (2000). Ovotoxicity in female Fischer rats and B6 mice induced by low-dose exposure to three polycyclic aromatic hydrocarbons: comparison through calculation of an ovotoxic index. *Toxicol. Appl. Pharmacol.* **167,** 191–198.

Cheng, S. W. Y., Fryer, L. G. D., Carling, D., and Shepherd, P. R. (2004). Thr2446 is a novel mammalian target of rapamycin (mTOR) phosphorylation site regulated by nutrient status. *J. Biol. Chem.* **279,** 15719–15722.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156–159.

Coulam, C. B., Adamson, S. C., and Annegers, J. F. (1986). Incidence of premature ovarian failure. *Obstet. Gynecol.* **67,** 604–606.

Crist, K. A., Zhang, Z., You, M., Gunning, W. T., Conran, P. B., Steele, V. E., and Lubet, R. A. (2005). Characterization of rat ovarian adenocarcinomas developed in response to direct instillation of 7, 12-dimethylbenz[a]-anthracene (DMBA) coated suture. *Carcinogenesis* **26,** 951–957.

Dai, Y. S., Xu, J., and Molkentin, J. D. (2005). The DnaJ-related factor Mrj interacts with nuclear factor of activated T cells c3 and mediates transcriptional repression through class II histone deacetylase recruitment. *Mol. Cell. Biol.* **25,** 9936–9948.

Diamond, R. H., Du, K., Lee, V. M., Mohn, K. L., Haber, B. A., Tewari, D. S., and Taub, R. (1993). Novel delayed-early and highly insulin-induced growth response genes. Identification of HRS, a potential regulator of alternative pre-mRNA splicing. *J. Biol. Chem.* **268,** 15185–15192.

Diehl, J. A., Yang, W., Rimerman, R. A., Xiao, H., and Emili, A. (2003). Hsc70 regulates accumulation of cyclin D1 and cyclin D1-dependent protein kinase. *Mol. Cell. Biol.* **23,** 1764–1774.

Du, X. L., Yang, H., Liu, S. G., Luo, M. L., Hao, J. J., Zhang, Y., Lin, D. C., Xu, X., Cai, Y., and Zhan, Q. M. (2009). Calreticulin promotes cell motility and enhances resistance to anoikis through STAT3–CTTN–Akt pathway in esophageal squamous cell carcinoma. *Oncogene* **28,** 3714–3722.

Fuller-Pace, F. V., and Moore, H. C. (2011). RNA helicases p68 and p72: multifunctional proteins with important implications for cancer development. *Future Oncol.* **7,** 239–251.

Gelboin, H. V. (1980). Benzo [alpha] pyrene metabolism, activation and carcinogenesis: role and regulation of mixed-function oxidases and related enzymes. *Physiol. Rev.* **60,** 1107–1166.

Hashimoto, T., Yanaihara, N., Okamoto, A., Nikaido, T., Saito, M., Takakura, S., Yasuda, M., Sasaki, H., Ochiai, K., and Tanaka, T. (2011). Cyclin D1 predicts the prognosis of advanced serous ovarian cancer. *Exp. Ther. Med.* **2,** 213–219.

Hirshfield, A. N. (1991). Development of follicles in the mammalian ovary. *Int. Rev. Cytol.* **124,** 43–101.

Hoyer, P. B., Davis, J. R., Bedrnicek, J. B., Marion, S. L., Christian, P. J., Barton, J. K., and Brewer, M. A. (2009). Ovarian neoplasm development by 7, 12-dimethylbenz [a] anthracene (DMBA) in a chemically-induced rat model of ovarian failure. *Gynecol. Oncol.* **112,** 610–615.

Hoyer, P. B., and Sipes, I. G. (1996). Assessment of follicle destruction in chemical-induced ovarian toxicity. *Annu. Rev. Pharmacol. Toxicol.* **36,** 307–331.

Huang, C. S., Shen, C. Y., Wang, H. W., Wu, P. E., and Cheng, C. W. (2007). Increased expression of SRp40 affecting CD44 splicing is associated with the clinical outcome of lymph node metastasis in human breast cancer. *Clin. Chim. Acta* **384,** 69–74.

Huez, I., Créancier, L., Audigier, S., Gensac, M. C., Prats, A. C., and Prats, H. (1998). Two independent internal ribosome entry sites are involved in translation initiation of vascular endothelial growth factor mRNA. *Mol. Cell. Biol.* **18,** 6178–6190.

Igawa, Y., Keating, A. F., Rajapaksa, K. S., Sipes, I. G., and Hoyer, P. B. (2009). Evaluation of ovotoxicity induced by 7, 12-dimethylbenz [a] anthracene and its 3, 4-diol metabolite utilizing a rat in vitro ovarian culture system. *Toxicol. Appl. Pharmacol.* **234,** 361–369.

Keating, A. F., Fernandez, S. M., Mark-Kappeler, C. J., Sen, N., Sipes, I. G., and Hoyer, P. B. (2010). Inhibition of PIK3 signaling pathway members by the ovotoxicant 4-vinylcyclohexene diepoxide in rats. *Biol. Reprod.* **84,** 743–751.

Kim, K. K., Shim, J. C., and Kim, J. R. (2003). Overexpression of p21, cyclin E and decreased expression of p27 in DMBA (7, 12 dimethylbenzanthracene) induced rat ovarian carcinogenesis. *Pathol. Int.* **53,** 291–296.

Koloteva-Levine, N., Pinchasi, D., Pereman, I., Zur, A., Brandeis, M., and Elroy-Stein, O. (2004). The Apc5 subunit of the anaphase-promoting complex/cyclosome interacts with poly (A) binding protein and represses internal ribosome entry site-mediated translation. *Mol. Cell. Biol.* **24,** 3577–3587.

Martherus, R. S. R. M., and Sluiter, W. (2010). Functional annotation of heart enriched mitochondrial genes GBAS and CHCHD10 through guilt by association. *Biochem. Biophys. Res. Commun.* **402**(2), 203–208.

Martineau, Y., Le Bec, C., Monbrun, L., Allo, V., Chiu, I. M., Danos, O., Moine, H., Prats, H., and Prats, A. C. (2004). Internal ribosome entry site structural motifs conserved among mammalian fibroblast growth factor 1 alternatively spliced mRNAs. *Mol. Cell. Biol.* **24,** 7622–7635.

Mattison, D. R., and Schulman, J. D. (1980). How xenobiotic chemicals can destroy oocytes. *Contemp. Obstet. Gynecol.* **15,** 157–169.

McGee, E. A., and Hsueh, A. J. W. (2000). Initial and cyclic recruitment of ovarian follicles. *Endocr. Rev.* **21,** 200–214.

McLaughlin, E. A., and McIver, S. C. (2009). Awakening the oocyte: controlling primordial follicle development. *Reproduction* **137,** 1–11.

Moskovitz, J., Berlett, B. S., Poston, J. M., and Stadtman, E. R. (1997). The yeast peptide-methionine sulfoxide reductase functions as an antioxidant in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 9585–9589.

Muñiz, L. C., Yehia, G., Mémin, E., Ratnakar, P. V. A. L., and Molina, C. A. (2006). Transcriptional regulation of cyclin D2 by the PKA pathway and inducible cAMP early repressor in granulosa cells. *Biol. Reprod.* **75,** 279–288.

Nauta, A. J., Daha, M. R., Kooten, C., and Roos, A. (2003). Recognition and clearance of apoptotic cells: a role for complement and pentraxins. *Trends Immunol.* **24,** 148–154.

Nicol, S. M., and Fuller-Pace, F. V. (2010). Analysis of the RNA helicase p68 (Ddx5) as a transcriptional regulator. *Methods Mol. Biol.* **587,** 265–279.

Pati, D., Keller, C., Groudine, M., and Plon, S. E. (1997). Reconstitution of a MEC1-independent checkpoint in yeast by expression of a novel human fork head cDNA. *Mol. Cell. Biol.* **17,** 3037–3046.

Polak, P., and Hall, M. N. (2006). mTORC2 caught in a SINful Akt. *Dev. Cell* **11,** 433–434.

Reddy, P., Adhikari, D., Zheng, W., Liang, S., Hamalainen, T., Tohonen, V., Ogawa, W., Noda, T., Volarevic, S., and Huhtaniemi, I. (2009). PDK1 signaling in oocytes controls reproductive aging and lifespan by manipulating the survival of primordial follicles. *Hum. Mol. Genet.* **18,** 2813–2824.

Reddy, P., Zheng, W., and Liu, K. (2010). Mechanisms maintaining the dormancy and survival of mammalian primordial follicles. *Trends Endocrinol. Metab.* **21,** 96–103.

Robker, R. L., and Richards, J. A. S. (1998). Hormone-induced proliferation and differentiation of granulosa cells: a coordinated balance of the cell cycle regulators cyclin D2 and p27Kip1. *Mol. Endocrinol.* **12,** 924–940.

Scott, K. L., and Plon, S. E. (2005). CHES1/FOXN3 interacts with Ski-interacting protein and acts as a transcriptional repressor. *Gene* **359,** 119–126.

Sobinoff, A. P., Pye, V., Nixon, B., Roman, S. D., and McLaughlin, E. A. (2010). Adding insult to injury: effects of xenobiotic induced preantral ovotoxicity on ovarian development and oocyte fusibility. *Toxicol. Sci* **118**(2), 653–666.

Tsai-Turton, M., Nakamura, B. N., and Luderer, U. (2007). Induction of apoptosis by 9, 10-dimethyl-1, 2-benzanthracene in cultured preovulatory rat follicles is preceded by a rise in reactive oxygen species and is prevented by glutathione. *Biol. Reprod.* **77,** 442–451.

Vanoverberghe, K., Abeele, F. V., Mariot, P., Lepage, G., Roudbaraki, M., Bonnal, J. L., Mauroy, B., Shuba, Y., Skryma, R., and Prevarskaya, N. (2003). Ca2+ homeostasis and apoptotic resistance of neuroendocrine-differentiated prostate cancer cells. *Cell Death Differ.* **11,** 321–330.

Vericat, J. A., Cheng, S. C., and Dipple, A. (1989). Absolute stereochemistry of the major 7, 12-dimethylbenz (a) anthracene-DNA adducts formed in mouse cells. *Carcinogenesis* **10,** 567–570.

Vougas, K., Gaitanarou, E., Marinos, E., Kittas, C., and Voloudakis-Baltatzis, I. E. (2008). Two-dimensional electrophoresis and immunohistochemical study of calreticulin in colorectal adenocarcinoma and mirror biopsies. *J. BUON* **13,** 101–107.

Wang, Q., Zhou, Y., Jackson, L. N., Johnson, S. M., Chow, C. W., and Evers, B. M. (2011). Nuclear factor of activated T cells (NFAT) signaling regulates PTEN expression and intestinal cell differentiation. *Mol. Biol. Cell* **22,** 412–420.

Wang, X. Y., Smith, D. I., Liu, W., and James, C. D. (1998). GBAS, a novel gene encoding a protein with tyrosine phosphorylation sites and a transmembrane domain, is co-amplified with EGFR. *Genomics* **49,** 448–451.

Zhang, D. X., Li, X. P., Sun, S. C., Shen, X. H., Cui, X. S., and Kim, N. H. (2010). Involvement of ER–calreticulin–Ca2+ signaling in the regulation of porcine oocyte meiotic maturation and maternal gene expression. *Mol. Reprod. Dev.* **77,** 462–471.

Chapter 3: Jumping the gun: Smoking constituent BaP causes premature primordial follicle activation and impairs oocyte fusibility through oxidative stress

Toxicology and Applied Pharmacology 260 (2012) 70–80

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Jumping the gun: Smoking constituent BaP causes premature primordial follicle activation and impairs oocyte fusibility through oxidative stress

A.P. Sobinoff [a], V. Pye [a], B. Nixon [a,b], S.D. Roman [a,b], E.A. McLaughlin [a,b,*]

[a] Reproductive Science Group, School of Environmental & Life Sciences, University of Newcastle, Callaghan, NSW2308, Australia
[b] ARC Centre of Excellence in Biotechnology & Development, University of Newcastle, Callaghan, NSW2308, Australia

ARTICLE INFO

Article history:
Received 6 January 2012
Revised 30 January 2012
Accepted 31 January 2012
Available online 8 February 2012

Keywords:
Benzo(a)pyrene
Ovary
Primordial follicle
Impaired fertilisation
Oxidative stress
Atresia

ABSTRACT

Benzo(a)pyrene (BaP) is an ovotoxic constituent of cigarette smoke associated with pre-mature ovarian failure and decreased rates of conception in IVF patients. Although the overall effect of BaP on female fertility has been documented, the exact molecular mechanisms behind its ovotoxicity remain elusive. In this study we examined the effects of BaP exposure on the ovarian transcriptome, and observed the effects of *in vivo* exposure on oocyte dysfunction. Microarray analysis of BaP cultured neonatal ovaries revealed a complex mechanism of ovotoxicity involving a small cohort of genes associated with follicular growth, cell cycle progression, and cell death. Histomorphological and immunohistochemical analysis supported these results, with BaP exposure causing increased primordial follicle activation and developing follicle atresia *in vitro* and *in vivo*. Functional analysis of oocytes obtained from adult Swiss mice treated neonatally revealed significantly increased levels of mitochondrial ROS/lipid peroxidation, and severely reduced sperm-egg binding and fusion in both low (1.5 mg/kg/daily) and high (3 mg/kg/daily) dose treatments. Our results reveal a complex mechanism of BaP induced ovotoxicity involving developing follicle atresia and accelerated primordial follicle activation, and suggest short term neonatal BaP exposure causes mitochondrial leakage resulting in reduced oolemma fluidity and impaired fertilisation in adulthood. This study highlights BaP as a key compound which may be partially responsible for the documented effects of cigarette smoke on follicular development and sub-fertility.

Introduction

Cigarette smoke is a known reproductive hazard associated with delayed conception, premature ovarian failure (POF), and lower fertilisation rates in women undergoing assisted conception (El-Nemr et al., 1998; Freour et al., 2008; Gruber et al., 2008; Howe et al., 1985; Sun et al., 2011; Windham et al., 2005). In addition to direct inhalation, sidestream cigarette exposure also adversely affects female fertility, resulting in reduced embryo implantation and pregnancy rates in IVF patients (Neal et al., 2005). Although we are aware of the destructive nature of cigarette smoke on female fertility, limited data exists on the chemical constituents which cause its reported ovotoxicity.

Cigarette smoke is composed of over 4,000 chemicals, including nicotine, nitroso compounds, aromatic amines, protein pyrolysates and polycyclic aromatic hydrocarbons (PAH) (Rustemeier et al., 2002). Of these chemicals, of particular interest is the polycyclic aromatic hydrocarbon Benzo(a)pyrene (BaP). BaP is a mutagenic toxicant produced via the incomplete combustion of carbon, and is present at relatively high levels in cigarette smoke (Lodovici et al., 2004). Studies of the effects of BaP on ovarian function have identified this chemical as a potent ovotoxicant capable of inducing POF through the rapid depletion of the primordial follicle pool (Mattison and Thorgeirsson, 1979). In the ovary, BaP is converted into a 7,8-dihydrodiol-9,10-epoxide bioactive metabolite by Aryl hydrocarbon receptor (Ahr) induced members of the cytochrome P450 family of oxidases (Bengtsson et al., 1983; Mattison et al., 1983). This bioactive metabolite then binds to the 2-amino group of DNA guanosine to form BaP-DNA adducts, resulting in mutagenesis and follicular atresia (Zhou et al., 2009). Interestingly, BaP-DNA adducts have been detected in the granulosa cells of women who smoke, indicating BaP may be a contributing factor in cigarette induced POF (Zenzes, 2000). Although the overall effect of BaP on follicular development has been well documented, the exact molecular mechanisms behind its ovotoxicity remain elusive. Recent studies conducted by ourselves and others have identified a novel mechanism of premature ovarian failure involving primordial follicle activation and developing follicle atresia for a number of ovotoxic xenobiotics, including the PAH 7,12-Dimethylbenz-[a]anthracene (DMBA) (Keating et al., 2010; Sobinoff et al., 2010, 2011). Given the similarities between DMBA

* Corresponding author at: Discipline of Biological Sciences, ARC Centre of Excellence in Biotechnology & Development, School of Environmental & Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia. Fax: +61 2 4921 6308.
E-mail address: eileen.mclaughlin@newcastle.edu.au (E.A. McLaughlin).

0041-008X/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2012.01.028

and BaP induced ovotoxicity, we sought to confirm that BaP also induces POF via a similar mechanism (Bengtsson et al., 1983; Mattison et al., 1983).

In addition to POF, evidence also suggests BaP may induce oocyte dysfunction. BaP is detected at high concentrations in the follicular fluid of female IVF patients who smoke, and was associated with a decreased rate of conception (Neal et al., 2008). One potential mechanism by which BaP may adversely affect oocyte quality is through the production of reactive oxygen species (ROS). Xenobiotic-enhanced ROS formation can occur via several mechanisms, including the unnatural "uncoupling" of phase I cytochrome P450 detoxification, quinone formation, and glutathione peroxidise depletion (Sobinoff et al., in press). Indeed, BaP is converted into 3,6-dione and 6,12-dion redox cycling quinones by cyp1A1, both of which have been detected in rodent ovaries after a single dose exposure of BaP (Ramesh et al., 2010). We have previously demonstrated that neonatal xenobiotic exposure severely reduces the ability of oocytes obtained in adulthood to undergo fertilisation, and that exposure to the same xenobiotics *in vitro* causes dose dependant lipid peroxidation in cultured MII oocytes (Sobinoff et al., 2010). Therefore, we hypothesised that BaP induces oocyte dysfunction through xenobiotic induced lipid peroxidation, resulting in impaired membrane fluidity and therefore sperm-oocyte fusion.

In order to gain a better understanding of the mechanisms behind BaP induced ovotoxicity, we examined the effects of BaP exposure on the ovarian transcriptome of cultured neonatal mouse ovaries, and observed the effects of *in vivo* exposure on oocyte dysfunction. Microarray analysis revealed a complex mechanism of BaP induced ovotoxicity targeting a small cohort of genes associated with follicular growth, cell cycle progression, and cell death. Histomorphological and immunohistochemical analysis supported these results, with BaP exposure causing increased primordial follicle activation and developing follicle atresia *in vitro* and *in vivo*. Mitochondrial ROS, lipid peroxidation, and sperm-oocyte fusion assays supported our hypothesis of BaP induced oocyte dysfunction, with short-term BaP exposure causing long term mitochondrial membrane damage, altered oolemma membrane fluidity, and impaired sperm/oocyte fusion and fertilisation.

Methods

Reagents. BaP (>95% purity), Menadione (MEN; >95% purity) and custom designed primers were purchased from Sigma Chemical Co. (St. Louis, MO). 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) were obtained from Abcam (Cambridge, MA). Mouse monoclonal anti-human Anti-Müllerian hormone (anti-AMH, MCA2246) was obtained from AbD Serotec (Kidlington, UK). Alexa Fluor 594 goat anti-rabbit IgG (A11012), Alexa Fluor 594 goat anti-mouse IgG (A11005), 4,4-difluoro-5-(4-phenyl-1,3-buttadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid 581/591C11 (BODIPY; D3861), MitoSOX red (M36008), fetal bovine serum (FBS), L-Glutamine, and Insulin-Transferrin-Selenium (ITS) were purchased from the Invitrogen Co. (Carlsbad, CA). L-Ascorbic Acid was obtained from MP Biomedicals (Solon, OH) and 0.4 μm Culture Plate Inserts were purchased from Millipore (Billerica, MA). All culture dishes and cell culture plates were obtained from Greiner Bio-One (Monroe, NC). Oligo(dT)15 primer, RNasin, dNTPs, M-MLV-Reverse Transcriptase, RQ1 DNAse, GoTaq Felxi, $MgCl_2$, GoTaq 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). 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 21–22 °C and supplied with food and water *ad libitum*.

Animal dosing. Female Swiss neonatal mice (day 4; 6–10 animals/treatment group) were weighed and administered (intraperitoneal; i.p.) 7 daily, consecutive doses of either sesame oil containing vehicle control (<10 μl/kg/daily Acetone) or sesame oil containing a low and high dose of BaP (1.5 mg/kg/daily; 3 mg/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. Half of the treated animals were euthanised by CO_2 asphyxiation 24 hours after the last injection. The remaining animals were weaned and then super-ovulated at 6 weeks of age via i.p. injection of 10 IU of Folligon (eCG; Intervet, Sydney, Australia), followed by i.p. administration of 10 IU of Chorulon (hCG; Intervet, Sydney, Australia) 48 h later and euthanasia by CO_2 asphyxiation 12 hrs later.

Ovarian culture. Ovaries from day 3–4 Swiss neonatal mice were cultured described previously (Sobinoff et al., 2010). Briefly, Swiss neonates were sacrificed by CO_2 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 DMEM/F12 medium containing 5% (v/v) fetal calf serum, 1 mg/ml bovine serum albumin, 50 μg/ml ascorbic acid, 27.5 μg/ml insulin–transferrin–selenium, 2.5 mM glutamine and 5U/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% CO_2 in air, with media changes every two days. Ovaries were treated with vehicle control medium (0.01% Acetone) or BaP (1 μM). The BaP 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 4 cuboidal granulosa cells in a single layer. Secondary follicles were identified as those with two layers of granulosa cells, and pre-antral 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 pre-antral stage.

Immunohistochemistry. Ovaries for immunohistochemistry were fixed in Bouin's and sectioned 4 μm thick. PCNA, active Casp2 and active Casp3 were probed for using the same protocol with the exception of the primary antibody. Slides were deparaffinised in xylene and rehydrated with subsequent washes in ethanol. Antigen retrieval was carried out by microwaving sections for 3×3 min in Tris buffer (50 mM, pH 10.6). Sections were then blocked in 3%BSA/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) or anti-Casp3 (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 IgG, Alexa Fluor 594 goat anti-mouse IgG; 1:200 dilution) for 1 h. Slides were then counter-stained 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) using epifluorescent optics and images captured using a Olympus DP70 camera (Olympus America, Center Valley, PA).

TUNEL analysis. Bouin's fixed sections were deparaffinised and rehydrated as described previously. Sections were then boiled in Tris buffer (50 mM, pH 10.6) for 20 min and treated with 20 μg/ml Proteinase K for 15 min in a humidified chamber. TUNEL analysis was then performed using an In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics Pty Ltd.; Dee Why, NSW) according to the manufacturer's instructions. Slides were then counter-stained with DAPI for 5 min, mounted in Mowiol and observed on an Axio Imager A1 fluorescent microscope (Carl Zeiss) using epifluorescent optics and images captured using a Olympus DP70 microscope camera (Olympus).

RNA extraction. Total RNA was isolated from ovaries using two rounds of a modified acid guanidinium thiocyanate–phenol–chloroform protocol (Chomczynski and Sacchi, 1987): washed cells resuspended in lysis buffer (4 M guanidinium thiocyanate, 25 mM 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, 0.5 mM 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, USA). Primer sequences along with annealing temperatures have been supplied as supplementary data (sTable 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 $2^{-\Delta\Delta C(t)}$, 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 BaP cultured neonatal ovaries and prepared for microarray analysis at the Australian Genome Research Facility (AGRF) using an Illumina Sentrix Mouse ref8v2 Beadchip. Labelling, hybridising, washing and array scanning were performed by the AGRF using the Illumina manual on an Illumina BeadArray Reader, and normalised according to the quantile normalisation 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 analysed using Ingenuity Pathways Analysis (Ingenuity Systems, Redwood City, CA) software to identify canonical signalling pathways influenced by BaP 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%BC%20GSE29263).

Sperm-oocyte fusion assay. Adult mice (6 weeks) treated with BaP over a 7 day period after birth were super ovulated as described previously under "animal dosing." Cumulus-intact oocytes were recovered 12–15 h after the final hCG injection by rupturing the oviductal ampullae of superovulated animals in M2 medium. Adherent cumulus cells were then dispersed by treating the collected oocytes with 300 IU/ml hyaluronidase solution and washing twice in M2 medium under oil. The zona pellucida was then removed from these oocytes by brief treatment with low-pH (2.5) Acid Tyrode's solution and allowed to recover at least 1 h at 37 °C in an atmosphere of 5% CO_2 in air. Sperm were collected from mature male mice by dissecting the cauda epididymides and squeezing out the dense sperm mass along the tube. The sperm were then allowed to disperse into 800 μl M2 medium, diluted to a final concentration of 2×10^5 sperm/ml in M2 medium, and allowed to capacitate for 3 h at 37 °C in 5% CO_2 in air. Following capacitation, zona free oocytes were preloaded with DAPI for 15 min; 12–25 oocytes were then added to the sperm suspensions and co-incubated for 15 min at 37 °C in 5% CO_2 in air. Using serial aspiration through a finely drawn pipette, unbound and loosely adhered spermatozoa were removed from oocytes. Oocytes were then mounted on slides, and the number of sperm bound to the oocyte membrane counted using phase-contrast microscopy. Sperm-oocyte fusion was then assayed by counting the number of DAPI stained sperm heads attached to the oocyte membrane using fluorescent microscopy.

Oocyte lipid peroxidation assay. Cumulus free oocytes were collected from adult mice (6 weeks) treated with BaP over a 7 day period as described previously under "sperm-oocyte fusion assay." Oocytes were then incubated under oil in 10 mM BODIPY stain for 30 min at 37 °C in 5% CO_2 in air. An oxidative stress positive control was also carried out by exposing oocytes from non-treated animals to 80 mM H_2O_2 for 30 min under oil at 37 °C and 5% CO_2 in air. The dye loaded oocytes were then washed twice in M2 medium and mounted on slides. Green and red fluorescence of BODIPY were determined using a LSM510 laser-scanning microscope (Carl Zeiss) equipped with Argon and Helium/Neon lasers at excitation wavelengths of 488 and 543 nm and emission spectra of 500–530 nm (green) and greater than 560 nm (red). Histogram analysis was then used to determine the ratio of green to red fluorescence.

Mitochondrial ROS assay. Cumulus free oocytes were collected from adult mice (6wks) treated with BaP over a 7 day period as described previously under "sperm-oocyte fusion assay". Oocytes were then incubated under oil in 5 μM MitoSOX red stain for 20 min at 37 °C in 5% CO_2 in air. An oxidative stress positive control was also carried out by exposing oocytes from non-treated animals to 80 mM H_2O_2 for 30 min under oil at 37 °C and 5% CO_2 in air. The dye loaded oocytes were then washed twice in M2 medium and mounted on slides. MitoSOX red fluorescence was determined using a LSM510 laser-scanning microscope (Carl Zeiss) equipped with Argon and Helium/Neon lasers at an excitation wavelength of 510 nm and emission maximum of 580 nm. Quantification of MitoSOX red was then carried out via histogram analysis.

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 $p<0.05$.

Experimental design. A summary of the experimental design can be found in supplementary data (sFig. 1).

Results

Effects of BaP exposure on the neonatal ovarian transcriptome

BaP exposure significantly altered the expression of a small cohort of genes representing 0.7% of the total number present on the array, suggesting a specific gene-regulatory response in the neonatal ovary (Fig. 1A). Significantly altered genes were then analysed for networks and molecular functions through the use of Ingenuity Pathway Analysis software. BaP altered genes were identified as components of molecular networks for gene expression, the cell cycle, cellular growth and proliferation, cellular development, and cell to cell signalling (Fig. 1B). In accordance with its reported role as an ovotoxicant/mutagen, BaP exposure also influenced a large number of genes implicated in cell death, cancer, and genetic disorder (Table 1).

Canonical pathways significantly up-regulated by BaP exposure

In order to gain further insight into the mechanisms behind BaP induced ovotoxicity, differentially expressed genes were also analysed for signalling pathways and molecular functions using Ingenuity Pathways Analysis (Fig. 2; sTable 2). We limited our analysis to those genes which demonstrated a significant increase (two fold, $p<0.05$) in expression to identify molecular signalling pathways that were significantly up-regulated by xenobiotic exposure. BaP exposure influenced five canonical pathways associated with follicular growth and development (Androgen and estrogen metabolism, EIF2 signaling, chromosomal replication, retinol metabolism, and growth hormone signaling), three pathways associated with DNA repair and follicular atresia (ATM signalling, Ahr signalling, and p53 signaling), one pathway associated with detoxification/bioactivation (Ahr signalling), and one pathway associated with oxidative stress (Nitric oxide and ROS production).

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 gene expression patterns were observed for all targets measured by QPCR when compared to the results of the microarray gene expression study. Consistent with a role in ovotoxicity/mutagenesis, three of these genes were associated with cell cycle arrest and apoptosis (*Cdkn1a*, *Ddx5* and *Cxcl12*), two were associated with increased tumorigenesis (*Ddx5* and *Cxcl12*), and two with xenobiotic metabolism (*Cyp1b1*, *Utg1a10*). The remaining five genes were all associated with increased cell cycle progression and follicular development (*Ccng1*, *Cdk2*, *Mcm7*, *Hspa8*, and *Igf2*). Interestingly, only three out of the 10 different genes have been previously associated with BaP induced toxicity (*Cyp1b1*, *Utg1a10*, *Cdkn1a*), suggesting BaP may have other mechanisms of ovotoxicity in addition to those already documented by the literature.

BaP causes primordial follicle activation and immature follicle atresia in vitro

To consolidate the data obtained from our microarray analysis and further characterise the ovarian response to BaP, cultured neonatal ovaries were fixed and probed by immunohistochemistry for markers associated with primordial follicular activation and atresia. PCNA staining was detected in both the granulosa cell and oocyte nuclei of BaP treated primordial follicles and absent in the control, indicating these follicle had committed to follicular activation (Fig. 3). Casp2, Casp3, and TUNEL staining were all detected in BaP treated primary and secondary follicles, but were absent from BaP treated primordial follicles, suggesting a selective mechanism of developing follicle atresia. AMH was localised to the granulosa cells of primary and secondary follicles in both control and BaP treated ovaries (Supplementary Fig. 3).

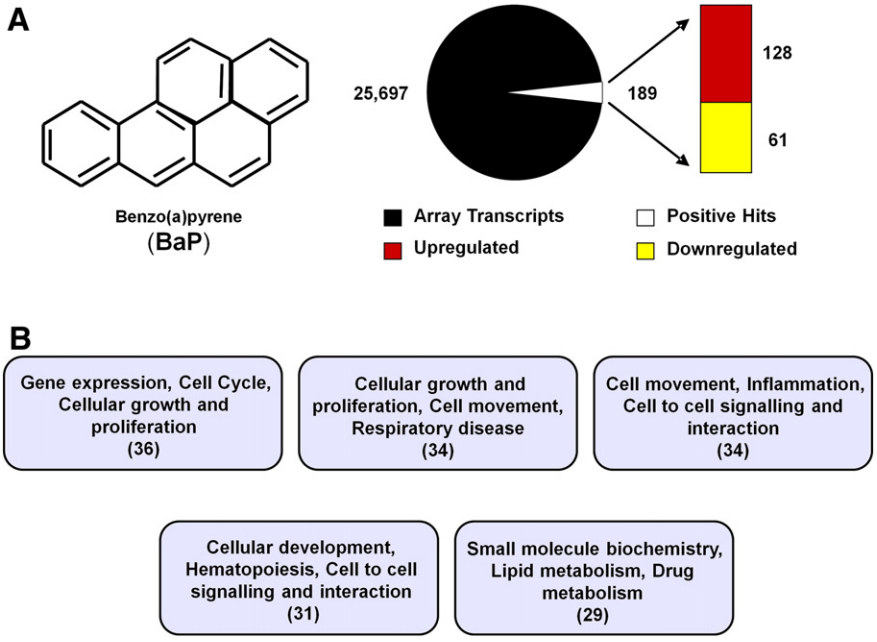


Fig. 1. Microarray analysis of control cultured ovaries verses BaP cultured ovaries. Ovaries were excised from neonatal mice (4 days old, n = 15) and cultured in xenobiotic treatment medium for 96 hours RNA extracted and subjected to microarray analysis as described in the materials and methods. (A) Summary of microarray results. Total number of genes found on an Illumina Sentrix Mouse ref8v2 Beadchip are presented as non-regulated (black) and regulated (white) genes with a significant change in expression (>2-fold change, $p<0.05$). The red bar represents the number of positively regulated genes, and the yellow bar represents the number of negatively regulated genes in xenobiotic cultured ovaries. (B) Molecular networks of significantly altered genes influenced by BaP exposure. Significantly altered genes were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base (Ingenuity Systems). Networks of significantly altered genes were then algorithmically generated based on their connectivity. The networks are ranked according to their scores, and the five highest-ranking networks of genes are displayed. The numbers of genes in each network are shown in brackets.

Table 1
Functional classification of genes that were up-regulated or down-regulated by BaP exposure in cultured neonatal ovaries. Genes were analysed using Ingenuity Pathways Analysis (Ingenuity Systems) for molecular and cellular functions. Only those genes exhibiting a greater than two-fold change in expression were categorised ($p<0.05$). Note that some genes are listed in multiple functional groups.

Molecular and cellular function	Up regulated	Down regulated
Cellular growth and proliferation	39	20
Tissue development	30	14
Cell-to-cell signalling and interaction	15	13
Gene expression	32	11
Cell death	30	13
Cell cycle	18	5
Genetic disorder	63	35
Cellular development	31	13
Cancer	51	22
Tissue morphology	25	9
Small molecule biochemistry	28	13

Effects of BaP exposure on primordial follicle activation and follicular atresia in vivo

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 either a high or low dose of BaP for seven days and their ovaries collected for analysis. Immunohistological examination of fixed ovaries did not detect any staining for PCNA in primordial follicle in the low dose BaP treatment (Fig. 4). However, PCNA staining was detected in large clusters of primordial follicles in the high dose treatment, with a staining pattern similar to that observed *in vitro* (Fig. 3). Histomorphological analysis revealed a slight but significant reduction in low dose treated primordial follicle population, mirrored by a comparable increase in the number of activating follicles (Fig. 5A). In agreement with our PCNA results, the follicular composition of ovaries from the high dose treated ovaries indicated a significant reduction (~2-fold) in primordial follicles, with a comparably large increase in the number of activating follicles (Fig. 5B). In addition, BaP also caused a significant increase in the percentage of primary (~2-fold) and secondary (~2-fold) follicles, and a significant decrease in the percentage of pre-antral follicles (~5-fold). Analysis of the average number of follicles per section also demonstrated a significant decrease in total follicular number in the high dose treated animals (52% of the control).

Short term neonatal BaP exposure causes long term mitochondrial damage, oolemma lipid peroxidation, and impaired oocyte fusibility in vivo

To investigate the effects of short term BaP exposure on long term oocyte dysfunction, adult Swiss mice treated with either a low or high dose of BaP after birth were superovulated and their oocytes collected for analysis. There was no difference between the number of retrievable oocytes from the control and BaP treated groups, indicating the process of folliculogenesis had not been disrupted long term (Supplementary Fig. 2). Mitochondrial ROS was detected using the fluorescent dye MitoSOX red, and revealed a significant increase in mitochondrial membrane leakage in both low and high dose treated oocytes (Fig. 6A). Lipid peroxidation studies using the fluorescent dye BODIPY also revealed significantly increased levels of peroxidative damage in the oolemma membrane of both low and high dose treated oocytes (Fig. 6B). Functional analysis of the treated oocytes ability to undergo fertilisation revealed significantly reduced sperm-egg binding in both high and low dose treatments (Fig. 7). Additionally, sperm-egg fusion was also severely reduced to almost negligible levels in both treatments.

Discussion

In this study we characterised the effects of the ovotoxic smoking constituent BaP on the ovarian transcriptome of neonatal mice *in vitro*. Microarray analysis revealed an elaborate mechanism of BaP induced ovotoxicity involving a small cohort of genes implicated in follicular growth and development, cell cycle regulation, cell to cell signalling, cell death, cancer, and genetic disorder (Fig. 1; Table 1). These results support previous studies which have identified BaP as a potent ovotoxicant and carcinogen (Lodovici et al., 2004; Mattison and Thorgeirsson, 1979). However, this is the first documented evidence of BaP stimulating follicular growth and development, with previous studies suggesting it is a potent inhibitor of antral follicle growth (Neal et al., 2007). In support of these observations, analysis of the signalling pathways up-regulated by BaP exposure identified a number of canonical pathways associated with the progression of folliculogenesis (Fig. 2). In particular, BaP exposure induced up-regulation of genes involved in growth hormone signalling, a pathway implicated in primordial follicle recruitment (Slot et al., 2006), and in estrogen metabolism, an essential process required for antral follicular growth (Couse and Korach, 1999). The induction of

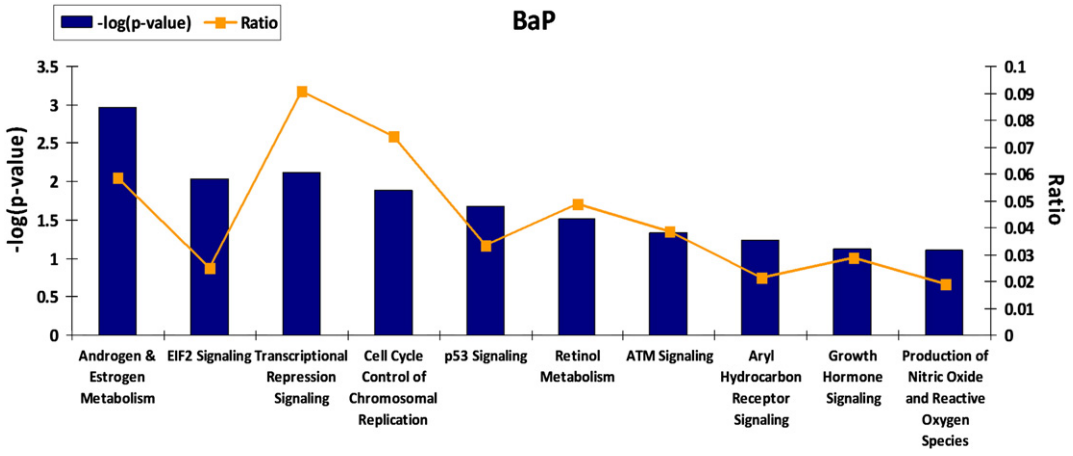


Fig. 2. Top canonical pathways that were significantly up-regulated by BaP cultured neonatal ovaries as identified by Ingenuity Pathways Analysis. The significance of the association between up-regulated genes and the canonical pathway was evaluated using a right-tailed Fisher's exact test to calculate a *p*-value determining the probability that the association is explained by chance alone (blue bars, y-axis). Ratios referring to the proportion of up-regulated genes from a pathway related to the total number of molecules that make up that particular pathway are also displayed (line graph, z-axis).

Table 2
QPCR validation of microarray results for select transcripts up-regulated by BaP cultured neonatal ovaries. 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. Fold change (mean ± SE) and summary of function relating to folliculogenesis are included. All fold changes were statistically significant ($p<0.05$).

Gene symbol	Gene name	Summary of function	Fold change
Cdkn1a	Cyclin-dependent kinase inhibitor 1A	Regulator of cell cycle progression at G1 phase; implicated in DNA damage repair, increased expression in response to BaP-DNA adduct formation (Binkova et al., 2000)	6.94 ± 0.8
Cyp1b1	Cytochrome P450, family 1, subfamily b, polypeptide 1	Phase I detoxifying enzyme; implicated in the bioactivation of BaP into DNA adduct forming metabolites in the ovary (Bengtsson et al., 1983; Mattison et al., 1983)	5.49 ± 0.4
Ccng1	Cyclin G1	Regulator of cell cycle progression at G2/M phase; implicated in DNA damage repair and apoptosis; associated with granulosa cell proliferation and differentiation (Kimura et al., 2001; Liu et al., 2006)	3.15 ± 0.2
Cxcl12	Chemokine (C-X-C motif) ligand 12	Chemokine; expressed in developing ovarian follicles; acts as an inhibitor of primordial follicle activation; overexpression associated with ER alpha tumorigenesis (Holt et al., 2006; Rhodes et al., 2011)	2.8 ± 0.2
Cdk2	Cyclin-dependent kinase 2	Protein kinase essential for G1/S cell cycle transition; Elevated Cdk2 associated with accelerated follicular activation and increased primordial follicle survival (Rajareddy et al., 2007)	2.18 ± 0.2
Mcm7	Minichromosome maintenance complex component 7	DNA replication factor essential for cellular proliferation; Increased expression associated with primordial follicle activation (Park et al., 2005)	2.05 ± 0.2
Hspa8	Heat shock protein 8	Stress related chaperone; expression increases during cell cycle G1 phase; regulates cyclin D1 accumulation and maintains its activity in the presence of inhibitory Cdkn1a (Diehl et al., 2003)	1.98 ± 0.4
Ddx5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	RNA helicase; stimulates cell proliferation, co-activator of p53 signalling, overexpression associated with ER alpha tumorigenesis (Nicol and Fuller-Pace, 2010)	1.77 ± 0.2
Igf2	Insulin-like growth factor 2	Growth factor expressed in rodent ovarian theca cells; acts as a autocrine signal and granulosa paracrine signal; stimulates proliferation (Adashi et al., 1985; Davoren et al., 1986; Hernandez et al., 1990)	1.76 ± 0.1
Ugt1a10	UDP glycosyltransferase 1 family, polypeptide A10	Phase II detoxifying enzyme; Implicated in the detoxification of bioactivated BaP metabolites (Dellinger et al., 2006)	1.59 ± 0.1

estrogenic metabolism is intriguing, as pre-antral follicular growth is estrogen independent. One explanation for the induction of this pathway may be the metabolism of estrogenic BaP metabolites, rather than estrogen itself, which are formed as a consequence of Ahr mediated bioactivation (van Lipzig et al., 2005). Indeed, one of the other pathways up-regulated by BaP exposure was aryl hydrocarbon receptor (Ahr) signalling, the primary mechanism responsible for BaP bioactivation in the ovary (Bengtsson et al., 1983; Mattison and Thorgeirsson, 1979). Analysis of Ahr-deficient mice has also revealed a physiological role for this receptor in regulating reproduction, growth and development (Robles et al., 2000). Robles et al. (2000) demonstrated that ovaries from Ahr deficient PND4 mice had a significant two-fold increase in the primordial follicle population, suggesting a developmental role for Ahr in regulating primordial follicle formation and atresia. Given that BaP is an Ahr ligand which causes follicular depletion, we hypothesise that part of its ovotoxicity may be due to perturbed Ahr developmental signalling, disrupting folliculogenesis. In accordance with its reported ovotoxicity, BaP exposure also up-regulated signalling pathways associated with DNA repair and follicular atresia (ATM signalling and p53 signalling).

In addition to stimulating pathways associated with follicular growth and atresia, BaP exposure also up-regulated genes involved in nitric oxide and ROS production. As described previously, BaP bioactivation can lead to the production of quinone-like metabolites in the ovary (Ramesh et al., 2010). These metabolites may undergo redox cycling to a corresponding semiquinone radical to produce superoxide anions, which then go on to form hydrogen peroxide and hydroxyl free radicals (Bolton et al., 2000). The up-regulation of this pathway, combined with

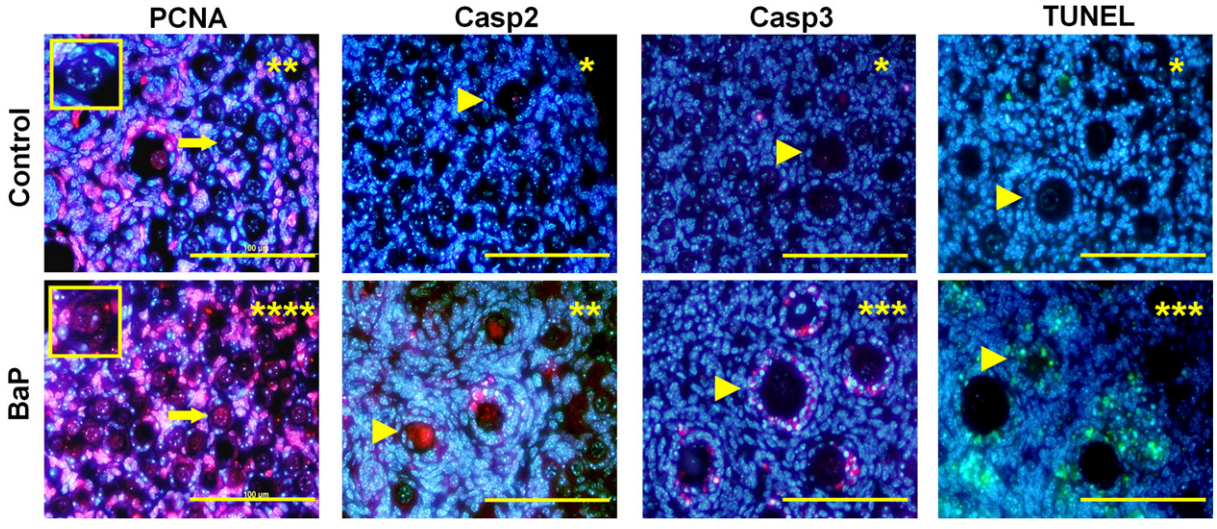


Fig. 3. BaP exposure causes immature follicular destruction and primordial follicle activation *in vitro*. Fluorescent immunohistological and TUNEL staining as visualised via epifluorescent microscopy. Ovaries excised from neonatal mice (4 days old) were cultured in BaP treated medium for 96 hours and processed for immunohistochemistry and TUNEL analysis as described in the materials and methods. Ovarian sections were probed 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 labelled follicles per section is represented by the following scale present in the top right hand corner; * = <25%, ** = 25–50%, *** = 51–75%, **** = 76–100%. Thin arrow = primordial follicle highlighted in insert at higher magnification; arrow head = primary follicle; scale bar is equal to 100 μm.

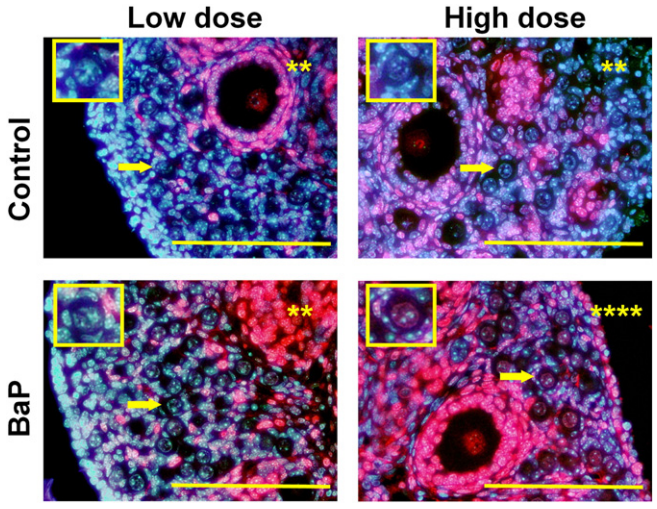


Fig. 4. Fluorescent immunolocalisation of PCNA protein in low and high dose BaP treated ovaries *in vivo*. Neonatal mice (4 days old) were treated with either a low or high dose of BaP over a seven day period, culled, and their ovaries extracted and processed of immunohistochemistry as outlined in materials and methods. The results presented here are representative of n = 3 experiments. Blue staining (DAPI) represents nuclear staining in all cells; red staining (Cy-5) represents specific staining for PCNA protein. The percentage of labelled follicles per section is represented by the following scale present in the top right hand corner; * = <10%, ** = 10–50%, *** = 51–75%, **** = 76–100%. Thin arrow = primordial follicle highlighted in insert at higher magnification; scale bar is equal to 100 μm.

the increased levels of mitochondrial ROS observed *in vivo* (Fig. 6A), suggest BaP may induce part of its ovotoxicity via oxidative stress.

In support of a complex mechanism of BaP induced ovotoxicity, we confirmed the up-regulation of 10 genes of interest involved in a diverse range of cellular processes (Table 2). Two of these genes were *Ddx5*, a member of the DEAD-box RNA helicase family, and *Cdkn1a*, a regulator of G1 cell cycle arrest (Binkova et al., 2000; Nicol and Fuller-Pace, 2010). Ddx5 acts as a co-factor for tumour suppressor p53 induced *Cdkn1a* gene expression in response to DNA damage (Nicol and Fuller-Pace, 2010). Interestingly, a previous study by our laboratory also identified an up-regulation of ovarian *Ddx5* and *Cdkn1a* in response to DMBA exposure, suggesting a common response to PAH induced DNA damage (Sobinoff et al., 2011). *Cxcl12*, a chemoattractive cytokine with receptors present in both the oocyte and granulosa cells (Holt et al., 2006), was also found to be up-regulated by BaP exposure. In terms of follicular development, *Cxcl12* expression increases during the primordial to primary follicle transition, and has been shown to act as an inhibitor of primordial follicle activation (Holt et al., 2006). Cxcl12 can also act as a pro-survival factor, stimulating Akt dependant anti-apoptotic pathways (Yano et al., 2007). Therefore, increased *Cxcl12* expression may represent an increase in the developing follicle number due to primordial follicle activation, thus acting to both repress overstimulated primordial follicle activation and as a pro-survival factor preventing BaP induced germ cell loss.

In addition to their roles in cell cycle arrest and primordial follicle regulation, both Ddx5 and Cxcl12 have been shown to induce tumorigenesis. Ddx5 acts as a co-activator of estrogen receptor alpha, up-regulating its expression and promoting proliferation/survival in cancer cell lines (Fuller-Pace and Moore, 2011). Cxcl12 has also been shown to regulate estrogen receptor alpha induced tumour growth, and promotes the transition of breast cancer cells into a more aggressive hormone independent phenotype (Rhodes et al., 2011). Given that BaP can be converted into estrogen like metabolites which stimulate estrogen receptor alpha, *Ddx5* and *Cxcl12* up-regulation provides insight into a potential mechanism by which BaP may induce ovarian tumorigenesis (Halon et al., 2011; van Lipzig et al., 2005).

Two detoxifying enzymes, *Cyp1b1* and *Ugt1a10*, were also up-regulated by BaP exposure. Cyp1b1 is a cytochrome p450 oxidase which acts as a phase I detoxification enzyme in Ahr induced xenobiotic metabolism (Bengtsson et al., 1983; Mattison et al., 1983).

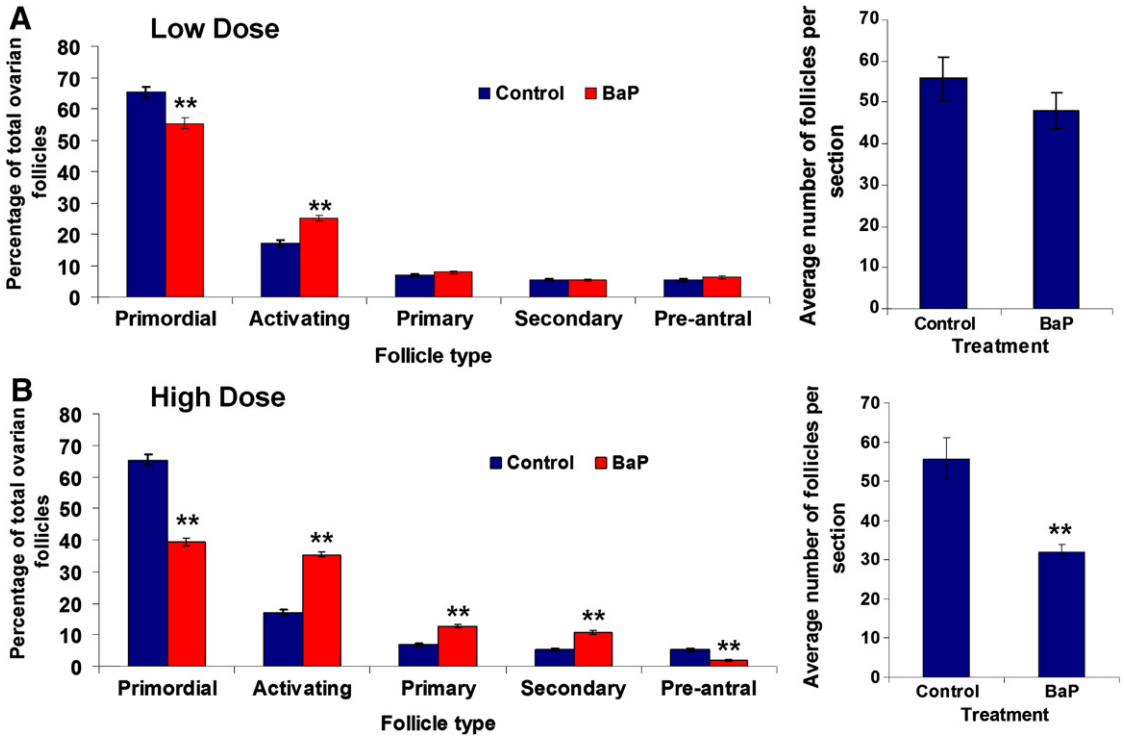


Fig. 5. Effect of BaP exposure on ovarian follicle composition and number *in vivo*. Neonatal mice (4 days old) were treated with either a low or high dose of BaP over a seven day period as described in materials and methods. Ovarian sections were stained with hematoxylin and eosin and healthy oocyte containing follicles were classified and counted under a microscope. (A) Low dose ovarian follicle composition (left panel) and average number of follicles per counted section (right panel). (B) High dose ovarian follicle composition (left panel) and average number of follicles per counted section (right panel). Values are mean ± SEM, n = 3–5 ovaries from 3 to 5 mice. The symbol ** represents $p < 0.01$ in comparison with control values.

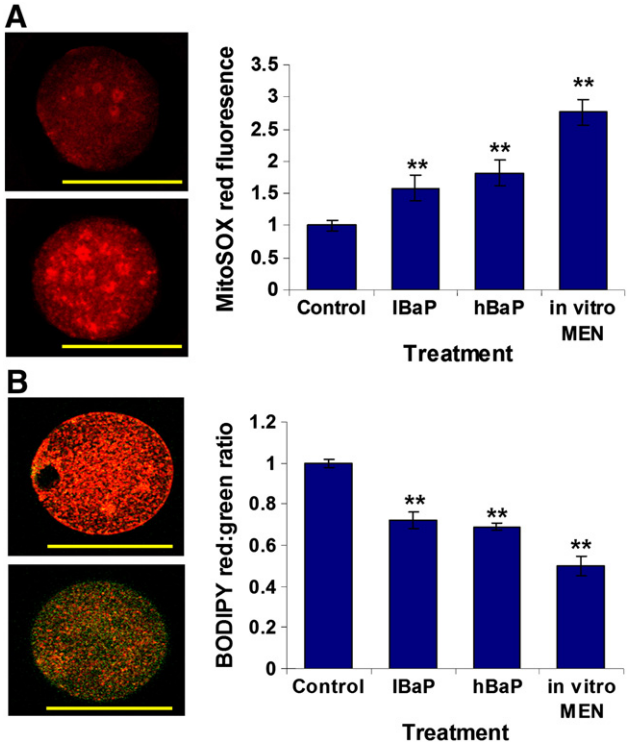


Fig. 6. Effect of neonatal BaP exposure on adult oocyte mitochondrial ROS production and lipid peroxidation *in vivo*. Neonatal mice (4 days old) were treated with either a low or high dose of BaP over a seven day period, weaned and super-ovulated at 6 weeks of age. Oocytes obtained from super-ovulated mice were then labelled with either MitoSOX red or BODIPY fluorescent dye as outlined in the materials and methods. (A) MitoSOX red results. Representative images of MitoSOX labelled control treated (top) and MEN treated (bottom) oocytes (left panel) and levels of mitochondrial ROS measured by red fluorescence (right panel). MEN (5 μM) was used as a representative of free radical ROS induced mitochondrial damage. Scale bar is equal to 100 μm. Results were normalised to control values for each experiment. Values are mean ± SE, n = 18–27 oocytes from 5 mice. IBaP = low dose; hBaP = high dose. The symbol ** represents $p<0.01$ in comparison with control values. (B) Representative images of BODIPY labelled control treated (top) and MEN treated (bottom) oocytes (left panel) and Levels of lipid peroxidation as measured by the ratio of red:green fluorescence (right panel). Scale bar is equal to 100 μm. Results were normalised to control values for each experiment. Values are mean ± SE, n = 18–27 oocytes from 5 mice. IBaP = low dose; hBaP = high dose. The symbol ** represents $p<0.01$ in comparison with control values.

Cyp1b1, along with cyp1a1, has been identified as the initial enzyme which results in the bioactivation of BaP into DNA adduct forming metabolites, estrogenic metabolites, and quinone like structures (Zhou et al., 2009).Ugt1a10 is a UDP-glucuronosyltransferase which acts as a phase II detoxification enzyme in the conversion of hydrophobic xenobiotics into water-soluble metabolites for excretion.Ugt1a10 metabolism results in the detoxification of Cyp1b1 bioactivated metabolites, and has been linked with BaP detoxification in the liver (Dellinger et al., 2006). Although increased Cyp1b1 expression has been detected in BaP exposed ovaries before, the identification of increased Ugt1a10 expression is novel, and provides further illumination of the detoxification of BaP metabolites in the ovary (Bengtsson et al., 1983; Mattison et al., 1983).

In support of BaP stimulating follicular growth and development, three genes associated with cellular proliferation (*Ccng1*, *Cdk2*, and *Mcm7*) were also up-regulated by BaP exposure. Ccng1 is a regulator of cell cycle progression at the G2/M phase associated with granulosa cell proliferation and differentiation (Kimura et al., 2001; Liu et al., 2006). Cdk2 is a cyclin dependant protein kinase responsible for G1/S cell cycle transition. Interestingly, increased Cdk2 expression has also been linked with accelerated primordial follicle activation, and increased primordial follicle survival (Rajareddy et al., 2007). Mcm7 is a DNA replication licensing factor essential for cellular proliferation and increased Mcm7 protein has also been linked to elevated levels of primordial follicle activation (Park et al., 2005).

Two other genes up-regulated by BaP exposure were *Hspa8*, and *Igf2*. Hspa8 is a heat shock protein which was also up-regulated in DMBA exposed neonatal ovaries (Sobinoff et al., 2011). Hspa8 has been shown to play a role in cyclin D1 maturation, and the formation of a catalytically active cyclin D1/CDK4/Cip/Kip protein complex implicated in cell cycle progression (Diehl et al., 2003). Igf2 is a growth factor hormone which shares structural similarities with insulin, and is instrumental in fetal and placental growth during gestation (Gicquel and Le Bouc, 2006). Ovarian expression of Igf2 has been localised to both the theca and granulose cells of developing follicles (Hernandez et al., 1990). It is thought Igf2 is produced in the theca cells, and acts as an autocrine and granulosa paracrine signal, promoting proliferation (Adashi et al., 1985; Davoren et al., 1986).

We have compiled our microarray results into a preliminary model of BaP induced ovotoxicity focusing on how the observed changes in gene expression relate to folliculogenesis (Fig. 8). The common themes seen throughout this model were apoptosis, tumorigenesis, and follicular growth/follicular activation. These results suggest an overlapping mechanism of BaP induced ovotoxicity involving follicular growth and atresia. In support of this PCNA, a marker of increased proliferation and follicular activation was up-regulated in the granulosa cells and oocytes of BaP treated primordial follicles (Fig. 3) (Picut et al., 2008; Tománek and Chronowska, 2006). However, markers of follicular atresia (Casp 2, Casp 3, TUNEL) were absent in BaP treated primordial follicles, and present in developing follicles (Fig. 3). Immunohistochemical and histomorphological analysis on BaP high dosed neonatal mice also detected increased follicular activation and developing follicular depletion *in vivo* (Figs. 4 and 5).

Collectively, these results support a mechanism of BaP induced ovotoxicity involving developing follicle atresia and increased primordial follicle recruitment. These observations coincide with our previous studies, in which we found DMBA and three other ovotoxic xenobiotics induced primordial follicle depletion via activation, not atresia (Sobinoff et al., 2010, 2011). In these studies we proposed that the observed increase in primordial follicle activation was due to a homeostatic mechanism of follicular replacement, whereby primordial follicle activation occurs in order to maintain the developing pool of developing follicles destroyed by xenobiotic exposure. To support this hypothesis we probed BaP cultured ovaries for AMH, a homeostatic regulator of primordial follicle growth secreted by developing follicles to prevent excessive follicular activation (Reddy et al., 2010). However, there was no difference in the staining pattern observed between control and BaP cultured ovaries (Supplementary Fig. 3). BaP induced primordial follicle activation may also be due to BaP induced ROS production. ROS play a physiological role in regulating signal transduction by selectively oxidising cysteine residues on proteins resulting in a variety of reversible molecular interactions (Wells et al., 2009). It is therefore conceivable that increased levels of BaP induced ROS could lead to abnormal cysteine oxidation and consequently dysregulated signal transduction. For example, the PI3K/Akt signalling pathway of primordial follicle activation has been shown to be up-regulated by increased levels of ROS through the H_2O_2 oxidation of phosphatases which negatively regulate the pathway (Kim et al., 2005; Naughton et al., 2009).

Another aim of this study was to determine if BaP *in vivo* exposure resulted in oocyte dysfunction via oxidative stress. According to the free radical hypothesis of mitochondrial ageing, non-renewing primordial follicles, which can remain quiescent for many years, gradually produce ROS through electron leakage from the mitochondrial electron transport chain (Tarin, 1996). Over time this ROS damages the mitochondrial membrane, leading to more electron leakage and further ROS production. It is hypothesised that in aged oocytes, mitochondrial ROS builds up to such high levels that it leads to oocyte dysfunction. It is therefore reasonable to assume that BaP induced ROS could exacerbate this process, damaging mitochondrial membrane

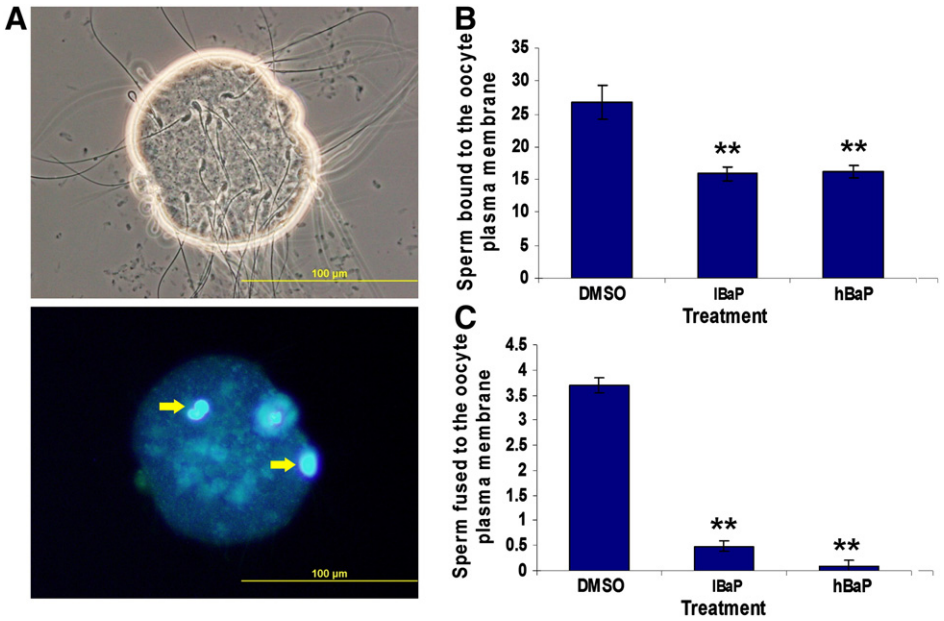


Fig. 7. Effect of neonatal BaP exposure on long term oocyte quality *in vivo*. Neonatal mice (4 days old) were treated with either a low or high dose of BaP over a seven day period, weaned and super-ovulated at 6 weeks of age. Collected zona-free oocytes were then co-incubated with sperm and assessed for impaired sperm egg binding and fusion as outlined in materials and methods. (A) Corresponding phase contrast and fluorescent microscopy images of control zona-free oocyte after sperm egg binding assay. Arrows = fused sperm nuclei. Scale bar is equal to 100 µm. (B) Number of sperm heads bound to zona-free oocytes after co-incubation. (C) Number of fused sperm observed after co-incubation. Values are mean ± SE, n = 12–25 oocytes from 4 mice. lBaP = low dose; hBaP = high dose. The symbol ** represents $p<0.01$ in comparison with control values.

integrity resulting in oocyte dysfunction. In support of this hypothesises, increased levels of mitochondrial ROS and oolemma lipid peroxidation were detected in oocytes obtained from adult mice treated with either a low or high dose of BaP over a 7 day period after birth (Fig. 6). Sperm-egg fusion assays also revealed severely reduced sperm-egg binding and fusion in both the high and low dose exposed groups (Fig. 7). Interestingly, there was no observable difference in oocyte dysfunction between exposed groups, indicating low level exposure which does not result in significant follicular depletion can induce the same level of oocyte dysfunction as the highest dose tested i.e. follicular depletion is independent of dysfunction (Fig. 5).

Taken together, these results suggest short term BaP exposure causes significant mitochondrial ROS leakage/dysfunction resulting in considerable plasma membrane lipid peroxidation and perturbed fertilisation. Interestingly, the severely decreased levels of sperm oocyte fusion observed in BaP exposed oocytes mimics the failure of fertilisation which occurs in oocytes obtained from female IVF

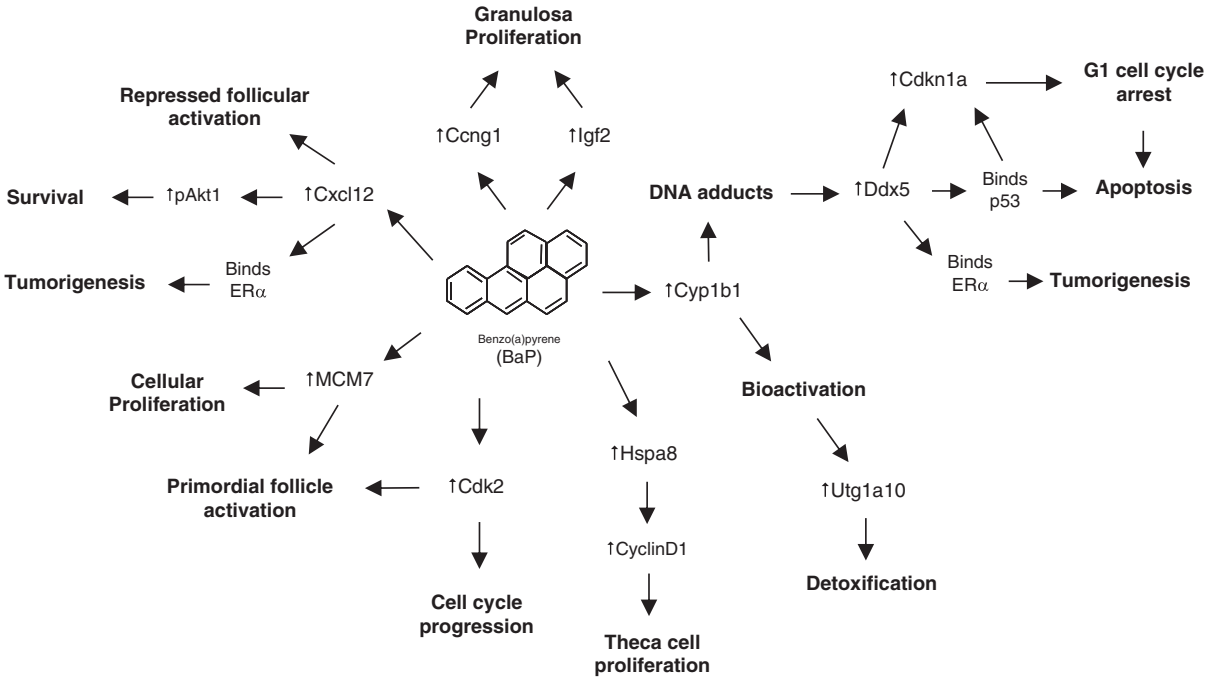


Fig. 8. Summary of qPCR results generating a preliminary model of the ovotoxic effects of BaP on ovarian folliculogenesis. BaP influenced the expression of a number of genes which either directly or non-directly associated with apoptosis, tumorigenesis, and follicular growth/development. ↑ represents up-regulation.

patients who smoke (Gruber et al., 2008). Given that BaP is detected at high concentrations in the follicular fluid of female IVF patients who smoke, we hypothesise BaP exposure may be a significant cause of cigarette smoke induced female sub-fertility. The results obtained in the sperm-egg fusion assay also correspond with that found previously in our studies with other ovotoxic xenobiotics, and suggest that short term xenobiotic exposure can cause lasting effects on female fertility in the form of xenobiotic ROS induced oocyte dysfunction, even after the offending toxin has been removed from the ovarian environment (Sobinoff et al., 2010).

In conclusion, the findings of this study suggest BaP exposure results in primordial follicle depletion via a complex mechanism of ovotoxicity involving developing follicle atresia and accelerated primordial follicle activation. This mechanism appears similar to that observed in other xenobiotic exposed models, and may represent a common mechanism by which some ovotoxic xenobiotics induce primordial follicle depletion. This study also demonstrates short term neonatal BaP exposure causes mitochondrial leakage resulting in reduced oolemma fluidity and impaired fertilisation in adulthood, a mechanism which may be partially responsible for the chronic levels of female sub-fertility observed in female smokers.

Supplementary materials related to this article can be found online at doi:10.1016/j.taap.2012.01.028.

Role of the funding source

Funding sources approved the overall study design as a condition of their support (Project grants).

Disclosure statement

The authors declare no conflict of interest.

Acknowledgments

The authors gratefully acknowledge the financial assistance to EAM by the Australian Research Council, Hunter Medical Research Institute and the Newcastle Permanent Building Society Charitable Trust. APS is the recipient of an Australian Postgraduate Award PhD scholarship. This work was supported by National Health and Medical Research Council (Project grant #510735) to EAM, SDR and BN.

References

Adashi, E.Y., Resnick, C.E., D'Ercole, A.J., Svoboda, M.E., Van Wyk, J.J., 1985. Insulin-like growth factors as intraovarian regulators of granulosa cell growth and function. Endocr. Rev. 6, 400–420.

Bengtsson, M., Montelius, J., Mankowitz, L., Rydstrom, J., 1983. Metabolism of polycyclic aromatic hydrocarbons in the rat ovary comparison with metabolism in adrenal and liver tissues. Biochem. Pharmacol. 32, 129–136.

Binkova, B., Giguere, Y., RossnerJr, P., Dostal, M., Sram, R.J., 2000. The effect of dibenzo [a, l] pyrene and benzo [a] pyrene on human diploid lung fibroblasts: the induction of DNA adducts, expression of p53 and p21WAF1 proteins and cell cycle distribution. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 471, 57–70.

Bolton, J.L., Trush, M.A., Trevor, M., Dryhurst, G., Monks, T.J., 2000. Role of quinones in toxicology. Chem. Res. Toxicol. 13, 135–160.

Borman, S.M., Christian, P.J., Sipes, I.G., Hoyer, P.B., 2000. Ovotoxicity in female Fischer rats and B6 mice induced by low-dose exposure to three polycyclic aromatic hydrocarbons: comparison through calculation of an ovotoxic index. Toxicol. Appl. Pharmacol. 167, 191–198.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Couse, J.F., Korach, K.S., 1999. Estrogen receptor null mice: what have we learned and where will they lead us? Endocr. Rev. 20, 358–417.

Davoren, J.B., Kasson, B.G., Li, C.H.A.O., Hsueh, A.J.W., 1986. Specific insulin-like growth factor (IGF) I-and II-binding sites on rat granulosa cells: relation to IGF action. Endocrinology 119, 2155–2162.

Dellinger, R.W., Fang, J.L., Chen, G., Weinberg, R., Lazarus, P., 2006. Importance of UDP-glucuronosyltransferase 1A10 (UGT1A10) in the detoxification of polycyclic aromatic hydrocarbons: decreased glucuronidative activity of the UGT1A10139Lys isoform. Drug Metab. Dispos. 34, 943–949.

Diehl, J.A., Yang, W., Rimerman, R.A., Xiao, H., Emili, A., 2003. Hsc70 regulates accumulation of cyclin D1 and cyclin D1-dependent protein kinase. Mol. Cell. Biol. 23, 1764–1774.

El-Nemr, A., Al-Shawaf, T., Sabatini, L., Wilson, C., Lower, A.M., Grudzinskas, J.G., 1998. Effect of smoking on ovarian reserve and ovarian stimulation in in-vitro fertilization and embryo transfer. Hum. Reprod. 13, 2192–2198.

Freour, T., Masson, D., Mirallie, S., Jean, M., Bach, K., Dejoie, T., Barriere, P., 2008. Active smoking compromises IVF outcome and affects ovarian reserve. Reprod. Biomed. Online 16, 96–102.

Fuller-Pace, F.V., Moore, H.C., 2011. RNA helicases p68 and p72: multifunctional proteins with important implications for cancer development. Future Oncol. 7, 239–251.

Gicquel, C., Le Bouc, Y., 2006. Hormonal regulation of fetal growth. Horm. Res. Paediatr. 65, 28–33.

Gruber, I., Just, A., Birner, M., Losch, A., 2008. Effect of a woman's smoking status on oocyte, zygote, and day 3 pre-embryo quality in in vitro fertilization and embryo transfer program. Fertil. Steril. 90, 1249–1252.

Halon, A., Materna, V., Drag-Zalesinska, M., Nowak-Markwitz, E., Gansukh, T., Donizy, P., Spaczynski, M., Zabel, M., Dietel, M., Lage, H., 2011. Estrogen receptor alpha expression in ovarian cancer predicts longer overall survival. Pathol. Oncol. Res. 17, 511–518.

Hernandez, E.R., Roberts, C.T., Hurwitz, A., LeRoith, D., Adashi, E.Y., 1990. Rat ovarian insulin-like growth factor II gene expression is theca-interstitial cell-exclusive: hormonal regulation and receptor distribution. Endocrinology 127, 3249–3251.

Holt, J.E., Jackson, A., Roman, S.D., Aitken, R.J., Koopman, P., McLaughlin, E.A., 2006. CXCR4/SDF1 interaction inhibits the primordial to primary follicle transition in the neonatal mouse ovary. Dev. Biol. 293, 449–460.

Howe, G., Westhoff, C., Vessey, M., Yeates, D., 1985. Effects of age, cigarette smoking, and other factors on fertility: findings in a large prospective study. Br. Med. J. (Clin. Res. Ed) 290, 1697–1700.

Keating, A.F., Fernandez, S.M., Mark-Kappeler, C.J., Sen, N., Sipes, I.G., Hoyer, P.B., 2010. Inhibition of PIK3 Signaling Pathway Members by the Ovotoxicant 4-Vinylcyclohexene Diepoxide in Rats. Biol. Reprod. 84, 743–751.

Kim, J.H., Chu, S.C., Gramlich, J.L., Pride, Y.B., Babendreier, E., Chauhan, D., Salgia, R., Podar, K., Griffin, J.D., Sattler, M., 2005. Activation of the PI3K/mTOR pathway by BCR-ABL contributes to increased production of reactive oxygen species. Blood 105, 1717–1723.

Kimura, S.H., Ikawa, M., Ito, A., Okabe, M., Nojima, H., 2001. Cyclin G1 is involved in G2/M arrest in response to DNA damage and in growth control after damage recovery. Oncogene 20, 3290–3300.

Liu, Z.H., Zhang, H., He, Y.P., Zhang, J.H., Yue, L.M., 2006. Cyclin G1 expressing in mouse ovary and relating to follicular development. Sichuan Da Xue Xue Bao Yi Xue Ban 37, 893–897.

Lodovici, M., Akpan, V., Evangelisti, C., Dolara, P., 2004. Sidestream tobacco smoke as the main predictor of exposure to polycyclic aromatic hydrocarbons. J. Appl. Toxicol. 24, 277–281.

Mattison, D.R., Thorgeirsson, S.S., 1979. Ovarian aryl hydrocarbon hydroxylase activity and primordial oocyte toxicity of polycyclic aromatic hydrocarbons in mice. Cancer Res. 39, 3471–3475.

Mattison, D.R., Shiromizu, K., Nightingale, M.S., 1983. Oocyte destruction by polycyclic aromatic hydrocarbons. Am. J. Ind. Med. 4, 191–202.

Naughton, R., Quiney, C., Turner, S.D., Cotter, T.G., 2009. Bcr-Abl-mediated redox regulation of the PI3K/AKT pathway. Leukemia 23, 1432–1440.

Neal, M.S., Hughes, E.G., Holloway, A.C., Foster, W.G., 2005. Sidestream smoking is equally as damaging as mainstream smoking on IVF outcomes. Hum. Reprod. 20, 2531–2535.

Neal, M.S., Zhu, J., Holloway, A.C., Foster, W.G., 2007. Follicle growth is inhibited by benzo-[a]-pyrene, at concentrations representative of human exposure, in an isolated rat follicle culture assay. Hum. Reprod. 22, 961–967.

Neal, M.S., Zhu, J., Foster, W.G., 2008. Quantification of benzo [a] pyrene and other PAHs in the serum and follicular fluid of smokers versus non-smokers. Reprod. Toxicol. 25, 100–106.

Nicol, S.M., Fuller-Pace, F.V., 2010. Analysis of the RNA helicase p68 (Ddx5) as a transcriptional regulator. Methods Mol. Biol. 587, 265–279.

Park, C.E., Cha, K.Y., Kim, K., Lee, K.A., 2005. Expression of cell cycle regulatory genes during primordial-primary follicle transition in the mouse ovary. Fertil. Steril. 83, 410–418.

Picut, C.A., Swanson, C.L., Scully, K.L., Roseman, V.C., Parker, R.F., Remick, A.K., 2008. Ovarian follicle counts using proliferating cell nuclear antigen (PCNA) and semi-automated image analysis in rats. Toxicol. Pathol. 36, 674.

Rajareddy, S., Reddy, P., Du, C., Liu, L., Jagarlamudi, K., Tang, W., Shen, Y., Berthet, C., Peng, S.L., Kaldis, P., 2007. p27kip1 (cyclin-dependent kinase inhibitor 1B) controls ovarian development by suppressing follicle endowment and activation and promoting follicle atresia in mice. Mol. Endocrinol. 21, 2189–2202.

Ramesh, A., Archibong, A.E., Niaz, M.S., 2010. Ovarian Susceptibility to Benzo [a] Pyrene: Tissue Burden of Metabolites and DNA Adducts in F-344 Rats. J. Toxicol. Environ. Health A 73, 1611–1625.

Reddy, P., Zheng, W., Liu, K., 2010. Mechanisms maintaining the dormancy and survival of mammalian primordial follicles. Trends Endocrinol. Metab. 21, 96–103.

Rhodes, L.V., Bratton, M.R., Zhu, Y., Tilghman, S.L., Muir, S.E., Salvo, V.A., Tate, C.R., Elliott, S., Nephew, K.P., Collins-Burow, B.M., 2011. Effects of SDF-1-CXCR4 signaling on microRNA expression and tumorigenesis in estrogen receptor-alpha (ER-alpha)-positive breast cancer cells. Exp. Cell Res. 371, 2573–2581.

Robles, R., Morita, Y., Mann, K.K., Perez, G.I., Yang, S., Matikainen, T., Sherr, D.H., Tilly, J.L., 2000. The aryl hydrocarbon receptor, a basic helix-loop-helix transcription

factor of the PAS gene family, is required for normal ovarian germ cell dynamics in the mouse. Endocrinology 141, 450–453.
Rustemeier, K., Stabbert, R., Haussmann, H.J., Roemer, E., Carmines, E.L., 2002. Evaluation of the potential effects of ingredients added to cigarettes. Part 2: Chemical composition of mainstream smoke* 1. Food Chem. Toxicol. 40, 93–104.
Slot, K.A., Kastelijn, J., Bachelot, A., Kelly, P.A., Binart, N., Teerds, K.J., 2006. Reduced recruitment and survival of primordial and growing follicles in GH receptor-deficient mice. Reproduction 131, 525–532.
Sobinoff, A.P., Pye, V., Nixon, B., Roman, S.D., McLaughlin, E.A., 2010. Adding Insult to Injury: Effects of Xenobiotic-Induced Preantral Ovotoxicity on Ovarian Development and Oocyte Fusibility. Toxicol. Sci. 118, 653–666.
Sobinoff, A.P., Mahony, M., Nixon, B., Roman, S.D., McLaughlin, E.A., 2011. Understanding the Villain: DMBA-Induced Preantral Ovotoxicity Involves Selective Follicular Destruction and Primordial Follicle Activation through PI3K/Akt and mTOR Signaling. Toxicol. Sci. 123, 563–575.
Sobinoff, A.P., Bernstein, I.R., McLaughlin, E.A., in press. All Your Eggs in One Basket; Mechanisms of Xenobiotic Induced Female Reproductive Senescence. In *Senescence* (T. Nagata, Ed.). InTech Press Rijeka, Croatia (accepted 20/09/11).
Sun, L., Tan, L., Yang, F., Luo, Y., Li, X., Deng, H.W., Dvornyk, V., 2011. Meta-analysis suggests that smoking is associated with an increased risk of early natural menopause. Menopause. doi:10.1097/gme.1090b1013e318224f318229ac.
Tarin, J.J., 1996. Potential effects of age-associated oxidative stress on mammalian oocytes/embryos. Mol. Hum. Reprod. 2, 717–724.
Tománek, M., Chronowska, E., 2006. Immunohistochemical localization of proliferating cell nuclear antigen (PCNA) in the pig ovary. Folia Histochem. Cytobiol. 44, 269.
van Lipzig, M.M.H., Vermeulen, N.P.E., Gusinu, R., Legler, J., Frank, H., Seidel, A., Meerman, J.H.N., 2005. Formation of estrogenic metabolites of benzo [a] pyrene and chrysene by cytochrome P450 activity and their combined and supramaximal estrogenic activity. Environ. Toxicol. Pharmacol. 19, 41–55.
Wells, P.G., McCallum, G.P., Chen, C.S., Henderson, J.T., Lee, C.J.J., Perstin, J., Preston, T.J., Wiley, M.J., Wong, A.W., 2009. Oxidative stress in developmental origins of disease: teratogenesis, neurodevelopmental deficits, and cancer. Toxicol. Sci. 108, 4–18.
Windham, G.C., Mitchell, P., Anderson, M., Lasley, B.L., 2005. Cigarette smoking and effects on hormone function in premenopausal women. Environ. Health Perspect. 113, 1285–1290.
Yano, T., Liu, Z., Donovan, J., Thomas, M.K., Habener, J.F., 2007. Stromal Cell Derived Factor-1 (SDF-1)/CXCL12 Attenuates Diabetes in Mice and Promotes Pancreatic Cell Survival by Activation of the Prosurvival Kinase Akt. Diabetes 56, 2946–2957.
Zenzes, M.T., 2000. Smoking and reproduction: gene damage to human gametes and embryos. Hum. Reprod. Update 6, 122–131.
Zhou, S.F., Liu, J.P., Chowbay, B., 2009. Polymorphism of human cytochrome P450 enzymes and its clinical impact. Drug Metab. Rev. 41, 89–295.

Chapter 4: Staying alive: PI3K pathway promotes primordial follicle activation and survival in response to 3-MC induced ovotoxicity

Toxicological Sciences
ToxSci Advance Access published April 12, 2012

Staying alive: PI3K pathway promotes primordial follicle activation and survival in response to 3-MC induced ovotoxicity

Sobinoff, A.P.[1], Nixon, B.[1,2], Roman, S.D.[1,2] and McLaughlin, E.A.[1,2]

[1]Reproductive Science Group, School of Environmental & Life Sciences and

[2]ARC Centre of Excellence in Biotechnology & Development, University of Newcastle, Callaghan, NSW2308, Australia

Running Title: 3MC induced follicle survival/growth/atresia

[3]Corresponding Author:

Professor Eileen McLaughlin

Discipline of Biological Sciences

ARC Centre of Excellence in Biotechnology & Development

School of Environmental & Life Sciences

University of Newcastle

Callaghan, NSW 2308, Australia

Phone: +61 2 4921 5708

Fax: +61 2 4921 6308

E-mail: eileen.mclaughlin@newcastle.edu.au

Abstract

3-methylcholanthrene (3MC) is a potent ovotoxicant capable of causing premature ovarian failure through primordial follicle depletion. Despite 3MCs ovotoxicity having been established for 30 years, relatively little information exists on the mechanisms. In this study we examined the effects of 3MC exposure on the immature ovarian follicle population. Microarray analysis revealed a complex mechanism of 3MC induced ovotoxicity involving a number of cellular processes associated with xenobiotic metabolism, ovarian cancer, cell cycle progression, and cell death. 3MC exposure was also found to induce developing follicle atresia and aberrant primordial follicle activation via the stimulation of PI3K/Akt and mTOR signalling pathways. Inhibition of PI3K/Akt signalling resulted in the severe depletion of the primordial follicle pool, with further analysis identifying increased Akt1 stimulated Bad phospho-inhibition in 3MC-treated primordial follicles. Our results suggest that the primordial follicle pool enters a "pro-survival" state upon 3MC exposure, and that its depletion is due to a vicious cycle of primordial follicle activation in an attempt to replace developing follicles undergoing follicular atresia.

Keywords: 3-methylcholanthrene, ovary, primordial follicle, cellular survival, PI3K/Akt/mTOR signalling

Introduction

The major function of the mammalian ovary is the development of mature oocytes for ovulation and fertilisation. Oocyte maturation occurs in ovarian follicles, which are first assembled within the primitive ovary, when primary oocytes are enveloped by a single layer of flattened pre-granulosa cells (McNatty *et al.* 2000). The resulting structure is known as the primordial follicle, the most immature stage of follicular development. These precious follicles cannot be regenerated post foetal development, and represent the total number of germ cells available to the mammalian female throughout her entire reproductive life (Edson *et al.* 2009). Primordial follicles remain in a quiescent state for many months or years until they are selectively recruited to join the growing population. This event occurs in regular waves, and is continuous from birth until the primordial follicle pool becomes depleted, resulting in menopause (McGee and Hsueh 2000). Overall, < 1% of all recruited follicles will complete maturation and undergo ovulation, with the vast majority being lost to an apoptotic process known as atresia (Hirshfield 1991). Therefore, the female reproductive lifespan can be shortened if anything untoward happens to the primordial follicle pool, such as aberrant activation and/or follicular atresia (McLaughlin and McIver 2009).

An increasing trend in the number of women opting to delay childbirth (>30 years) has led to an increased awareness of the impact of environmental chemicals on reproductive function (Hamilton *et al.* 2010). Ongoing research has identified a number of ovotoxic xenobiotics which specifically target ovarian follicles for destruction, leading to perturbed fertility (Mark-Kappeler *et al.* 2011). Of chief interest are those ovotoxic xenobiotics which specifically target irreplaceable primordial follicles, resulting in premature ovarian failure (POF). Evidence suggests that these ovotoxic chemicals cause POF by inducing pro-apoptotic signalling events (Caspase activation, pro-apoptotic Bcl2 signalling) resulting in primordial follicle atresia, and by stimulating members of the PI3K/Akt and mTOR signalling pathways, resulting in accelerated primordial follicle activation (Hu *et al.* 2001; Matikainen *et al.* 2001; Sobinoff *et al.* 2012a; Sobinoff *et al.* 2011). Although patterns are starting to emerge as to how environmental ovotoxicants cause primordial follicle depletion, the mechanisms underlying their ovotoxicity remain largely unknown (Sobinoff *et al.* 2012a).

One group of environmental chemicals which are notorious reproductive toxicants are the polycyclic aromatic hydrocarbons (PAH). PAH are major constituents of cigarette smoke, and are produced via various combustion reactions (including automotive exhaust and charbroiled foods), making these compounds ubiquitous pollutants (CDC 2009). The three PAH Benzo(a)pyrene (BaP), 9:10-dimethyl-1:2-benzanthracene (DMBA), and 3-methylcholanthrene (3MC) are all thought to specifically target immature follicles for destruction, causing POF (Borman *et al.* 2000; Mattison *et al.* 1983). Although there are a number of studies characterising the ovarian response to both BaP and DMBA exposure, relatively little information exists on the mechanisms behind 3MC induced

ovotoxicity (Bhattacharya and Keating 2011; Neal *et al.* 2007; Sobinoff *et al.* 2011; Sobinoff *et al.* 2012b). Initial studies in rodents suggest that 3MC specifically targets immature primordial follicle oocytes for destruction (Borman *et al.* 2000; Mattison 1980). Inhibition of the aryl-hydrocarbon receptor by alpha-naphthoflavone nullifies 3MC induced primordial follicle depletion, revealing its ovotoxicity is dependant upon metabolic bioactivation (Shiromizu and Mattison 1985).

In order to further characterise the mechanisms behind 3MC induced ovotoxicity we examined its effects on the ovarian transcriptome of cultured neonatal mouse ovaries. Microarray analysis revealed 3MC significantly altered the expression of genes involved in cancer, cellular growth and proliferation, and cell death. In contrast to the current literature, histomorphological and immunohistological analysis revealed 3MC specifically targeted developing follicles for destruction, not primordial follicles. Our results suggest that 3MC exposure induces primordial follicle depletion via an overstimulation of primordial follicle activation involving downstream members of the PI3K/Akt and mTOR signalling pathways (McLaughlin and Sobinoff 2010). However, inhibition of PI3K using the inhibitor LY294002 resulted in the severe depletion of the primordial follicle pool, suggesting that PI3K/Akt signalling prevents 3MC induced primordial follicle destruction in addition to promoting primordial follicle activation. We also provide evidence of increased pro-survival BAD S136 phosphorylation in 3MC exposed primordial follicles, indicating the population enters a "pro-survival state" upon exposure to 3MC.

Methods

Reagents: 3MC (≥95% purity), LY294002 (L9908), Rapamycin (R0395) 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) Rabbit polyclonal anti-Foxo3a (anti-Foxo3a, ab47409), Rabbit polyclonal anti-Tsc2 (phospho S939) (anti-pTsc2 (S939), ab59269), and Rabbit polyclonal anti-Bad (phosphor S139) (anti-pBad (S139), ab28824) were obtained from Abcam (Cambridge, MA). Mouse monoclonal anti-human Anti-Müllerian hormone (anti-AMH, MCA2246) was obtained from AbD Serotec (Kidlington, UK). Rabbit polyclonal anti-Akt (anti-Akt, #9272) was obtained from Cell Signalling Technologies (Beverly, MA). Alexa Fluor 594 goat anti-rabbit IgG (A11012), Alexa Fluor 594 goat anti-mouse IgG (A11005), fetal bovine serum (FBS), L-Glutamine, and Insulin-Transferrin-Selenium (ITS) were purchased from the Invitrogen Co. (Carlsbad, CA). L-Ascorbic Acid was obtained from MP Biomedicals (Solon, OH). Rabbit polyclonal anti-phospho-mTOR (phosphor S2448) (anti-pmTOR (S2448), 09-213SP), Rabbit polyclonal anti-phospho-mTOR (phosphor T2446)

(anti-pmTOR (T2446), 09-345SP), and 0.4μm Culture Plate Inserts were purchased from Millipore (Billerica, MA). All culture dishes and cell culture plates were obtained from Greiner Bio-One (Monroe, NC). Oligo(dT)15 primer, RNasin, dNTPs, M-MLV-Reverse Transcriptase, RQ1 DNase, GoTaq Flexi, $MgCl_2$, GoTaq 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). 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 21–22°C and supplied with food and water *ad libitum*.

Animal dosing: Female Swiss neonatal mice (day 4; 6-10 animals/treatment group) were weighed and administered (intraperitoneal; i.p.) 7 daily, consecutive doses of either sesame oil containing vehicle control (<10μl/kg/daily DMSO) or sesame oil containing a low and high dose of 3MC (5mg and 10mg/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 CO_2 asphyxiation 24 hours after the last injection.

Ovarian Culture: Ovaries from day 3-4 Swiss neonatal mice were cultured as described previously (Sobinoff *et al.* 2010). Briefly, Swiss neonates were sacrificed by CO_2 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 1.5ml DMEM/F12 medium containing 5% (v/v) fetal calf serum, 1mg/ml bovine serum albumin, 50μg/ml ascorbic acid, 27.5 μg/ml insulin–transferrin–selenium, 2.5 mM glutamine and 5U/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% CO_2 in air, with media changes every two days. Pilot studies performed in our laboratory identified this time point as a "middle ground" between the initial and end stages of 3MC induced ovotoxicity (i.e. after exposure and before complete follicular depletion). This would allow us to identify both causative and responsive changes in ovarian gene expression induced by 3MC exposure. Ovaries were treated with vehicle control medium (0.01% DMSO) or 3MC (5 μM) ± LY294002 (15 μM) or Rapamycin (50 nM). The 3MC and LY294002/Rapamycin culture concentrations were 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 (4μm 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 4 cuboidal granulosa cells in a single layer. Secondary follicles were identified as those with two layers of granulosa cells, and pre-antral 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 pre-antral stage.

Immunohistochemistry: Ovaries for immunohistochemistry were fixed in Bouin's and sectioned 4 µm thick. PCNA, active Casp2, active Casp3, AMH, Akt, pAkt1, pmTOR, pTsc2, pBad and Foxo3a were stained using the same protocol with the exception of the primary antibody. Slides were deparaffinised in xylene and rehydrated with subsequent washes in ethanol. Antigen retrieval was carried out by microwaving sections for 3×3min in Tris buffer (50 mM, pH 10.6). Sections were then blocked in 3% BSA/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 IgG, Alexa Fluor 594 goat anti-mouse IgG; 1:200 dilution) for 1 h. Slides were then counter-stained with 4'-6-Diamidino-2-phenylindole (DAPI) for 5 min, mounted in Mowiol and observed on an Axio Imager A1 fluorescence microscope (Carl Zeiss MicroImaging, Inc, Thornwood, NY) under fluorescence optics and pictures taken using a Olympus DP70 microscope camera (Olympus America, Center Valley, PA). Protein staining was quantified according to Cy5 intensity in primordial follicle oocytes using ImageJ software (NCBI). Negative controls lacking the primary antibody were performed alongside each experiment (Supplementary Figure 3).

TUNEL Analysis: Bouin's fixed sections were deparaffinised 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. TUNEL analysis was then performed using an In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics Pty Ltd.; Dee Why, NSW) according to the manufacturer's instructions. Slides were then counter-stained with DAPI for 5min, mounted in Mowiol and observed using an Axio Imager A1 epifluorescent microscope (Carl Zeiss) and images captured using a Olympus DP70 microscope camera (Olympus). Negative controls lacking the TUNEL enzyme and positive controls pre-treated with DNase enzyme were performed alongside each experiment (Supplementary Figure 4).

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

Real Time PCR (QPCR): Reverse transcription was performed with 2 µg of isolated RNA, 500 ng oligo(dT)15 primer, 40 U of RNasin, 0.5 mM 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, USA). Primer sequences along with annealing temperatures have been supplied as supplementary data (sTable3). 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 $2^{-\Delta C(t)}$, where $C(t)$ is the cycle at which fluorescence was first detected above background fluorescence. Data were normalized to *cyclophilin*, 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 3MC-cultured neonatal ovaries and prepared for microarray analysis at the Australian Genome Research Facility (AGRF) using an Illumina Sentrix Mouse ref8v2 Beadchip. Labelling, hybridising, washing and array scanning were performed by the AGRF using the Illumina manual on an Illumina BeadArray Reader, and normalised according to the quantile normalisation 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 1.5 fold difference in gene expression ($p<0.05$) determined through the use of a 'volcano plot' were then analysed using Ingenuity Pathways Analysis (Ingenuity Systems, Redwood City, CA) software to identify canonical signalling pathways influenced by 3MC exposure. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE35836 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35836).

Statistics: Comparisons between the control and 3MC-treated groups were performed using one-way analysis of variance (ANOVA), Student's t-test, and Tukey's Honestly Significant Difference test. The assigned level of significance for all tests was $p<0.05$.

Results

Effects of 3MC exposure on the neonatal ovarian transcriptome

3MC exposure caused a significant change in ovarian gene expression, altering the expression of 428 genes representing 1.7% of the total number present on the array (Fig. 1A). Though the use of Ingenuity Pathway Analysis (IPA) software these altered genes were identified as components of molecular networks implicated in cell cycle regulation, cancer, cell death, cell to cell signalling, free radical scavenging, and drug metabolism (Fig. 1B). Grouping according to their molecular and cellular function revealed a large proportion of significantly altered genes were also implicated in cellular growth and proliferation, genetic disorder, and reproductive system disease (Table 1; supplementary table 1). These results suggest that 3MC exposure alters a small subset of genes involved in a variety of cellular processes which contribute to its ovotoxicity.

Canonical pathways significantly up-regulated by 3MC exposure

In order to further characterise the mechanisms behind 3MC induced ovotoxicity, differentially expressed genes were also analysed for signalling pathways and molecular functions using IPA (Fig 2; sTable 2). 3MC exposure influenced genes belonging to pathways involved in xenobiotic metabolism (Aryl hydrocarbon receptor signalling, xenobiotic metabolism signalling), tumorigenesis (molecular mechanisms of cancer, ovarian cancer signalling), cell death and DNA damage repair (p53 signalling, cell cycle G2/M DNA damage checkpoint regulation, cell cycle G1/S checkpoint regulation), cellular proliferation (cyclins and cell cycle regulation), and folliculogenesis (Aryl hydrocarbon receptor signalling, ILK signalling, PI3K/Akt signalling). The up-regulation of pathways associated with increased proliferation and follicular development is intriguing, as it suggests 3MC induced ovotoxicity is not limited to primordial follicle atresia.

QPCR validation of microarray results

Validation of microarray results was performed by examining the levels of expression for different genes using QPCR (Table 2). Similar gene expression patterns were observed for all targets measured by QPCR when compared to the results of the microarray gene expression study. Of these genes, five were associated with xenobiotic detoxification/metabolism (*Cyp1b1*, *Aldh1l2*, *Aldh4a1*, *Nog1*, and *Ugt1a10*), three with increased cellular proliferation and follicular development (*Ccng1*, *Ccnd1*, and *Hspa8*), two with cell cycle arrest and apoptosis (*Cdkn1a*, and *Aldh1l2*), and one with increased cell stress (*Hspa8*). These results provide insight into ovarian 3MC metabolism, and support the notion 3MC exposure is not limited to primordial follicle destruction.

3MC exposure induces developing follicle atresia and primordial follicle activation in vitro and in vivo

In order to gain a better understanding of the effects of 3MC exposure on the primordial follicle pool we cultured neonatal ovaries in the presence of 3MC and probed for markers of follicular atresia and proliferation (Fig. 3). The early markers of apoptosis, Casp2 and Casp3, were both detected in 3MC-treated developing follicle oocytes and granulosa cells, but were absent in 3MC-treated primordial follicles. TUNEL, a technique used to detect DNA strand breaks and therefore end stage atresia, was also detected in 3MC-treated developing follicles and absent from treated primordial follicles. These results suggest that 3MC specifically targets developing follicles for destruction, not primordial follicles. PCNA, a marker of cellular proliferation and primordial follicle activation, was detected in the majority of 3MC-treated primordial follicle oocyte and granulosa cells, suggesting increased levels of primordial follicle activation (Fig 3). Again these results are intriguing; as they suggest that 3MC induced primordial follicle depletion may be due to excessive activation, not atresia.

To confirm the observed effects of 3MC exposure on folliculogenesis *in vivo*, we treated PND4 neonatal mice with both low (5mg/kg/daily) and high (10mg/kg/daily) dose exposures over a period of seven days and collected their ovaries for analysis. PCNA was detected in large groups of primordial follicles reminiscent of localisation patterns observed *in vitro* in the high dose treated primordial follicles, but not in the low dose and control-treated primordial follicles (Fig. 4). Histomorphological analysis revealed a slight reduction in primordial follicle composition coupled with a comparable increase in activating and primary follicles (Fig. 5A). There was no difference between the average number of follicles per section between the vehicle control (DMSO) and low dose treated ovaries. The high dose treatment induced a significant decrease in primordial follicle composition (~2-fold) and a significant increase in activating follicle composition (~3-fold) (Fig. 5B). There was also a significant decrease in the total number of follicles per section between the high dose and control-treatment (69% of the control). These results suggest that considerable 3MC induced follicular depletion occurs alongside the profound primordial follicle activation observed *in vivo*.

3MC exposure increases primordial follicle Akt1 phosphorylation

To investigate the mechanisms behind 3MC induced primordial follicle activation, we probed cultured neonatal ovaries for total Akt protein, pAkt1 (S473), and pAkt1 (T308) (Fig. 6).Total Akt protein was detected in both the oocyte (cytoplasm and nucleus) and granulosa cells of 3MC and control-treated follicles at all stages of folliculogenesis, with no distinguishable differences between the two treatments (Fig. 6A). Both pAkt1 (S473) and pAkt1 (T308) were also detected in the oocyte and granulosa cells at all stages of folliculogenesis (Fig. 6B and 6C). However, quantitative analysis revealed a significant up-regulation in pAkt1 (S473) and pAkt1 (T308) phosphorylation in 3MC-treated primordial follicle oocytes (~1.5-fold) (Fig. 6B and 6C). Levels of pAkt1 (S473) and pAkt1

(T308) did not differ between 3MC-treated and control developing follicles (Supplementary figure. 5). These results suggest that 3MC exposure causes increased primordial follicle Akt1 phosphorylation; an event which is crucial for PI3K/Akt induced follicular activation.

3MC exposure up-regulates positive mTOR signalling in primordial follicle oocytes

Given the positive up-regulation of Akt1 phosphorylation in 3MC-treated primordial follicles, we next observed the levels of downstream targets pTsc2 (S930), pmTOR (S2448), and pmTOR (T2446) in primordial follicle oocytes (Fig. 7). pTsc2 (S930) phosphorylation was predominantly localised in the oocyte nucleus of developing follicles in control-treated ovaries, but was significantly up-regulated in 3MC-treated primordial follicles (~1.8 fold) (Fig.7A). pmTOR (S2448) phosphorylation was localised to both the oocyte nucleus and cytoplasm, and was also significantly up-regulated in 3MC-treated primordial follicles (~1.8 fold) (Fig.7B). pmTOR (T2446) phosphorylation was primarily localised to the nucleus of control-treated primordial and primary follicle oocytes, and was severely reduced in 3MC-treated primordial follicles (~5 fold) (Fig.7C). These events correspond with the positive up-regulation of the mTOR pathway, which also plays an essential role in primordial follicle activation.

PI3K inhibition causes complete primordial follicle depletion in 3MC-cultured neonatal ovaries

In order to examine the role of PI3K/Akt signalling in 3MC induced primordial follicle ovotoxicity, neonatal ovaries were cultured in vehicle control and 3MC supplemented medium in the presence of the PI3K inhibitor LY294002 and probed for markers of primordial follicle activation and atresia (Fig. 8). PCNA was detected in both the oocyte and granulosa cells of LY294002 treated ovaries from the primary stage onwards, and was absent from primordial follicles. Although PCNA was also localised to the oocyte and granulosa cells of developing follicles in 3MC +LY294002 cultured ovaries, almost no primordial follicles were detected, suggesting a complete lack of the primordial follicle pool. This was confirmed via histomorphological analysis, which revealed a 20-fold reduction in primordial follicle number compared to the LY294002 control-treatment (Supplementary Fig. 1). Casp2, Casp3, and TUNEL were all localised to the majority of primary and secondary follicles present in 3MC +LY294002 cultured ovaries, suggesting follicular atresia. In addition, Casp2 and TUNEL staining were also detected in the sparse primordial follicle population of 3MC +LY294002 cultured ovaries (Fig. 8B). These results suggest that PI3K/Akt signalling is essential for primordial follicle survival when exposed to 3MC, but does not play a role in 3MC induced developing follicle ovotoxicity. Akt1 stimulates phosphorylation of pro-survival pBad on S136.Therefore we examined 3MC-cultured neonatal ovaries for pBad (136) phosphorylation (Fig. 9). Phosphorylation was significantly increased in 3MC exposed primordial follicles (~8 fold), suggesting that these follicles have entered a "pro-survival state".

Discussion

The primary focus of this study was to characterise the mechanisms behind 3MC induced ovotoxicity. Microarray analysis revealed a diverse response in ovarian gene expression to 3MC exposure. 3MC influenced the expression of a number of genes involved in cellular processes consistent with its bioactivation into DNA adduct forming compounds, such as drug metabolism, cell death, genetic disorder, and cancer (Fig. 1;Table 1) (Wood *et al.* 1978). In addition, 3MC also caused the up regulation of a number of genes involved in cellular processes associated with positive follicular development, such as cellular growth/proliferation, cellular development, and cell cycle regulation. This was surprising, as these developmental processes have not been previously associated with 3MC induced ovotoxicity. Canonical pathway analysis also identified a number of significantly up-regulated signalling pathways associated with positive proliferation and folliculogenesis (Fig. 2). These pathways included PI3K/Akt and ILK signalling, both of which are essential for primordial follicle activation/growth and survival (Reddy *et al.* 2010; Troussard *et al.* 2003), and Ahr signalling, which plays an important physiological role in the regulation of follicular growth and development (Robles *et al.* 2000). In the context of its reported ovotoxicity, Ahr signalling is also essential for 3MC bioactivation, with Ahr receptor inhibition nullifying 3MC induced primordial follicle depletion (Shiromizu and Mattison 1985). Pathways associated with DNA damage and programmed cell death (p53 signalling, G2/M and G1/S checkpoint control) were also up-regulated by 3MC exposure, supporting follicular atresia as a mechanism of its ovotoxicity. Ovarian cancer signalling was also significantly up-regulated by 3MC exposure, suggesting that the ovotoxicant may also stimulate ovarian tumorigenesis in addition to causing POF.

QPCR analysis on genes up-regulated in our microarray data confirmed an increase in nine individual genes associated with a variety of cellular processes (Table 2). One of these genes was *cyp1b1*, a cytochrome p450 oxidase phase I detoxifying enzyme up-regulated by Ahr signalling in response to xenobiotic exposure. Cyp1b1 has been identified as one of the initial enzymes which results in the bioactivation of a number of PAH into DNA adduct forming metabolites, resulting in toxicity (Shimada and Fujii-Kuriyama 2004). As inhibition of Ahr nullifies 3MC ovotoxicity, cyp1b1 most likely plays a role in ovarian 3MC bioactivation (Shiromizu and Mattison 1985). 3MC exposure also up regulated *aldh1l2* and *aldh4a1*, two members of the aldehyde dehydrogenase (ALDH) superfamily of $NAD(P)^+$-dependent phase I aldehyde oxidisers (Marchitti *et al.* 2008). Aldehydes are highly reactive molecules which are pathogenically produced as a consequence of oxidative stress (Esterbauer *et al.* 1991). As xenobiotics generate reactive oxygen species as a consequence of their detoxification, the up-regulation of these two detoxifying enzymes may be a consequence of 3MC induced oxidative stress (Sobinoff *et al.* 2012a). Indeed, the ALDH *Aldh3a1* contains multiple

xenobiotic response elements in its promoter region, and is up-regulated by 3MC exposure (Reisdorph and Lindahl 2007). Individually, aldh1l2 is a mitochondrial homolog of pro-apoptotic 10-formyltetrahydrofolate dehydrogenase, while aldh4a1 is a mitochondrial matrix protein implicated in p53 mediated protection against oxidative stress, DNA repair and cell survival (Ghose *et al.* 2009; Krupenko *et al.* 2010; Yoon *et al.* 2004). In addition to phase I detoxifying enzymes, 3MC exposure also up-regulated two phase II enzymes, *ugt1a10* and *nqo1*. *Ugt1a10* is a UDP-glucuronosyltransferase which converts phase I bioactivated xenobiotics into water-soluble metabolites for excretion. Ugt1a10 metabolism results in the detoxification of Cyp1b1 bioactivated metabolites, and is up-regulated by 3MC exposure (Dellinger *et al.* 2006; Horio and Horie 1997). Nqo1 is a anti-oxidant flavoprotein that detoxifies quinones into quinols, and has been previously implicated in 3MC metabolism (Kondraganti *et al.* 2008; Ross 2004). In addition, Nqo1 has also been associated with p53 dependent apoptosis (Ross 2004). The up-regulation of these Phase I and II genes in response to 3MC exposure provides insight into its ovarian bioactivation into ovotoxic compounds.

3MC exposure also up-regulated a number of genes involved in cell cycle regulation. One of these genes was *cdkn1a*, a well known regulator of cell cycle progression at G1 phase, involved in p53 induced apoptosis and DNA damage repair (Bartek and Lukas 2001). The up-regulation of this cell cycle checkpoint protein would suggest that 3MC induced ovotoxicity involves reduced cellular proliferation alongside DNA damage and apoptosis. However, 3MC exposure also up-regulated genes associated with positive cell cycle progression, such as the cyclins *ccnd1* and *ccng1*. Ccnd1 promotes cell cycle progression from G1-S phase, and is exclusively expressed in theca cells within the ovary (Robker and Richards 1998). As theca cells are only present in developing follicles, ccnd1 represents a marker of developing follicle growth and an indicator of increased follicular activation. Intriguingly, ccnd1 binds to cdk4 and promotes cell cycle progression from the G1-S phase, while cdkn1a binds to ccnd1/cdk4 complexes to inhibit G1 phase progression (Harris and Levine 2005). Another gene up-regulated by 3MC was *hspa8*, a heat shock protein responsible for regulating protein function and maturation (Agashe and Hartl 2000). It is now known that hspa8 expression increases during the G1 phase where it binds to ccnd1 complexes and prevents the inhibitory effect of cdkn1a, allowing the formation of an active ccnd1/cdk4/cdkn1a complex (Diehl *et al.* 2003). Therefore *hspa8* up-regulation in response to 3MC exposure may prevent cdkn1a repression, and allow ccnd1 mediated cell cycle progression. Increased *ccnd1* expression has also been associated with an increased incidence in ovarian cancer, providing further support for a hypothesis of 3MC induced ovarian tumorigenesis (Bali *et al.* 2004). Ccng1, a regulator of cell cycle progression at the G2/M phase, was also up-regulated by 3MC exposure (Kimura *et al.* 2001). In terms of ovarian function and development, ccng1 is essential for granulosa cell proliferation and differentiation, and may therefore represent a marker of primordial follicle activation (Liu *et al.* 2006).

Overall our microarray results reveal a complex mechanism of 3MC induced ovotoxicity involving a number of cellular processes. These results are comparable to what we observed in other studies investigating the mechanisms of DMBA and BaP induced ovotoxicity, and imply a similar mechanism of follicular depletion (Sobinoff *et al.* 2011; Sobinoff *et al.* 2012b). However, our results are at odds with previous studies, as they suggest that 3MC induced ovotoxicity is limited to primordial follicle oocyte atresia, a hypothesis based exclusively on histomorphological analysis (Borman *et al.* 2000; Mattison *et al.* 1983). Therefore, we re-examined the effects of 3MC exposure on folliculogenesis using markers of follicular activation and atresia. PCNA, a marker of cellular proliferation and primordial follicle activation, was detected in both the oocyte and granulosa cells of primordial follicles in both cultured and *in vivo* high dose treated ovaries (Fig. 3; Fig. 4). Conversely, markers of follicular atresia (Casp 2, Casp3, and TUNEL) were all detected in developing follicles, but not primordial follicles (Fig. 3). Although these markers of apoptosis do not appear to be involved physiological primordial follicle atresia, they do participate in stress induced primordial follicle cell death (Hanoux *et al.* 2006; Sobinoff *et al.* 2010; Tingen *et al.* 2009). Therefore, these results suggest that 3MC does not specifically target primordial follicle oocytes for atresia, and instead causes developing follicle atresia and primordial follicle activation. These results coincide with our previous studies, in which we found ovotoxic xenobiotics cause primordial follicle loss through activation, and not atresia (Sobinoff *et al.* 2011; Sobinoff *et al.* 2010, 2012b). As primordial follicle activation occurred alongside developing follicle atresia, we hypothesised that 3MC induced follicular activation was due to a homeostatic mechanism of developing follicle replacement. To support this hypothesis we probed 3MC-cultured ovaries for AMH, a negative regulator of follicular activation secreted by developing follicles (Supplementary Fig. 2) (Reddy *et al.* 2010). Histomorphological analysis revealed a significant reduction in the number of developing follicles expressing AMH. These results support a homeostatic replacement hypothesis of primordial follicle depletion, whereby 3MC directly targets developing follicles for atresia, resulting aberrant primordial follicle activation due to a decrease in negative signalling (Sobinoff *et al.* 2012a). Therefore previous investigations into 3MC ovotoxicity relying solely on histomorphological analysis may have seen no evidence of atresia in the developing follicular pool due to its successful maintenance through primordial follicle activation, with the resulting depletion in the primordial follicle pool being put down to atresia (Borman *et al.* 2000; Mattison *et al.* 1983).Histomorphological analysis supports this *in vivo*, as significant 3MC induced follicular depletion occurred alongside significant primordial follicle activation, with no apparent change in the developing pools composition (Fig. 5B).

To further investigate the mechanisms behind 3MC induced primordial follicle activation we probed 3MC-cultured ovaries for members of the PI3K/Akt and mTOR pathways of primordial follicle activation. Akt1 is a serine-threonine protein kinase central to the PI3K signalling pathway, and is phosphorylated upon PI3K stimulation (Reddy *et al.* 2010). We observed increased levels of

both pAkt1 (T308) and pAkt1 (S437) in 3MC-treated primordial follicle oocytes (Fig. 6). Akt1 is phosphorylated on its T308 residue by Pdpk1 (3-phosphoinositide-dependent protein kinase), an indirect measurement of PI3K activity and a marker of primordial follicle activation (Reddy *et al.* 2009). Akt1 is also phosphorylated on its S437 residue by activated mTORC2, an event which coincides with Akt1 T308 phosphorylation during follicular activation (Polak and Hall 2006). In addition to Akt1 phosphorylation levels, we also investigated Tsc2 and mTOR phosphorylation (Fig. 7). Tsc2 is a tumour suppressor protein which forms a heterodimer with Tsc1 to suppress mTOR phospoactivation, and therefore prevent the overstimulation of primordial follicle recruitment (Adhikari *et al.* 2009). Tsc2 is phosphorylated on its S939 residue by pAkt1 as part of the PI3K/Akt signalling cascade, resulting in its sequestration from Tsc1 by 14-3-3 proteins to allow mTOR activation (Cai *et al.* 2006). Tsc2 was localised to the oocyte of developing follicles in control-treated ovaries, but was significantly increased in 3MC-treated primordial follicles, suggesting a promotion of mTOR activation. mTOR itself is a central component in the multimeric kinase mTORC1, a key regulator of primordial follicle activation (Adhikari *et al.* 2010). Its activity is regulated via a phosphorylation-dependent molecular switch, whereby mTOR S2448 phosphorylation results in activation, and mTOR T2446 phosphorylation causes repression (Cheng *et al.* 2004). mTOR S2448 phosphorylation was significantly increased in 3MC-treated primordial follicle oocytes, while mTOR T2446 phosphorylation was significantly decreased (Fig. 7). This suggests an up-regulation of mTOR activation upon 3MC exposure, and therefore primordial follicle activation. Overall, these results suggest that 3MC induces primordial follicle depletion via activation through synergistic PI3K/Akt1 and mTOR signalling.

To confirm a role for PI3K signalling in 3MC induced primordial follicle depletion we exposed neonatal ovaries to both 3MC and the PI3K inhibitor LY294002 (Keating *et al.* 2011). Immunohistological analysis for markers of follicular activation and follicular atresia revealed a severely reduced population of primordial follicles showing signs of atresia (Casp2, TUNEL) in 3MC +LY294002 cultured ovaries (Fig. 8) (Supplementary Fig. 1). These results suggest that the up-regulation of the PI3K pathway may play a role in preventing 3MC induced primordial follicle depletion by promoting cell survival. Indeed, there is extensive documentation detailing PI3K and its downstream targets involvement in the promotion of cell survival by inhibiting apoptosis (Franke *et al.* 2003). To confirm this hypothesis of PI3K induced primordial follicle survival, we probed 3MC exposed neonatal ovaries for pBad S136 phosphorylation. Bad is a member of the BCL-2 family of cell death regulators which binds to, and inhibits pro-apoptotic members from the same family (Kim *et al.* 2006). Upon stimulation of PI3K signalling, activated Akt1 phosphorylates Bad on its S136 residue, resulting in its sequestration from pro-survival factors by 14-3-3 proteins to prevent apoptosis (Blume-Jensen *et al.* 1998; Masters *et al.* 2001). pBAD (S136) was significantly increased in 3MC exposed primordial follicles, suggesting the inhibition of apoptosis via Akt1 and therefore PI3K

signalling (Fig. 9). These results are intriguing, as they directly conflict with the current ovarian toxicology literature in suggesting that exposure to 3MC actually activates pro-survival pathways in primordial follicle, not atresia (Borman *et al.* 2000; Mattison 1980; Mattison *et al.* 1983). To confirm a role for mTOR signalling in 3MC induced primordial follicle depletion we exposed neonatal ovaries to both 3MC and the mTORC1 complex inhibitor Rapamycin (Kim *et al.* 2002). Immunohistological analysis for markers of follicular activation and follicular atresia suggest that inhibition of mTORC1 does not alter 3MC induced primordial follicle activation and developing follicle atresia, as evidenced by primordial follicle PCNA staining in the oocyte and granulosa cells and markers of follicular atresia (Casp 2, Casp3, and TUNEL) being detected in developing follicles (Supplementary Fig. 6). It is now known that mTOR signalling acts synergistically with PI3K/Akt signalling to induce primordial follicle activation, and that the inhibition of one pathway is not sufficient to prevent activation (Tingen *et al.* 2009). Therefore, these results suggest that mTORC1 does not play a role in 3MC induced primordial follicle survival, and act in a synergistic role with PI3K signalling in promoting 3MC induced primordial follicle growth.

In conclusion, this study represents one of the first comprehensive studies into the mechanism of 3MC induced ovotoxicity. Our results suggest that the ovary directly combats 3MC induced primordial follicle depletion by promoting cellular survival, and that the resulting depletion of the primordial follicle pool occurs due to a homeostatic mechanism of developing follicle replacement in an attempt to replace maturing follicles lost due to 3MC induced follicular atresia.

Acknowledgments

The authors gratefully acknowledge the financial assistance to EAM by the Australian Research Council, Hunter Medical Research Institute and the Newcastle Permanent Building Society Charitable Trust. APS is the recipient of a Australian Postgraduate Award PhD scholarship. This work was supported by National Health and Medical Research Council (Project grant #510735) to EAM, SDR and BN.

Supplementary data

Supplementary data includes fluorescence immunolocalisation of AMH in 3MC-cultured ovaries; microarray data for control vs. 3MC-treated neonatal ovaries, primordial follicle counts in 3MC + LY294002-cultured ovaries, negative controls for antibodies, positive and negative control TUNEL analysis, quantification of Akt phosphorylation in control and 3MC cultured developing follicles, and fluorescence immunolocalisation of PCNA, Casp2, Casp3, and TUNEL in 3MC+Rapamycin cultured ovaries.

References

Adhikari, D., Flohr, G., Gorre, N., Shen, Y., Yang, H., Lundin, E., Lan, Z., Gambello, M. J., and Liu, K. (2009). Disruption of Tsc2 in oocytes leads to overactivation of the entire pool of primordial follicles. *Molecular human reproduction* **15**, 765-770.

Adhikari, D., Zheng, W., Shen, Y., Gorre, N., Hamalainen, T., Cooney, A. J., Huhtaniemi, I., Lan, Z. J., and Liu, K. (2010). Tsc/mTORC1 signaling in oocytes governs the quiescence and activation of primordial follicles. *Human molecular genetics* **19**, 397-410.

Agashe, V. R., and Hartl, F. (2000). Roles of molecular chaperones in cytoplasmic protein folding. In Semin. Cell Dev. Biol., Vol. 11, pp. 15-25. Elsevier.

Bali, A., O'Brien, P. M., Edwards, L. S., Sutherland, R. L., Hacker, N. F., and Henshall, S. M. (2004). Cyclin D1, p53, and p21Waf1/Cip1 expression is predictive of poor clinical outcome in serous epithelial ovarian cancer. *Clinical cancer research* **10**, 5168-5177.

Bartek, J., and Lukas, J. (2001). Pathways governing G1/S transition and their response to DNA damage. *FEBS letters* **490**, 117-122.

Bhattacharya, P., and Keating, A. F. (2011). Ovarian metabolism of xenobiotics. *Experimental Biology and Medicine* **236**, 765-771.

Blume-Jensen, P., Janknecht, R., and Hunter, T. (1998). The kit receptor promotes cell survival via activation of PI 3-kinase and subsequent Akt-mediated phosphorylation of Bad on Ser136. *Current biology* **8**, 779-785.

Borman, S. M., Christian, P. J., Sipes, I. G., and Hoyer, P. B. (2000). Ovotoxicity in female Fischer rats and B6 mice induced by low-dose exposure to three polycyclic aromatic hydrocarbons: comparison through calculation of an ovotoxic index. *Toxicology and applied pharmacology* **167**, 191-198.

Cai, S. L., Tee, A. R., Short, J. D., Bergeron, J. M., Kim, J., Shen, J., Guo, R., Johnson, C. L., Kiguchi, K., and Walker, C. L. (2006). Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane partitioning. *The Journal of cell biology* **173**, 279-289.

CDC (2009). Fourth National Report on Human Exposure to Environmental Chemicals. National Center for Environmental Health Atlanta, GA.

Cheng, S. W. Y., Fryer, L. G. D., Carling, D., and Shepherd, P. R. (2004). Thr2446 is a novel mammalian target of rapamycin (mTOR) phosphorylation site regulated by nutrient status. *Journal of Biological Chemistry* **279**, 15719-15722.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical biochemistry* **162**, 156-159.

Dellinger, R. W., Fang, J. L., Chen, G., Weinberg, R., and Lazarus, P. (2006). Importance of UDP-glucuronosyltransferase 1A10 (UGT1A10) in the detoxification of polycyclic aromatic hydrocarbons: decreased glucuronidative activity of the UGT1A10139Lys isoform. *Drug metabolism and disposition* **34**, 943-949.

Diehl, J. A., Yang, W., Rimerman, R. A., Xiao, H., and Emili, A. (2003). Hsc70 regulates accumulation of cyclin D1 and cyclin D1-dependent protein kinase. *Molecular and cellular biology* **23**, 1764-1774.

Edson, M. A., Nagaraja, A. K., and Matzuk, M. M. (2009). The mammalian ovary from genesis to revelation. *Endocrine reviews* **30**, 624-712.

Esterbauer, H., Schaur, R. J., and Zollner, H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biology and Medicine* **11**, 81-128.

Franke, T. F., Hornik, C. P., Segev, L., Shostak, G. A., and Sugimoto, C. (2003). PI3K/Akt and apoptosis: size matters. *Oncogene* **22**, 8983-8998.

Ghose, S., Oleinik, N. V., Krupenko, N. I., and Krupenko, S. A. (2009). FDH-induced JNK pathways diverge at the JNK substrate level in cells with different p53 status. *Molecular cancer research: MCR* **7**, 99-107.

Hamilton, B. E., Martin, J. A., and Ventura, M. A. (2010). Births: preliminary data for 2009. Hyattsville, MD: US Department of Health and Human Services, CDC. *National Center for Health Statistics*.

Hanoux, V., Pairault, C., Bakalska, M., Habert, R., and Livera, G. (2006). Caspase-2 involvement during ionizing radiation-induced oocyte death in the mouse ovary. *Cell Death & Differentiation* **14**, 671-681.

Harris, S. L., and Levine, A. J. (2005). The p53 pathway: positive and negative feedback loops. *Oncogene* **24**, 2899-2908.

Hirshfield, A. N. (1991). Development of follicles in the mammalian ovary. *International review of cytology* **124**, 43-101.

Horio, F., and Horie, T. (1997). The role of microsomal beta-glucuronidase in ascorbic acid biosynthesis stimulated by xenobiotics in rats. *Bioscience, Biotechnology, and Biochemistry* **61**, 109-112.

Hu, X., Christian, P., Sipes, I. G., and Hoyer, P. B. (2001). Expression and redistribution of cellular Bad, Bax, and Bcl-xL protein is associated with VCD-induced ovotoxicity in rats. *Biology of reproduction* **65**, 1489-1495.

Keating, A. F., Fernandez, S. M., Mark-Kappeler, C. J., Sen, N., Sipes, I. G., and Hoyer, P. B. (2011). Inhibition of PIK3 signaling pathway members by the ovotoxicant 4-vinylcyclohexene diepoxide in rats. *Biology of reproduction* **84**, 743-751.

Kim, D. H., Sarbassov, D. D., Ali, S. M., King, J. E., Latek, R. R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D. M. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* **110**, 163-175.

Kim, H., Rafiuddin-Shah, M., Tu, H. C., Jeffers, J. R., Zambetti, G. P., Hsieh, J. J. D., and Cheng, E. H. Y. (2006). Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. *Nature cell biology* **8**, 1348-1358.

Kimura, S. H., Ikawa, M., Ito, A., Okabe, M., and Nojima, H. (2001). Cyclin G1 is involved in G2/M arrest in response to DNA damage and in growth control after damage recovery. *Oncogene* **20**, 3290-3300.

Kondraganti, S. R., Jiang, W., Jaiswal, A. K., and Moorthy, B. (2008). Persistent induction of hepatic and pulmonary phase II enzymes by 3-methylcholanthrene in rats. *Toxicological sciences* **102**, 337-344.

Krupenko, N. I., Dubard, M. E., Strickland, K. C., Moxley, K. M., Oleinik, N. V., and Krupenko, S. A. (2010). ALDH1L2 is the mitochondrial homolog of 10-formyltetrahydrofolate dehydrogenase. *Journal of Biological Chemistry* **285**, 23056-23063.

Liu, Z. H., Zhang, H., He, Y. P., Zhang, J. H., and Yue, L. M. (2006). Cyclin G1 expressing in mouse ovary and relating to follicular development. *Sichuan da xue xue bao. Yi xue ban Journal of Sichuan University. Medical science edition* **37**, 893-897.

Marchitti, S. A., Brocker, C., Stagos, D., and Vasiliou, V. (2008). Non-P450 aldehyde oxidizing enzymes: the aldehyde dehydrogenase superfamily. *Expert Opinion on Drug Metabolism & Toxicology* **4**, 697–720.

Mark-Kappeler, C. J., Hoyer, P. B., and Devine, P. J. (2011). Xenobiotic Effects on Ovarian Preantral Follicles. *Biology of Reproduction* **85**, 871-883.

Masters, S. C., Yang, H., Datta, S. R., Greenberg, M. E., and Fu, H. (2001). 14-3-3 inhibits Bad-induced cell death through interaction with serine-136. *Molecular Pharmacology* **60**, 1325-1331.

Matikainen, T., Perez, G. I., Jurisicova, A., Pru, J. K., Schlezinger, J. J., Ryu, H. Y., Laine, J., Sakai, T., Korsmeyer, S. J., and Casper, R. F. (2001). Aromatic hydrocarbon receptor-driven Bax gene expression is required for premature ovarian failure caused by biohazardous environmental chemicals. *Nature genetics* **28**, 355-360.

Mattison, D. R. (1980). Morphology of oocyte and follicle destruction by polycyclic aromatic hydrocarbons in mice. *Toxicology and applied pharmacology* **53**, 249-259.

Mattison, D. R., Shiromizu, K., and Nightingale, M. S. (1983). Oocyte destruction by polycyclic aromatic hydrocarbons. *American journal of industrial medicine* **4**, 191-202.

McGee, E. A., and Hsueh, A. J. W. (2000). Initial and cyclic recruitment of ovarian follicles. *Endocrine reviews* **21**, 200-214.

McLaughlin, E. A., and McIver, S. C. (2009). Awakening the oocyte: controlling primordial follicle development. *Reproduction* **137**, 1-11.

McLaughlin, E. A., and Sobinoff, A. P. (2010). Contraception Targets in Mammalian Ovarian Development. In Handbook of Experimental Pharmacology – Contraception & Fertility Control – Today and in the Future, Eds. (U. Habenicht and R. J. Aitken, eds.), Vol. 198, pp. 45-66. Springer-Verlag, Berlin.

McNatty, K. P., Fidler, A. E., Juengel, J. L., Quirke, L. D., Smith, P. R., Heath, D. A., Lundy, T., O'Connell, A., and Tisdall, D. J. (2000). Growth and paracrine factors regulating follicular formation and cellular function. *Molecular and Cellular Endocrinology* **163**, 11-20.

Neal, M. S., Zhu, J., Holloway, A. C., and Foster, W. G. (2007). Follicle growth is inhibited by benzo-[a]-pyrene, at concentrations representative of human exposure, in an isolated rat follicle culture assay. *Human reproduction* **22**, 961-967.

Polak, P., and Hall, M. N. (2006). mTORC2 caught in a SINful Akt. *Developmental cell* **11**, 433-434.

Reddy, P., Adhikari, D., Zheng, W., Liang, S., HÃ¤mÃ¤lÃ¤inen, T., Tohonen, V., Ogawa, W., Noda, T., Volarevic, S., and Huhtaniemi, I. (2009). PDK1 signaling in oocytes controls reproductive aging and lifespan by manipulating the survival of primordial follicles. *Human molecular genetics* **18**, 2813-2824.

Reddy, P., Zheng, W., and Liu, K. (2010). Mechanisms maintaining the dormancy and survival of mammalian primordial follicles. *Trends in Endocrinology & Metabolism* **21**, 96-103.

Reisdorph, R., and Lindahl, R. (2007). Constitutive and 3-methylcholanthrene-induced rat ALDH3A1 expression is mediated by multiple xenobiotic response elements. *Drug metabolism and disposition* **35**, 386-393.

Robker, R. L., and Richards, J. A. S. (1998). Hormone-induced proliferation and differentiation of granulosa cells: a coordinated balance of the cell cycle regulators cyclin D2 and p27Kip1. *molecular endocrinology* **12**, 924-940.

Robles, R., Morita, Y., Mann, K. K., Perez, G. I., Yang, S., Matikainen, T., Sherr, D. H., and Tilly, J. L. (2000). The aryl hydrocarbon receptor, a basic helix-loop-helix transcription factor of the PAS gene family, is required for normal ovarian germ cell dynamics in the mouse. *Endocrinology* **141**, 450-453.

Ross, D. (2004). Quinone reductases multitasking in the metabolic world. *Drug metabolism reviews* **36**, 639-654.

Shimada, T., and Fujii-Kuriyama, Y. (2004). Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and1B1. *Cancer science* **95**, 1-6.

Shiromizu, K., and Mattison, D. R. (1985). Murine oocyte destruction following intraovarian treatment with 3-methylcholanthrene or 7, 12-dimethylbenz (a) anthracene: Protection by alpha naphthoflavone. *Teratogenesis, carcinogenesis, and mutagenesis* **5**, 463-472.

Sobinoff, A. P., Bernstein, I. R., and McLaughlin, E. A. (2012a). All Your Eggs in One Basket; Mechanisms of Xenobiotic Induced Female Reproductive Senescence. In Senescence (T. Nagata, ed., pp. 559-584. InTech Press Rijeka, Croatia.

Sobinoff, A. P., Mahony, M., Nixon, B., Roman, S. D., and McLaughlin, E. A. (2011). Understanding the Villain: DMBA-Induced Preantral Ovotoxicity Involves Selective Follicular Destruction and Primordial Follicle Activation through PI3K/Akt and mTOR Signaling. *Toxicological Sciences* **123**, 563-575.

Sobinoff, A. P., Pye, V., Nixon, B., Roman, S. D., and McLaughlin, E. A. (2010). Adding Insult to Injury: Effects of Xenobiotic-Induced Preantral Ovotoxicity on Ovarian Development and Oocyte Fusibility. *Toxicological Sciences* **118**, 653-666.

Sobinoff, A. P., Pye, V., Nixon, B., Roman, S. D., and McLaughlin, E. A. (2012b). Jumping the gun: Smoking constituent BaP causes premature primordial follicle activation and impairs oocyte fusibility through oxidative stress. *Toxicology and Applied Pharmacology* **260**, 70-80.

Tingen, C. M., Bristol-Gould, S. K., Kiesewetter, S. E., Wellington, J. T., Shea, L., and Woodruff, T. K. (2009). Prepubertal primordial follicle loss in mice is not due to classical apoptotic pathways. *Biology of reproduction* **81**, 16-25.

Troussard, A. A., Mawji, N. M., Ong, C., Mui, A., St-Arnaud, R., and Dedhar, S. (2003). Conditional knock-out of integrin-linked kinase demonstrates an essential role in protein kinase B/Akt activation. *Journal of Biological Chemistry* **278**, 22374-22378.

Wood, A. W., Chang, R. L., Levin, W., Thomas, P. E., Ryan, D., Stoming, T. A., Thakker, D. R., Jerina, D. M., and Conney, A. H. (1978). Metabolic activation of 3-methylcholanthrene and its metabolites to products mutagenic to bacterial and mammalian cells. *Cancer Research* **38**, 3398-3404.

Yoon, K. A., Nakamura, Y., and Arakawa, H. (2004). Identification of ALDH4 as a p53-inducible gene and its protective role in cellular stresses. *Journal of human genetics* **49**, 134-140.

Figure legend:

Figure 1. Microarray analysis of control-cultured ovaries verses 3MC-cultured ovaries. Ovaries were excised from neonatal mice (4 days old, n=15) and cultured in xenobiotic treated medium for 96 hours RNA extracted and subjected to microarray analysis as described in the materials and methods. (A) Summary of microarray results. Total number of genes found on an Illumina Sentrix Mouse ref8v2 Beadchip are presented as non-regulated (black) and regulated (white) genes with a significant change in expression (>1.5-fold change, $p<0.05$). The red bar represents the number of positively regulated genes, and the yellow bar represents the number of negatively regulated genes in xenobiotic cultured ovaries. (B) Molecular networks of significantly altered genes influenced by 3MC exposure. Significantly altered genes were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base (Ingenuity Systems). Networks of significantly altered genes were then algorithmically generated based on their connectivity. The networks are ranked according to their scores, and the five highest-ranking networks of genes are displayed. The numbers of genes in each network are shown in brackets.

Figure 2. Top canonical pathways that were significantly up-regulated by 3MC-cultured neonatal ovaries as identified by IPA. The significance of the association between up-regulated genes and the canonical pathway was evaluated using a right-tailed Fisher's exact test to calculate a *p*-value determining the probability that the association is explained by chance alone (blue bars, y-axis). Ratios referring to the proportion of up-regulated genes from a pathway related to the total number of molecules that make up that particular pathway are also displayed (line graph, x-axis).

Figure 3. 3MC exposure causes immature follicular destruction and primordial follicle activation *in vitro*. Fluorescent immunohistological and TUNEL staining as visualised via epifluorescent microscopy. Ovaries excised from neonatal mice (4 days old) were cultured in 3MC-treated medium for 96 hours and processed for immunohistochemistry and TUNEL analysis as described in the materials and methods. Ovarian sections were probed 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 DNA strand breaks (TUNEL). The results presented here are representative of n = 3 experiments. The percentage of labelled follicles per section is represented by the following scale present in the top right hand corner; *=<25%, **=25-50%, ***=51-75%, ****=76-100%. Thin arrow=primordial follicle highlighted in insert at higher magnification; arrow head=primary follicle; scale bar is equal to 100 μm.

Figure 4. Fluorescent immunolocalisation of PCNA protein in low and high dose 3MC-treated ovaries *in vivo*. Neonatal mice (4 days old) were treated with either a low or high dose of 3MC over a seven day period, culled, and their ovaries extracted and processed of immunohistochemistry as outlined in materials and methods. The results presented here are representative of n=3 experiments. Blue staining

(DAPI) represents nuclear staining in all cells; red staining (Cy-5) represents specific staining for PCNA protein. The percentage of labelled follicles per section is represented by the following scale present in the top right hand corner; *=<10%, **=10-50%, ***=51-75%, ****=76-100%. Thin arrow=primordial follicle highlighted in insert at higher magnification; scale bar is equal to 100μm.

Figure 5. Effect of 3MC exposure on ovarian follicle composition and number *in vivo*. Neonatal mice (4 days old) were treated with either a low or high dose of 3MC over a seven day period as described in materials and methods. Ovarian sections were stained with hematoxylin and eosin and healthy oocyte containing follicles were classified and counted under a microscope. (A) Low dose ovarian follicle composition (left panel) and average number of follicles per counted section (right panel). (B) High dose ovarian follicle composition (left panel) and average number of follicles per counted section (right panel). Values are mean ± SEM, n=3-5 ovaries from 3-5 mice. The symbol ** represents $p<0.01$ in comparison with control values.

Figure 6. Fluorescent immunolocalisation of Akt (A), pAkt1 (S473)(B), and pAkt1 (T308)(C) in 3MC-cultured ovaries. Ovaries excised from neonatal mice (4 days old) were cultured in 3MC-treated medium for 96 hours and processed for immunohistochemistry as outlined in materials and methods. Representative image of both control and 3MC-treated ovaries (left panel) and quantification of oocyte nuclear staining (right panel). The results presented here are representative of n=3 experiments. Blue staining (DAPI) represents nuclear staining in all cells; red staining (Cy-5) represents specific staining for the described protein. Arrow=primordial follicle; scale bar is equal to 100 μm. The symbol ** represents $p<0.01$ in comparison with control values.

Figure 7. Fluorescent immunolocalisation of pTsc2 (S939)(A), pmTOR (S2448)(B), and pmTOR (T2446)(C) in 3MC-cultured ovaries. Ovaries excised from neonatal mice (4 days old) were cultured in 3MC-treated medium for 96 hours and processed for immunohistochemistry as outlined in materials and methods. Representative image of both control and 3MC-treated ovaries (left panel) and quantification of oocyte nuclear staining (right panel). The results presented here are representative of n=3 experiments. Blue staining (DAPI) represents nuclear staining in all cells; red staining (Cy-5) represents specific staining for the described protein. Arrow=primordial follicle; scale bar is equal to 100 μm. The symbol ** represents $p<0.01$ in comparison with control values.

Figure 8. PI3K inhibition causes complete primordial follicle depletion in 3MC exposed ovaries *in vitro*. (A) Fluorescent immunohistological and TUNEL staining as visualised via epifluorescent microscopy. (B) Primordial follicle Casp2 and TUNEL staining in 3MC+LY294002 treated ovaries. Ovaries excised from neonatal mice (4 days old) were cultured in 3MC-treated medium + LY294002 for 96 hours and processed for immunohistochemistry and TUNEL analysis as described in the materials and methods. Ovarian sections were probed 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 labelled follicles per section is represented by the following scale present in the top right hand corner; *=<25%, **=25-50%, ***=51-75%, ****=76-100%. Thin arrow=primordial follicle highlighted in insert at higher magnification; arrow head=primary follicle; scale bar is equal to 100 μm.

Figure 9. Fluorescent immunolocalisation of pBad (S136) in 3MC-cultured ovaries. Ovaries excised from neonatal mice (4 days old) were cultured in 3MC-treated medium for 96 hours and processed for immunohistochemistry as outlined in materials and methods. Representative image of both control and 3MC-treated ovaries (left panel) and quantification of oocyte nuclear staining (right panel). The results presented here are representative of n=3 experiments. Blue staining (DAPI) represents nuclear staining in all cells; red staining (Cy-5) represents specific staining for the described protein. Arrow head=primordial follicle; scale bar is equal to 100 μm. The symbol ** represents $p<0.01$ in comparison with control values.

Tables

Table 1. Functional classification of genes up-regulated or down-regulated by 3MC exposure in cultured neonatal ovaries. Genes were analysed using Ingenuity Pathways Analysis (Ingenuity Systems) for molecular and cellular functions. Only those genes exhibiting a greater than two-fold change in expression were categorised ($p<0.05$). Note that some genes are listed in multiple functional groups.

Molecular and Cellular Function	Up regulated	Down regulated
Cellular growth and proliferation	102	55
Genetic disorder	88	55
Cellular development	86	43
Tissue development	82	51
Reproductive system disease	77	19
Cell death	77	49
Cell to cell signalling and interaction	59	46
Inflammatory response	52	58

Cell cycle	43	18
Cellular function	40	48
Organismal development	58	1

Table 2. QPCR validation of microarray results for select transcripts up-regulated by 3MC-cultured neonatal ovaries. 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. 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 ($p<0.05$).

Gene symbol	Gene name	Summary of function	Fold change
Cdkn1a	Cyclin-dependent kinase inhibitor 1A	Regulator of cell cycle progression at G1 phase; implicated in DNA damage repair (Binkova et al., 2000)	8.64±1.9
Ccng1	Cyclin G1	Regulator of cell cycle progression at G2/M phase; implicated in DNA damage repair and apoptosis; associated with granulosa cell proliferation and differentiation (Kimura et al. 2001; Liu et al. 2006)	3.9±0.4
Cyp1b1	Cytochrome P450, family 1, subfamily b, polypeptide 1	Phase I detoxifying enzyme; implicated in the bioactivation of PAH into DNA adduct forming metabolites (Shimada and Fujii-Kuriyama, 2004)	3.78±0.4
Aldh1l2	Aldehyde dehydrogenase 1 family, member L2	Mitochondrial homolog of pro-apoptotic 10-formyltetrahydrofolate dehydrogenase (Ghose et al., 2009; Krupenko et al., 2010).	3.16±0.2
Ngo1	NAD(P)H-quinone oxidoreductase	Phase II detoxifying enzyme; detoxifies quinones into quinols; implicated in 3MC metabolism (Ross, 2004; Kondraganti et al., 2008)	2.61±0.2
Aldh4a1	Aldehyde dehydrogenase 4 family, member A1	Mitochondrial matrix protein essential for proline degradation; implicated in p53 mediated protection against oxidative stress (Yoon et al., 2004)	2.45±0.1
Ugt1a10	UDP glycosyltransferase 1 family, polypeptide	Phase II detoxifying enzyme; Implicated in the detoxification of bioactivated PAH metabolites (Dellinger	1.87±0.3

	A10	et al. 2006)	
nd1	Cyclin D1	Promotes cell cycle progression from G1-S phase; overexpression associated with breast/ovarian cancer (Robker and Richards 1998; Bali et al., 2004).	1.7±0.4
pa8	Heat shock protein 8	Stress related chaperone; expression increases during cell cycle G1 phase; regulates cyclin D1 accumulation and maintains its activity in the presence of inhibitory Cdkn1a (Diehl et al., 2003)	1.51±0.1
lkn1a	Cyclin-dependent kinase inhibitor 1A	Regulator of cell cycle progression at G1 phase; implicated in DNA damage repair (Binkova et al., 2000)	8.64±1.9

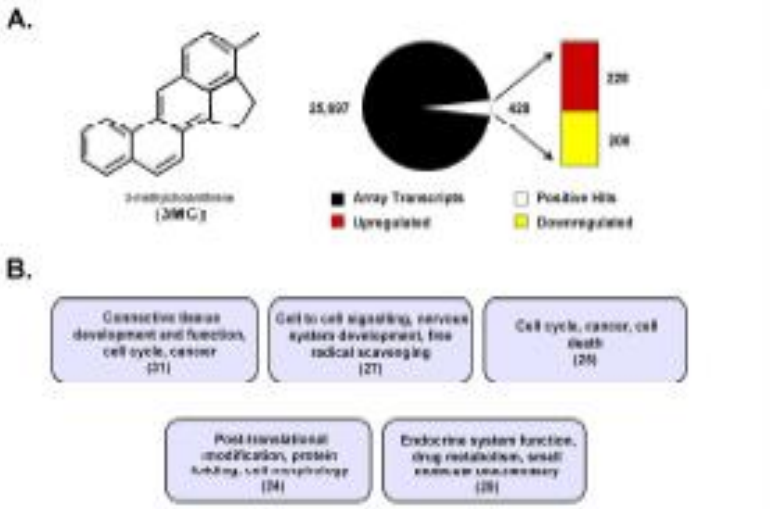


Figure 1. Microarray analysis of control cultured ovaries verses 3MC cultured ovaries. Ovaries were excised from neonatal mice (4 days old, n=15) and cultured in xenobiotic treated medium for 96 hours RNA extracted and subjected to microarray analysis as described in the materials and methods. (A) Summary of microarray results. Total number of genes found on an Illumina Sentrix Mouse ref8v2 Beadchip are presented as non-regulated (black) and regulated (white) genes with a significant change in expression (>1.5-fold change, $p<0.05$). The red bar represents the number of positively regulated genes, and the yellow bar represents the number of negatively regulated genes in xenobiotic cultured ovaries. (B) Molecular networks of significantly altered genes influenced by 3MC exposure. Significantly altered genes were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base (Ingenuity Systems). Networks of significantly altered genes were then algorithmically generated based on their connectivity. The networks are ranked according to their scores, and the five highest-ranking networks of genes are displayed. The numbers of genes in each network are shown in brackets.
190x142mm (300 x 300 DPI)

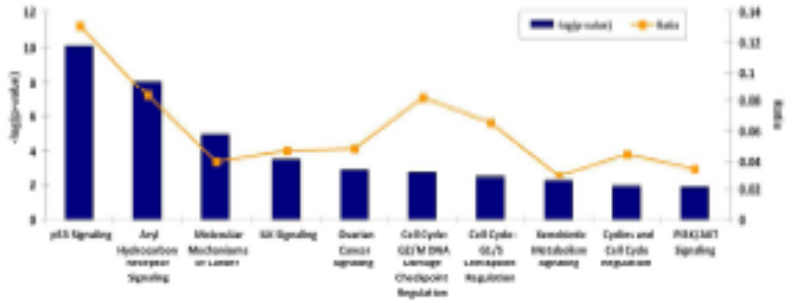


Figure 2. Top canonical pathways that were significantly up-regulated by 3MC cultured neonatal ovaries as identified by IPA. The significance of the association between up-regulated genes and the canonical pathway was evaluated using a right-tailed Fisher's exact test to calculate a p-value determining the probability that the association is explained by chance alone (blue bars, y-axis). Ratios referring to the proportion of up-regulated genes from a pathway related to the total number of molecules that make up that particular pathway are also displayed (line graph, z-axis).
190x142mm (300 x 300 DPI)

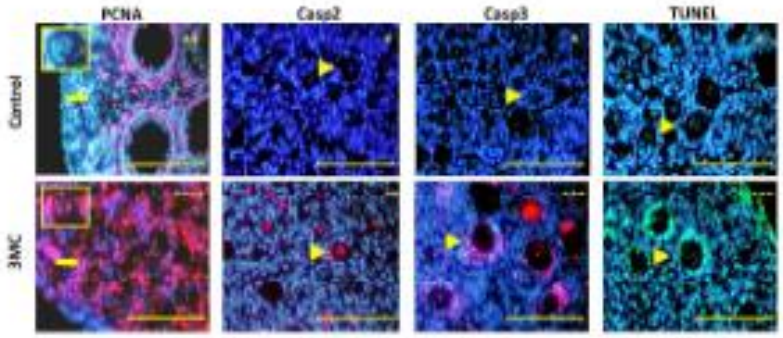


Figure 3. 3MC exposure causes immature follicular destruction and primordial follicle activation in vitro. Fluorescent immunohistological and TUNEL staining as visualised via epifluorescent microscopy. Ovaries dissected from neonatal mice (4 days old) were cultured in 3MC treated medium for 96 hours and processed for immunohistochemistry and TUNEL analysis as described in the materials and methods. Ovarian sections were probed 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 DNA strand breaks (TUNEL). The results presented here are representative of n = 3 experiments. The percentage of labelled follicles per section is represented by the following scale present in the top right hand corner; *=<25%, **=25-50%, ***=51-75%, ****=76-100%. Thin arrow=primordial follicle highlighted in insert at higher magnification; arrow head=primary follicle; scale bar is equal to 100 µm.
190x142mm (300 x 300 DPI)

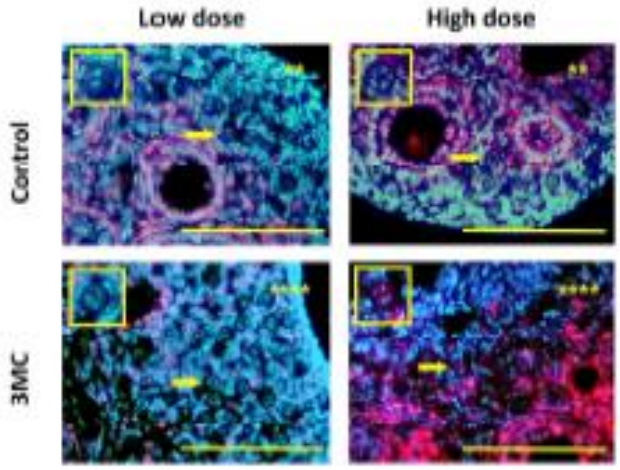


Figure 4. Fluorescent immunolocalisation of PCNA protein in low and high dose 3MC treated ovaries in vivo. Neonatal mice (4 days old) were treated with either a low or high dose of 3MC over a seven day period, culled, and their ovaries extracted and processed of immunohistochemistry as outlined in materials and methods. The results presented here are representative of n=3 experiments. Blue staining (DAPI) represents nuclear staining in all cells; red staining (Cy-5) represents specific staining for PCNA protein. The percentage of labelled follicles per section is represented by the following scale present in the top right hand corner; *=<10%, **=10-50%, ***=51-75%, ****=76-100%. Thin arrow=primordial follicle highlighted in insert at higher magnification; scale bar is equal to 100μm.
190x142mm (300 x 300 DPI)

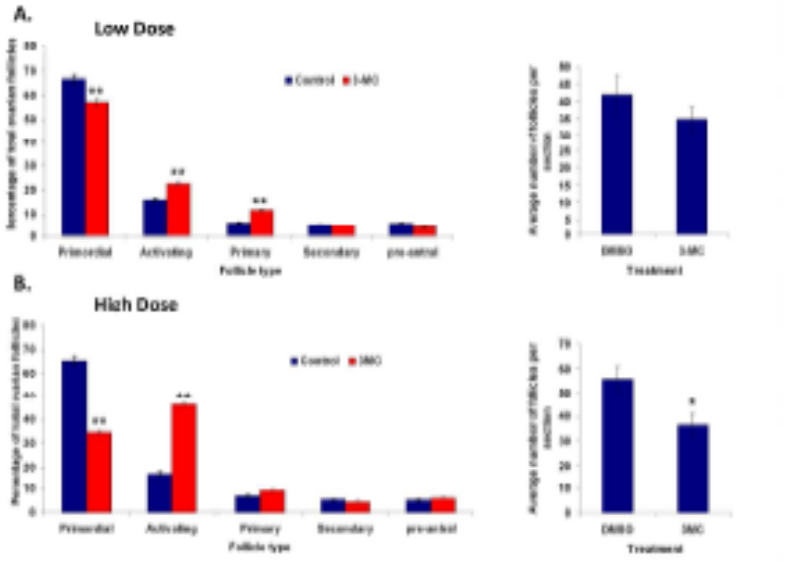


Figure 5. Effect of 3MC exposure on ovarian follicle composition and number in vivo. Neonatal mice (4 days old) were treated with either a low or high dose of 3MC over a seven day period as described in materials and methods. Ovarian sections were stained with hematoxylin and eosin and healthy oocyte containing follicles were classified and counted under a microscope. (A) Low dose ovarian follicle composition (left panel) and average number of follicles per counted section (right panel). (B) High dose ovarian follicle composition (left panel) and average number of follicles per counted section (right panel). Values are mean ± SEM, n=3-5 ovaries from 3-5 mice. The symbol ** represents p<0.01 in comparison with control values.
190x142mm (300 x 300 DPI)

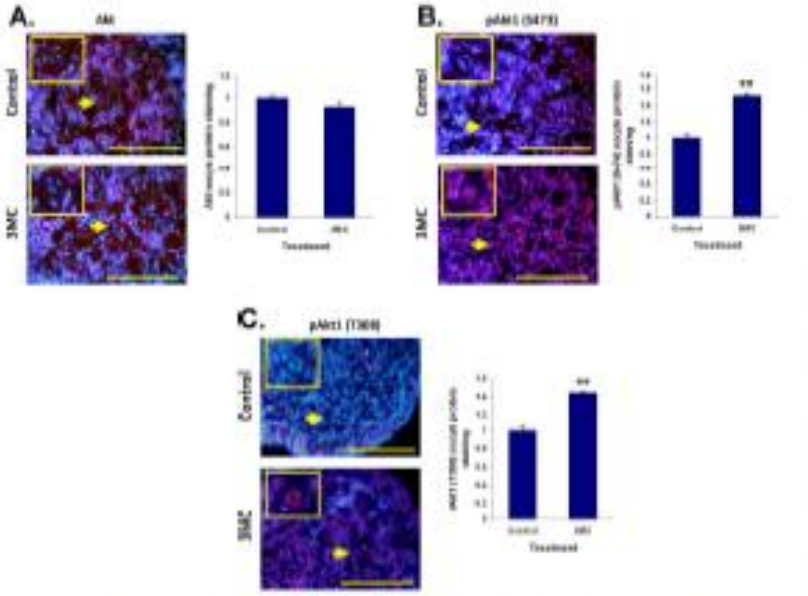


Figure 6. Fluorescent immunolocalisation of Akt (A), pAkt1 (S473)(B), and pAkt1 (T308)(C) in 3MC cultured ovaries. Ovaries excised from neonatal mice (4 days old) were cultured in 3MC treated medium for 96 hours and processed for immunohistochemistry as outlined in materials and methods. Representative image of both control and 3MC treated ovaries (left panel) and quantification of oocyte nuclear staining (right panel). The results presented here are representative of n=3 experiments. Blue staining (DAPI) represents nuclear staining in all cells; red staining (Cy-5) represents specific staining for the described protein. Arrow=primordial follicle; scale bar is equal to 100 µm. The symbol ** represents $p<0.01$ in comparison with control values.
190x142mm (300 x 300 DPI)

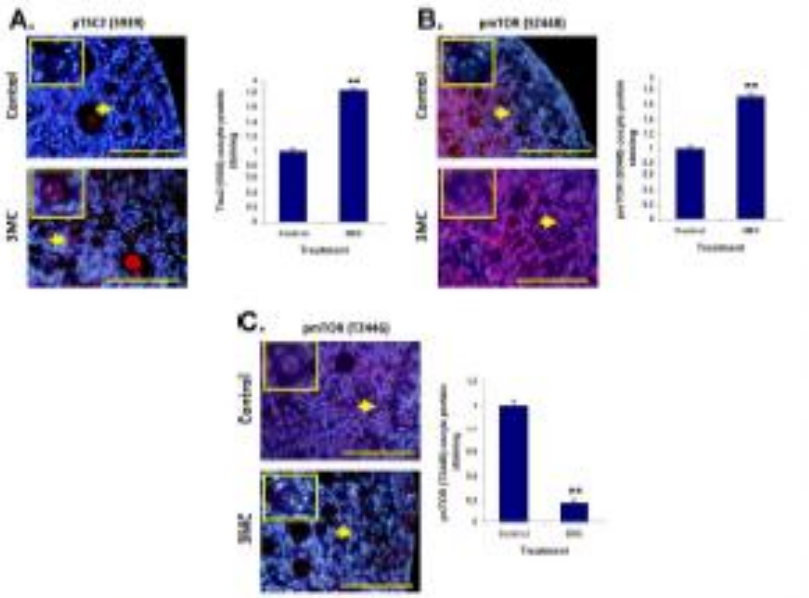


Figure 7. Fluorescent immunolocalisation of pTsc2 (S939)(A), pmTOR (S2448)(B), and pmTOR (T2446)(C) in 3MC cultured ovaries. Ovaries excised from neonatal mice (4 days old) were cultured in 3MC treated medium for 96 hours and processed for immunohistochemistry as outlined in materials and methods. Representative image of both control and 3MC treated ovaries (left panel) and quantification of oocyte nuclear staining (right panel). The results presented here are representative of n=3 experiments. Blue staining (DAPI) represents nuclear staining in all cells; red staining (Cy-5) represents specific staining for the described protein. Arrow=primordial follicle; scale bar is equal to 100 µm. The symbol ** represents $p<0.01$ in comparison with control values.
190x142mm (300 x 300 DPI)

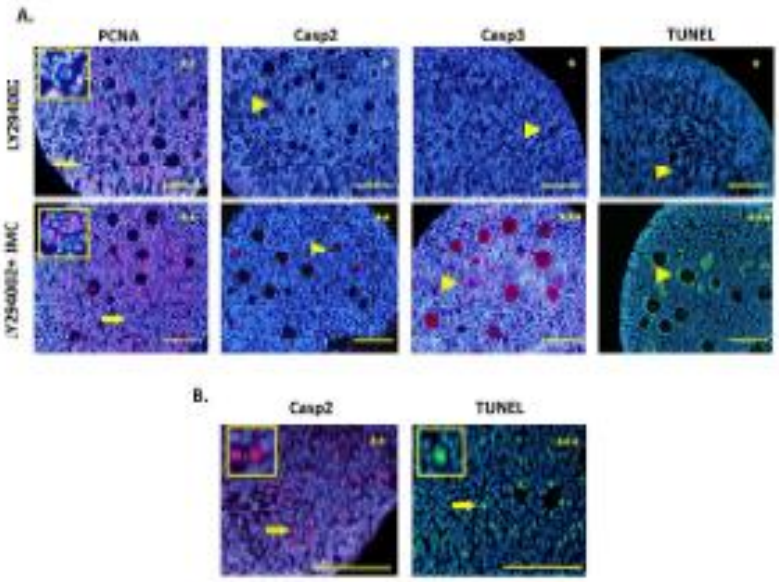


Figure 8. PI3K inhibition causes complete primordial follicle depletion in 3MC exposed ovaries in vitro. (A) Fluorescent immunohistological and TUNEL staining as visualised via epifluorescent microscopy. (B) Primordial follicle Casp2 and TUNEL staining in 3MC+LY294002 treated ovaries. Ovaries excised from neonatal mice (4 days old) were cultured in 3MC-treated medium + LY294002 for 96 hours and processed for immunohistochemistry and TUNEL analysis as described in the materials and methods. Ovarian sections were probed 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 labelled follicles per section is represented by the following scale present in the top right hand corner; *=<25%, **=25-50%, ***=51-75%, ****=76-100%. Thin arrow=primordial follicle highlighted in insert at higher magnification; arrow head=primary follicle; scale bar is equal to 100 µm.
190x142mm (300 x 300 DPI)

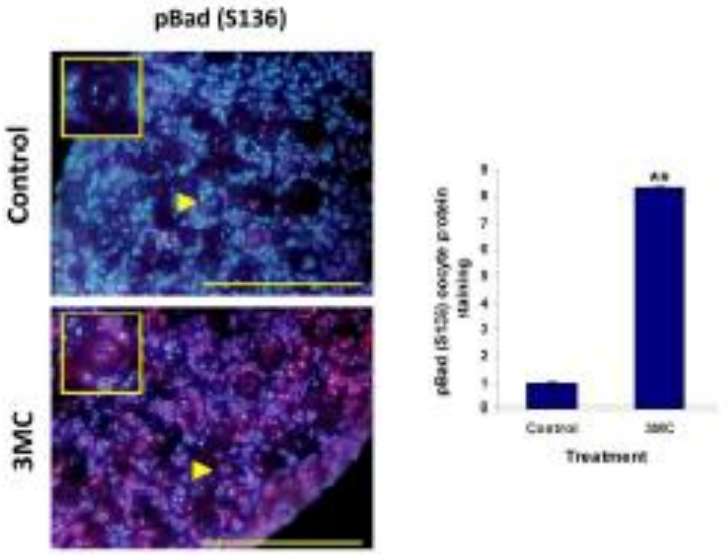


Figure 9. Fluorescent immunolocalisation of pBad (S136) in 3MC cultured ovaries. Ovaries excised from neonatal mice (4 days old) were cultured in 3MC treated medium for 96 hours and processed for immunohistochemistry as outlined in materials and methods. Representative image of both control and 3MC treated ovaries (left panel) and quantification of oocyte nuclear staining (right panel). The results presented here are representative of n=3 experiments. Blue staining (DAPI) represents nuclear staining in all cells; red staining (Cy-5) represents specific staining for the described protein. Arrow head=primordial follicle: scale bar is equal to 100 µm. The symbol ** represents p<0.01 in comparison with control values.
190x142mm (300 x 300 DPI)

Chapter 5: Final Discussion

Chapter 5: Final Discussion

5.1 Introduction

The mammalian ovary is a complex organ which contains the entire supply of female germ cells available for reproduction. The primordial follicle represents the basic functional unit of the ovary, and consists of a single oocyte surrounded by a layer of immature somatic support cells (McNatty *et al.* 2000). Due to the nature of ovarian follicular development, the number of primordial follicles established around the time of birth is finite, representing the total population of germ cells available to the mammalian female throughout her entire life (Edson *et al.* 2009). Once the primordial follicle pool becomes exhausted, the mammalian female enters reproductive senescence, or menopause. Therefore, this precious ovarian reserve dictates the length of the female reproductive lifespan. Female fertility is also highly dependant on oocyte quality. Long lived species such as humans and primates experience a decline in oocyte quality with age, with higher incidences of chromosomal abnormalities, cellular fragmentation, abnormal morphology and poor fertilization being reported in "older" women (>30 years) (Battaglia *et al.* 1996; Broekmans *et al.* 2009; Miao *et al.* 2009).

Exposure to specific xenobiotics and other reproductive hazards has been shown to adversely affect female fertility, including inducing oocyte dysfunction and primordial follicle loss resulting in pre-mature ovarian failure (POF) (Borman *et al.* 2000; Mark-Kappeler *et al.* 2011a; Mattison *et al.* 1983; Sobinoff *et al.* 2010, 2012c). In addition to the inability to conceive, exposure to ovotoxic hazards also represents a significant health risk for females, as the loss of ovarian hormones which accompanies POF has been linked with an increased incidence of cardiovascular disease, osteoporosis, Alzheimer's disease, and ovarian cancer (Cenci *et al.* 2003; Pike *et al.* 2009; Rosano *et al.* 2007; Shuster *et al.* 2010). Unfortunately, female exposure to these xenobiotics often goes unnoticed due to the primordial follicle's gonadotropin independent nature and the subtlety of oocyte dysfunction, making effective diagnosis and treatment difficult (Edson *et al.* 2009; Hooser *et al.* 1994).

Over the last 5 years there has been a growing body of evidence leading to the characterisation of the molecular mechanisms underpinning xenobiotic induced primordial follicle depletion and oocyte dysfunction (Bhattacharya *et al.* 2012; Keating *et al.* 2011; Keating *et al.* 2009; Mark-Kappeler *et al.* 2010; Sobinoff *et al.* 2011, 2012b; Sobinoff *et al.* 2010, 2012c). This has led to new approaches in studying this type of ovotoxicity, increasing awareness and producing novel insights, which may lead to the development of better

reproductive strategies to assist women exposed to these compounds. The following is a brief review summarising the contribution this thesis has made to the advancements in understanding pre-antral/long term ovotoxicity and the implications of this research for future study.

5.2 Primordial follicle depletion; not as simple as atresia

The ability of specific xenobiotics to cause POF via primordial follicle depletion has been studied in depth over the last 30 years. Some of the most extensive studies have been performed on the polycyclic aromatic hydrocarbon (PAH) family of potent environmental toxicants (Borman *et al.* 2000; Mattison 1980; Mattison *et al.* 1983). Particular attention has been paid to the three ovotoxic PAHs benzo[a]pyrene (BaP), 9:10-dimethyl-1:2-benzanthracene (DMBA), and 3-methylcholanthrene (3-MC). Early investigations into their mechanisms of ovotoxicity found that *in vitro* and *in vivo* exposure to these three PAH resulted in a dose related depletion of the primordial follicle pool (Borman *et al.* 2000; Rajapaksa *et al.* 2007). These early studies relied heavily on histomorphological analysis to determine the effects of these xenobiotics on folliculogenesis. Follicle counts revealed reduced numbers of primordial and primary follicles in treated ovaries, with the remaining follicles showing signs of pre-mature follicular atresia (or apoptosis) and necrosis (Borman *et al.* 2000; Mattison 1980; Mattison *et al.* 1983). Subsequent metabolic studies revealed that these PAH were non-toxic in their native format, and that the observed primordial and primary follicle depletion was dependant on their bioactivation by the Aryl Hydrocarbon Receptor (Ahr) (Jurisicova *et al.* 2007; Shiromizu and Mattison 1985). It was therefore hypothesised that these PAH are bioactivated into ovotoxic constituents by the body, and are then capable of causing DNA damage in small developing follicle oocytes, resulting in pre-mature primordial follicle atresia and consequently POF. In the case of DMBA, latter studies also identified increased expression of pro-apoptotic protein Bax in exposed pre-antral oocytes, further supporting an apoptotic based mechanism of primordial follicle depletion (Matikainen *et al.* 2001).

Although the hypothesis of xenobiotic induced primordial follicle atresia has remained unchanged since the initial studies investigating BaP, DMBA, and 3MC induced ovotoxicity in the early 1980's, recent evidence acquired by our laboratory has supported an alternative hypothesis of primordial depletion via activation (Sobinoff *et al.* 2011, 2012b; Sobinoff *et al.* 2012c). All three PAH up-regulated molecular pathways associated with

follicular growth/development in neonatal ovaries cultured *in vitro* (BaP 1μM; DMBA 50nM; 3MC 5μM), including PI3K/Akt, mTOR, and growth hormone signalling (Reddy *et al.* 2010). PCNA, a marker of primordial follicle activation, was also significantly up-regulated in PAH treated primordial follicles *in vitro* and *in vivo*, suggesting activation (Picut *et al.* 2008). Conversely, markers of follicular atresia (activated Caspase 2/3 and TUNEL) were absent in PAH treated primordial follicles, and were instead localised exclusively to the developing follicle pool. Studies incorporating multiple intraperitoneal (i.p.) injections (BaP 3mg/kg/daily; DMBA 1mg/kg/daily; 3MC 10 mg/kg/daily) over a period of 7 days also revealed significant primordial follicle activation alongside follicular depletion *in vivo* (Sobinoff *et al.* 2011, 2012b; Sobinoff *et al.* 2012c). As primordial follicle activation occurred alongside developing follicle atresia for each xenobiotic, it can be hypothesised that these PAH act in a similar way to directly target developing follicles for atresia, resulting in the homeostatic activation of the primordial follicle pool to replace those targeted for destruction (Sobinoff *et al.* 2012a). Immunocontraception studies targeting developing follicles in rabbits and primates have identified a similar mechanism of primordial follicle depletion, and may be due to the loss of negative regulators of primordial follicle activation secreted by the developing pool (Paterson *et al.* 1992; Skinner *et al.* 1984). However, Anti-Müllerian hormone (AMH), a member of the TGF-β superfamily secreted by developing follicles to prevent excessive primordial follicle activation, was only found to be significantly decreased by 3MC exposure, and was unaffected in BaP and DMBA treated ovaries (Reddy *et al.* 2010; Sobinoff *et al.* 2011, 2012b; Sobinoff *et al.* 2012c). Therefore, although each PAH induced similar levels of developing follicle atresia alongside primordial follicle activation, their mechanisms of ovotoxicity remain unique. An alternative mechanism for BaP and DMBA related primordial follicle activation could be perturbed signal transduction caused by xenobiotic induced oxidative stress (Wells *et al.* 2009). BaP itself is converted into the o-quinones benzo[a]pyrene-3,6-dione and benzo[a]pyrene-6,12-dion by cyp1a1, and caused significant levels of oxidative stress in adult oocytes exposed to the xenobiotic *in vivo* (Schwarz *et al.* 2001; Trevor *et al.* 1996). DMBA metabolism has also been shown to induce oxidative stress in the follicular population of the ovary (Tsai-Turton *et al.* 2007).

In further support of excessive primordial follicle activation in response to PAH exposure, DMBA and 3MC have both been found to up-regulate members of the PI3K/Akt and mTOR pathways of primordial follicle activation (Fig. 1) (Reddy *et al.* 2010; Sobinoff *et al.* 2011, 2012b). Inhibition studies performed in 3MC cultured ovaries also revealed a role

for PI3K/Akt signalling in preserving the primordial follicle pathway by stimulating pro-survival events, such as the phospho-inhibition of pro-apoptotic Bad (Sobinoff *et al.* 2012b).

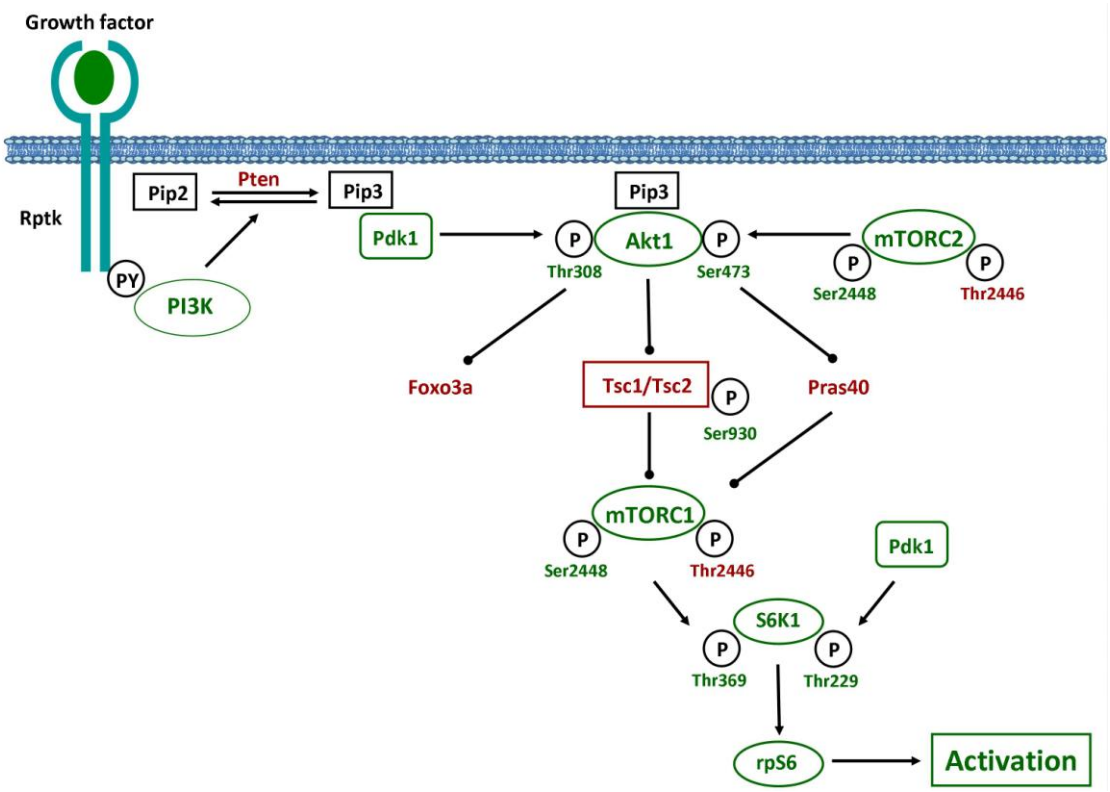


Figure 1. Diagrammatic representation of primordial follicle PI3K/Akt/mTOR signalling adapted from Reddy et al., 2010. Molecules and phosphorylation sites which maintain primordial follicle senescence are labelled red, while molecules and phosphorylation sites which induce primordial follicle activation are labelled green. Briefly, Rptk activation by growth factors results in PI3K activation, which subsequently facilitates the conversion of Pip2 to Pip3 via phosphorylation, a reversible event catalysed by Pten. Pip3 provides a docking site for Pdk1 and Akt1, resulting in Pdk1 induced Akt1 Thr308 phosphorylation and mTORC2 induced Akt1 Ser473 phosphorylation. Akt1 then phosphorylates Foxo3a, resulting in its translocation from the nucleus to the cytoplasm, stimulating follicular activation. Akt1 also phosphorylates Tsc2 on its Ser930 residue, causing Tsc1/Tsc2 complex repression and allowing mTORC1 activation. Akt1 also negatively regulates Pras40, another inhibitor of mTORC1 activation. Akt1 activated mTORC1 then enhances S6K1-rpS6 signalling through S6K1 Thr369 phosphorylation. PI3K activated Pdk1 also phosphorylates S6K1 on its Thr229 residue, further stimulating S6K1-rpS6 signalling. (Rptk: Receptor Protein Tyrosine Kinase; PI3K: Phosphoinositide 3-kinase; Pip2/3: Phosphatidylinositol 4,5-bisphosphate 2/3; Pten: Phosphatase and tensin homolog; Pdk1: Phosphoinositide-dependent kinase-1; Akt1: RAC-alpha serine/threonine-protein kinase; mTORC1/2: mammalian target of Rapamycin complex 1/2; Foxo3a: Forkhead box O3; Tsc1/2: Tuberous sclerosis protein 1/2; Proline-rich Akt substrate of 40 kilodaltons; S6K1: 70-kDa 40 S ribosomal protein S6 kinase-1; rpS6: Ribosomal protein S6)

Inhibition of the PI3K/Akt signalling pathway in DMBA cultured ovaries has also been shown to cause a similar effect, exacerbating the loss of primordial and small primary follicles in neonatal rats (Keating *et al.* 2009). We have repeated these studies using our ovarian *in vitro* culture system and observed similar results in mice, with DMBA causing

complete primordial follicle depletion reminiscent of 3MC exposure (Fig. 2A). In addition, we also found DMBA to stimulate the phospho-inhibition of Bad (S136), suggesting DMBA induced PI3K/Akt signalling also stimulated pro-survival of the primordial follicle pool (Fig. 2B). These results have lead to the generation of a novel hypothesis of DMBA and 3MC induced ovotoxicity in which the ovary attempts to preserve the primordial follicle pool when faced with xenobiotic insult by stimulating pro-survival pathways (Fig. 3). Unfortunately, this attempt ultimately proves futile due to the excessive stimulation of primordial follicle activation to preserve the developing pool. This hypothesis is novel as it refutes the conventional view that 3MC and DMBA cause direct primordial follicle atresia (Mattison 1980; Mattison *et al.* 1983).

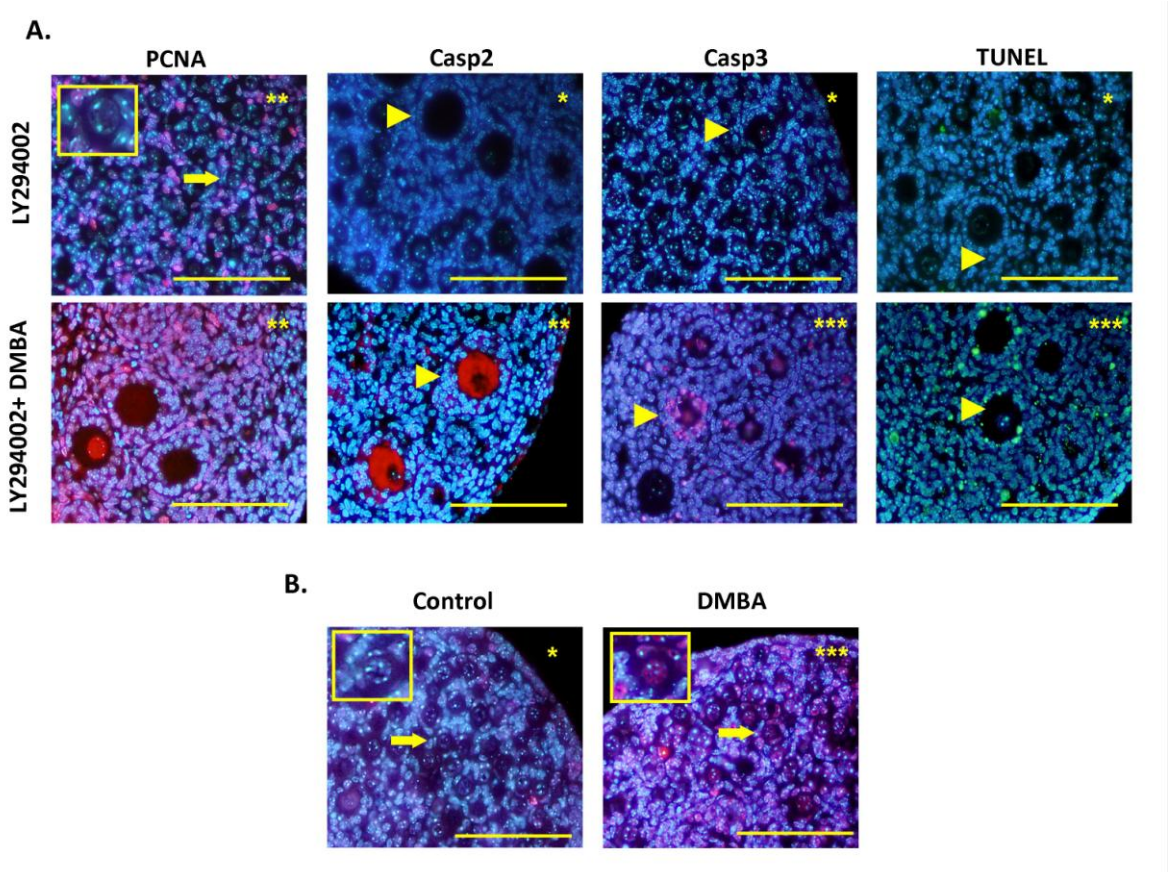


Figure 2. Role of PI3K signalling in DMBA induced ovotoxicity. (A) PI3K inhibition causes complete primordial follicle depletion in 3MC exposed ovaries *in vitro*. (B) Fluorescent immunolocalisation of pBad (S136) in 3MC-cultured ovaries . Ovaries excised from neonatal mice (4 days old) were cultured in 3MC-treated medium + LY294002 for 96 hours and processed for immunohistochemistry and TUNEL analysis as described previously (Sobinoff *et al*., 2012b). Ovarian sections were probed with antibodies against PCNA, active caspase 2, active caspase 3 and pBad (S136), 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 labelled follicles per section is represented by the following scale present in the top right hand corner; *=<25%, **=25-50%, ***=51-75%. Thin arrow=primordial follicle highlighted in insert at higher magnification; arrow head=primary follicle; scale bar is equal to 100 µm.

Recent investigations into the molecular mechanisms behind other xenobiotic induced ovotoxicity have also identified activation as a method of primordial follicle depletion. The industrial chemical 4-vinylcyclohexene diepoxide (VCD) is a commercially available chemical diluent of diepoxides and epoxy resins, and a well known ovotoxicant (Hoyer and Sipes 2007; Huff 2001). Early studies involving VCD demonstrated its potent ovotoxicity towards primordial and primary follicles in rodents, with *in vitro* and *in vivo* exposures resulting in POF (Devine *et al.* 2002; Kao *et al.* 1999; Springer *et al.* 1996). Follicle counts and immunohistological analysis combined with mechanistic studies suggested that VCD directly targets these follicles for destruction by inducing members of the BCL2 and mitogen-activated protein kinase families (Hu *et al.* 2001a; Hu *et al.* 2001b; Hu *et al.* 2002). Recent studies performed by ourselves and others have now also demonstrated that VCD exposure causes primordial follicle depletion via activation (Keating *et al.* 2011; Sobinoff *et al.* 2010). Neonatal mouse ovaries cultured in VCD (25μM) for 96 hours up-regulated key genes and pathways involved in primordial follicle activation, including *Akt1, Akt2, Rarg*, PI3K/Akt and mTOR signalling. VCD neonatal mouse cultured ovaries also showed signs of developing follicle atresia and primordial follicle activation. Studies performed in rat ovaries *in vitro* also identified a role for *Kit* and *Kitl* in VCD induced follicle loss, with the addition of exogenous Kitl attenuating VCD induced primordial follicle loss (Fernandez *et al.* 2008; Mark-Kappeler *et al.* 2011b). Binding of the granulosa cell secreted growth factor Kitl to primordial follicle oocytes has been shown to localise to oocyte cell surface Kit receptor and activate the PI3K/Akt signalling pathway, promoting primordial follicle activation and survival (Reddy *et al.* 2010). PI3K inhibition in VCD cultured rat ovaries prevented primordial follicle depletion, but had no effect on primary and developing follicle destruction (Keating *et al.* 2009). The observed increases in primordial follicle activation in mice coupled with the "protective" effects of PI3K inhibition suggests that primordial follicles may be resistant to VCD induced ovotoxicity, and only become destroyed once committed to activation (Keating *et al.* 2009). However, in a follow up study performed by Keating *et al*, VCD exposure did not positively regulate Akt or Foxo3a expression/phosphorylation in rat cultured primordial follicles, both of which are integral events during PI3K induced follicular activation (Keating *et al.* 2011). The aforementioned study observed these changes in events after two and four days of VCD exposure, while significant primordial follicle loss only occurs after six days of culture (Keating *et al.* 2011; Keating *et al.* 2009). Therefore, the time points chosen may have been too early to observe significant changes representative of the PI3k/Akt signalling.

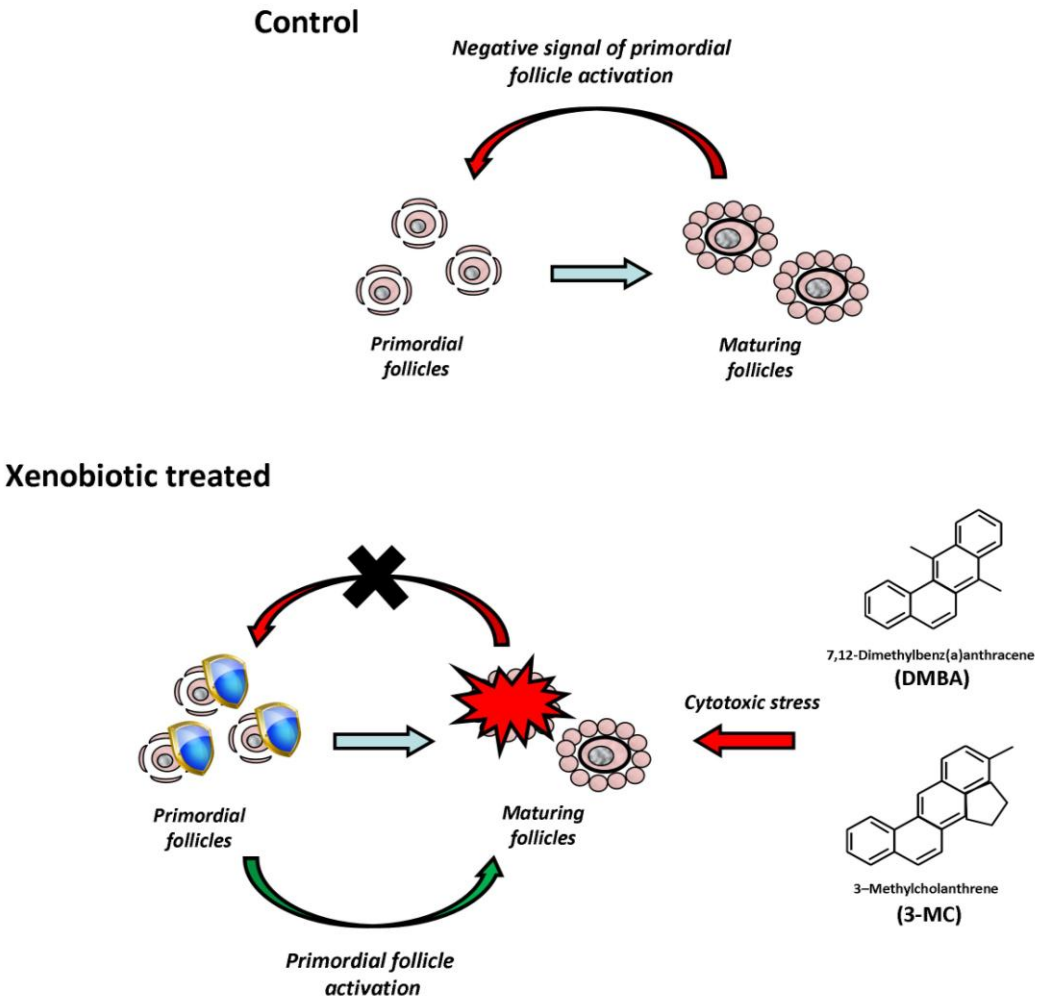


Figure 3. Hypothesised mechanism of DMBA and 3MC induced primordial follicle depletion. Under control conditions, developing follicles secrete negative regulators of primordial follicle activation to preserve the quiescent pool. DMBA and 3MC exposure both cause cytotoxic stress, which leads to the destruction of the developing pool, and an increase in primordial follicle pro-survival signalling. However, the removal of the negative signals produced by the maturing follicle population results in excessive primordial follicle activation, ultimately leading to a vicious cycle of primordial follicle depletion.

Our research has also identified xenobiotics which are capable of depleting primordial follicles via activation neonatally, but have a different mechanism of ovotoxicity in adulthood. Methoxychlor (MXC) is an organochlorine pesticide intended as a replacement for dichlorodiphenyltrichloroethane (DDT), but has since been banned in the United States and Australia (APVMA 2009; Edwards 2004). It is now used as a model compound for environmental estrogens due to its pro- and antiestrogenic activity (Bulger *et al.* 1985; Cummings and Laskey 1993). Studies performed in adult rodents have revealed that MXC directly impacts female reproduction by targeting the antral follicular pool. In adult mice, dosing regimes of 8, 16 and 32mg/kg/day were shown to significantly increase antral

follicular atresia and decrease markers of follicular growth over a 20 day time period (Borgeest *et al.* 2002; Gupta *et al.* 2009). Culture experiments using antral follicles obtained from 39-day-old mice confirmed the of ability MXC (1-100 µg/ml) to induce antral follicular atresia and prevent follicular growth, both of which were inhibited by the addition of the anti-oxidant N-acetyl cysteine (1-10 mM) (Gupta *et al.* 2006). Antral follicles isolated from adult Esr1 overexpression mice were also shown to be more sensitive to toxicity caused by MXC (1-100 µg/ml) and its metabolites mono-hydroxy MXC (MOH) (0.1–10 µg/ml) and bis-hydroxy MXC (HPTE) (0.1–10 µg/ml) (Paulose *et al.* 2011). Combined, these results suggest a method of ovotoxicity dependant on both xenobiotic induced oxidative stress and the suite of genes activated by Esr1. In neonatal mice, whole ovarian culture revealed MXC (25µM) upregulated genes and pathways involved in cell death and proliferation, including *Ccnd2*, *Bcl2l1*, *Cdkn2a*, PI3K/Akt and p53 signalling (Sobinoff *et al.* 2010). Additionally, MXC was shown to directly target primary and secondary follicles for follicular atresia, and showed evidence of increased primordial follicle activation and therefore granulosa cell proliferation (Sobinoff *et al.* 2010). Neonatal mice given 100mg/kg/day of MXC over a period of seven days also showed signs of primordial follicle activation and developing follicle atresia. Therefore, the ovotoxic effects of MXC on the follicular pool differ in both neonates and adults, with neonatal exposure causing developing follicle activation and small developing follicle atresia, and adult exposure repressing antral follicular growth and inducing pre-mature follicular atresia. Although both exposures have different effects on the ovary, they may both be caused by a similar mechanism. Adult pre-antral follicular toxicity seemed to be dependant on xenobiotic induced oxidative stress, while neonatal MXC exposure also increased signs of oxidative stress within the whole ovary (Gupta *et al.* 2006; Sobinoff *et al.* 2010). This suggests xenobiotic induced oxidative stress as a primary mechanism of MXC induced ovotoxicity. The differences in effect could be due to several reasons, including age-dependent differences between the disposition and metabolism of xenobiotics. For example, MXC ovotoxicity was found to increase with higher expression levels of Ers1 (Paulose *et al.* 2011). However, the neonatal ovary is estrogen independent; removing the possibility of activating the Ers1 gene battery partially credited with MXC induced pre-antral ovotoxicity (Edson *et al.* 2009). Levels of antioxidant potential in neonates, particularly if born pre-term, are generally lower than that observed in adults, and may impact on the effects of MXC induced oxidative stress on the ovary (Sullivan and Newton 1988).

5.3 Long term impacts of ovotoxic exposure; adding insult to injury

A recent avenue of ovotoxic research has focused on the effects short term xenobiotic exposure on long term female fertility. One of the currently held theories of xenobiotic induced follicular destruction is that once the offending xenobiotic is removed from the environment, female fertility can revert back to "normal". Although this may be the case in regard to folliculogenesis, it has been hypothesised that xenobiotic exposure may lead to decreased oocyte quality through the production of xenobiotic induced reactive oxygen species (ROS), causing female sub-fertility. The basis for this hypothesis revolves around the theory of mitochondrial induced ageing through free radical production (Beckman and Ames 1998). According to this theory, cells gradually produce excess ROS over time due to electron leakage from the mitochondrial electron transport chain. Eventually this excess ROS can build up to such levels that it damages the mitochondrial membrane, leading to further electron leakage and ROS production. When coupled with the already "redox sensitive" nature of the mammalian ovary, this makes the oocyte particularly vulnerable to excess ROS exposure (Tarin 1996). It is now thought that xenobiotic induced ROS production caused by detoxification can exacerbate this process, leading to accelerated mitochondrial ROS production (Sobinoff *et al.* 2012a). In terms of oocyte functionality, excess ROS production can result in oxidative adducts leading to DNA damage/mutation, abnormal cytoskeletal alterations which increase the frequency of aneuploidy and gross morphological abnormalities, and lipid peroxidation resulting in a change of oolemma membrane fluidity and therefore reduced fertilisation (Tarin 1996) (Fig. 4).

Studies conducted in our own laboratory have focused mainly on the effects of short term xenobiotic exposure on long term oolemma membrane fluidity. The PAH BaP is a potent ovotoxicant and one of the most prevalent chemicals found in cigarette smoke (Lodovici *et al.* 2004; Mattison *et al.* 1983). In the context of oocyte dysfunction, BaP is detected in high concentrations in the follicular fluid of women who smoke, and has been positively linked with reduced fertilisation rates in female smokers undergoing *in vitro* fertilisation (IVF) (Neal *et al.* 2008). Oocytes obtained from adult mice treated neonatally with either a "low dose" (1.5 mg/kg/daily) or "high dose" (3 mg/kg/daily) of BaP over a period of several days had

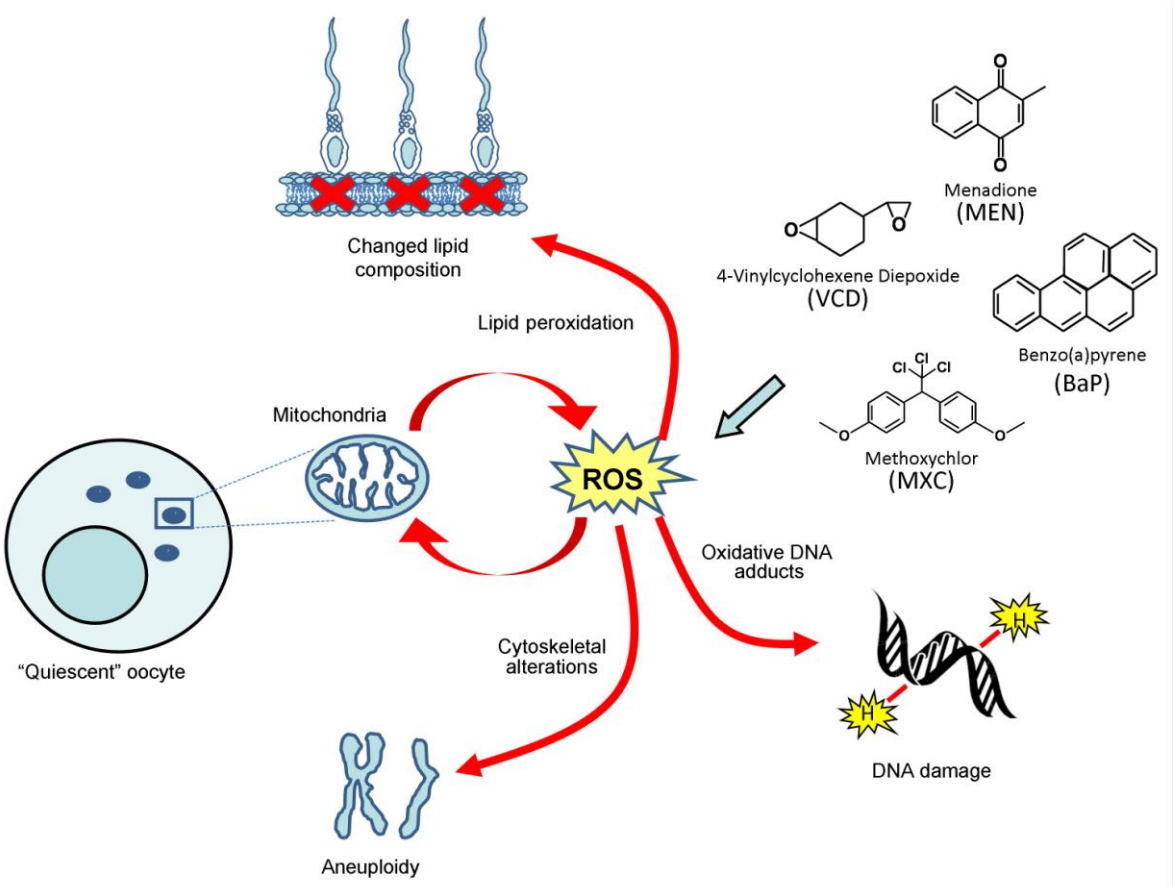


Figure 4. Potential effects of xenobiotic induced ROS on oocyte functionality. Excess ROS produced by xenobiotic metabolism results in mitochondrial membrane damage and electron transport chain leakage, resulting in a vicious cycle of ROS production. Accumulated ROS eventually results in oxidative DNA adducts/damage, abnormal cytoskeletal alterations causing aneuploidy, and increased lipid peroxidation resulting in reduced oolemma membrane fluidity and fertilisation.

significantly increased mitochondrial ROS production and lipid peroxidation compared to control treated animals (Sobinoff *et al.* 2012c). This increase in oxidative damage to the oolemma membrane was accompanied by severely reduced levels of sperm-egg binding and fusion in both doses. It was hypothesised that BaP exposure damaged the mitochondrial membrane in the neonatal animals, with the resulting electron transport chain leakage causing significant mitochondrial ROS leakage/dysfunction over time, and ultimately increased lipid peroxidation and perturbed fertilisation (Sobinoff *et al.* 2012c). This study also reported no neonatal follicle loss in the "low dose" treated animals, and no difference in the number of super-ovulated oocytes between the control and both BaP treatments (Sobinoff *et al.* 2012c). It would have therefore been difficult to detect any abnormalities caused by the "low dose" BaP exposure without comprehensive oocyte analysis, making this type of ovotoxicity insidious.

Further evidence of this type of ovotoxicity was seen with the xenobiotics VCD, MXC, and Menadione (MEN). Each of these xenobiotics, or their bioactivated products, caused significant oolemma lipid peroxidation in a dose dependant manner *in vitro* (Sobinoff *et al.* 2010). Adult mice treated neonatally with either a "low dose" (VCD 40mg/kg/day, MXC 50mg/kg/day, MEN 7.5mg/kg/day) or "high dose" (VCD 80mg/kg/day, MXC 100mg/kg/day, MEN 15 mg/kg/day) of the xenobiotics over several days also had dysfunctional oocytes which displayed a severely reduced capacity to interact with spermatozoa and undergo fertilisation (Sobinoff *et al.* 2010). As in the BaP study, the "low dose" VCD and MXC treatments did not induce significant follicle loss in neonatal mice, with defective oocytes only being detected through in depth individual analysis. This study further supports a hypothesis of xenobiotic induced long term oocyte dysfunction through oxidative damage, and suggests a common mechanism of ovotoxicity shared between various chemical compounds.

5.4 Future Directions

Although the research contained within this thesis provides a comprehensive view of the mechanisms of pre-antral ovotoxicity and the long term consequences of xenobiotic exposure, there are a number of limitations which must be addressed and improved upon in future research. For example, the doses and modes of exposure used in our *in vivo* model of ovotoxicity do not take into account real life doses and modes of exposure; women are more likely to become exposed a cocktail of environmental pollutants at chronic doses rather than acute, and will rarely inject these compounds intraperitoneally. Therefore, future research should take into account "real life" doses and modes of exposure. Cigarette smoke represents a common source of human xenobiotic exposure and contains over 4,000 potentially harmful chemical compounds including PAH, nicotine, nitroso compounds, protein pyrolysates, and aromatic amines (Carmines 2002). Although there is evidence of cigarette induced female infertility and sub fertility, the mechanisms behind these outcomes remains largely unknown, and should be the subject of future investigations (Freour *et al.* 2008; Gruber *et al.* 2008; Windham *et al.* 2005). The majority of investigations into the effects of ovotoxic compounds on female fertility have based their conclusion on their effects on follicle populations and germ cells analysis (Hoyer and Sipes 2007; Neal *et al.* 2008; Sobinoff *et al.* 2011, 2012b; Sobinoff *et al.* 2010, 2012c). However, these investigations lack corresponding breeding trials to confirm the hypothesised female infertility and sub-fertility. Although these

experiments in polyovular mouse models may not directly relate to monovular humans, these trials would help deduce the effects of these ovotoxicants on pregnancy rates and embryo development. In addition, these studies would permit the investigation of transgenerational effects of ovotoxic exposure, as the reported oxidative stress and DNA-adducts could result in genetic mutation.

Experiments conducted by ourselves have also been limited to the effects of short and long term ovotoxic neonatal exposure, and do not take into account the effects of adult exposure (Sobinoff *et al.* 2011, 2012b; Sobinoff *et al.* 2010, 2012c). Due to the differences between adults and neonates in xenobiotic defence mechanisms and overall metabolism, our investigations are only indicative of early life exposure which may be different in adults. Therefore, future experiments should focus on the short and long term effects of adult exposure on female fertility. In addition, our novel investigations into the effects of short term neonatal xenobiotic exposure on long term oocyte viability have exclusively focused fertilisation potential (Sobinoff *et al.* 2010, 2012c). As xenobiotic induced oxidative stress could also cause oxidative adducts and abnormal effects on cytoskeletal organisation, future investigations should examine the levels of aneuploidy and 8-oxoguanine formation in neonatally treated adult oocytes (Klaunig and Kamendulis 2004; Tarin 1996). Xenobiotic macromolecular adducts have also been detected in the follicular fluid and granulosa cells of women who smoke, and therefore may also be present in neonatally exposed adult oocytes, resulting in dysfunction (Lodovici *et al.* 2004; Neal *et al.* 2008). Microarray analysis has also revealed the up-regulation of many genes and signalling pathways involved in tumorigenesis in response to xenobiotic exposure (Sobinoff *et al.* 2011, 2012b; Sobinoff *et al.* 2010, 2012c). Although there is evidence of xenobiotics inducing ovarian cancer, further characterisation of these pathways may increase our understanding of the causes and symptoms of this disease, leading to novel treatments and diagnostic tests.

5.5 Conclusion

The findings contained within this thesis have a number of implications in the field of reproductive toxicology, environmental health management, and biomedical research. The identification of follicular activation as a mechanism of xenobiotic induced follicular depletion has increased our knowledge of the mechanisms behind xenobiotic induced POF, and lead to new approaches in studying ovotoxicity which are not limited to atresia. In addition, our research has helped increase awareness of the effects of these ovotoxic

compounds on female fertility. In particular, the discovery of short term xenobiotic exposure causing long term oocyte dysfunction at concentrations which do not cause overt follicular destruction calls for a re-evaluation of the level of xenobiotic exposure deemed to be “safe”. A better understanding of the molecular mechanisms behind xenobiotic induced ovotoxicity will also assist in the development of novel reproductive strategies to help women exposed to these compounds. For example, the identification of ROS as a significant factor in xenobiotic induced oocyte dysfunction and potentially primordial follicle depletion in the smoking constituent BaP could help women who recently quit smoking through antioxidant therapy.

6.5 References

APVMA (2009). The History of "Organochlorine Pesticides" in Australia. In Australian Pesticides and Veterinary Medicines Authority, Kingston, ACT.

Battaglia, D. E., Goodwin, P., Klein, N. A., and Soules, M. R. (1996). Fertilization and early embryology: Influence of maternal age on meiotic spindle assembly oocytes from naturally cycling women. *Human Reproduction* **11**, 2217-2222.

Beckman, K. B., and Ames, B. N. (1998). The free radical theory of aging matures. *Physiological reviews* **78**, 547-581.

Bhattacharya, P., Sen, N., Hoyer, P. B., and Keating, A. F. (2012). Ovarian expressed microsomal epoxide hydrolase: Role in detoxification of 4-vinylcyclohexene diepoxide and regulation by phosphatidylinositol-3 kinase signaling. *Toxicology and Applied Pharmacology* **258**, 118-123.

Borgeest, C., Symonds, D., Mayer, L. P., Hoyer, P. B., and Flaws, J. A. (2002). Methoxychlor may cause ovarian follicular atresia and proliferation of the ovarian epithelium in the mouse. *Toxicological sciences* **68**, 473-478.

Borman, S. M., Christian, P. J., Sipes, I. G., and Hoyer, P. B. (2000). Ovotoxicity in female Fischer rats and B6 mice induced by low-dose exposure to three polycyclic aromatic hydrocarbons: comparison through calculation of an ovotoxic index. *Toxicology and applied pharmacology* **167**, 191-198.

Broekmans, F. J., Soules, M. R., and Fauser, B. C. (2009). Ovarian aging: mechanisms and clinical consequences. *Endocrine reviews* **30**, 465-493.

Bulger, W. H., Feil, V. J., and Kupfer, D. (1985). Role of hepatic monooxygenases in generating estrogenic metabolites from methoxychlor and from its identified contaminants. *Molecular pharmacology* **27**, 115-124.

Carmines, E. L. (2002). Evaluation of the potential effects of ingredients added to cigarettes. Part 1: Cigarette design, testing approach, and review of results. *Food and Chemical Toxicology* **40**, 77-91.

Cenci, S., Toraldo, G., Weitzmann, M. N., Roggia, C., Gao, Y., Qian, W. P., Sierra, O., and Pacifici, R. (2003). Estrogen deficiency induces bone loss by increasing T cell proliferation and lifespan through IFN- -induced class II transactivator. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 10405-10410.

Cummings, A. M., and Laskey, J. (1993). Effect of methoxychlor on ovarian steroidogenesis: role in early pregnancy loss. *Reproductive Toxicology* **7**, 17-23.

Devine, P. J., Sipes, I. G., Skinner, M. K., and Hoyer, P. B. (2002). Characterization of a rat in vitro ovarian culture system to study the ovarian toxicant 4-vinylcyclohexene diepoxide. *Toxicology and applied pharmacology* **184**, 107-115.

Edson, M. A., Nagaraja, A. K., and Matzuk, M. M. (2009). The mammalian ovary from genesis to revelation. *Endocrine reviews* **30**, 624-712.

Edwards, D. (2004). Methoxychlor Reregistration Eligibility Decision (RED). EPA738-R-04-010. In US Environmental Protection Agency, Office of Pesticide Programs, Washington, DC, p. 9.

Fernandez, S. M., Keating, A. F., Christian, P. J., Sen, N., Hoying, J. B., Brooks, H. L., and Hoyer, P. B. (2008). Involvement of the KIT/KITL signaling pathway in 4-vinylcyclohexene diepoxide-induced ovarian follicle loss in rats. *Biology of reproduction* **79**, 318-327.

Freour, T., Masson, D., Mirallie, S., Jean, M., Bach, K., Dejoie, T., and Barriere, P. (2008). Active smoking compromises IVF outcome and affects ovarian reserve. *Reproductive biomedicine online* **16**, 96-102.

Gruber, I., Just, A., Birner, M., and Lasch, A. (2008). Effect of a woman's smoking status on oocyte, zygote, and day 3 pre-embryo quality in in vitro fertilization and embryo transfer program. *Fertility and sterility* **90**, 1249-1252.

Gupta, R. K., Meachum, S., Hernandez-Ochoa, I., Peretz, J., Yao, H. H., and Flaws, J. A. (2009). Methoxychlor inhibits growth of antral follicles by altering cell cycle regulators. *Toxicology and applied pharmacology* **240**, 1-7.

Gupta, R. K., Miller, K. P., Babus, J. K., and Flaws, J. A. (2006). Methoxychlor inhibits growth and induces atresia of antral follicles through an oxidative stress pathway. *Toxicological sciences* **93**, 382-389.

Hooser, S. B., Douds, D. P., DeMerell, D. G., Hoyer, P. B., and Sipes, I. G. (1994). Long-term ovarian and gonadotropin changes in mice exposed to 4-vinylcyclohexene. *Reproductive Toxicology* **8**, 315-323.

Hoyer, P. B., and Sipes, I. G. (2007). Development of an animal model for ovotoxicity using 4â€• vinylcyclohexene: a case study. *Birth Defects Research Part B: Developmental and Reproductive Toxicology* **80**, 113-125.

Hu, X., Christian, P., Sipes, I. G., and Hoyer, P. B. (2001a). Expression and redistribution of cellular Bad, Bax, and Bcl-xL protein is associated with VCD-induced ovotoxicity in rats. *Biology of reproduction* **65**, 1489-1495.

Hu, X., Christian, P. J., Thompson, K. E., Sipes, I. G., and Hoyer, P. B. (2001b). Apoptosis induced in rats by 4-vinylcyclohexene diepoxide is associated with activation of the caspase cascades. *Biology of reproduction* **65**, 87-93.

Hu, X., Flaws, J. A., Sipes, I. G., and Hoyer, P. B. (2002). Activation of mitogen-activated protein kinases and AP-1 transcription factor in ovotoxicity induced by 4-vinylcyclohexene diepoxide in rats. *Biology of reproduction* **67**, 718-724.

Huff, J. (2001). Carcinogencity Bioassays of Bisphenol A, 4-Vinylcyclohexene Diepoxide, and 4-Vinycyclohexene. *Toxicological Sciences* **64**, 282-283.

Jurisicova, A., Taniuchi, A., Li, H., Shang, Y., Antenos, M., Detmar, J., Xu, J., Matikainen, T., HernÃ¡ndez, A. B., and Nunez, G. (2007). Maternal exposure to polycyclic aromatic hydrocarbons diminishes murine ovarian reserve via induction of Harakiri. *Journal of Clinical Investigation* **117**, 3971-3978.

Kao, S. W., Sipes, I. G., and Hoyer, P. B. (1999). Early effects of ovotoxicity induced by 4-vinylcyclohexene diepoxide in rats and mice. *Reproductive Toxicology* **13**, 67-75.

Keating, A. F., Fernandez, S. M., Mark-Kappeler, C. J., Sen, N., Sipes, I. G., and Hoyer, P. B. (2011). Inhibition of PIK3 signaling pathway members by the ovotoxicant 4-vinylcyclohexene diepoxide in rats. *Biology of reproduction* **84**, 743-751.

Keating, A. F., Sen, N., Sipes, I. G., and Hoyer, P. B. (2009). Effect of phosphatidylinositol-3 kinase inhibition on ovotoxicity caused by 4-vinylcyclohexene diepoxide and 7, 12-dimethylbenz [a] anthracene in neonatal rat ovaries. *Toxicology and applied pharmacology* **241**, 127-134.

Klaunig, J. E., and Kamendulis, L. M. (2004). The role of oxidative stress in carcinogenesis. *Annu. Rev. Pharmacol. Toxicol.* **44**, 239-267.

Lodovici, M., Akpan, V., Evangelisti, C., and Dolara, P. (2004). Sidestream tobacco smoke as the main predictor of exposure to polycyclic aromatic hydrocarbons. *Journal of applied toxicology* **24**, 277-281.

Mark-Kappeler, C. J., Hoyer, P. B., and Devine, P. J. (2011a). Xenobiotic Effects on Ovarian Preantral Follicles. *Biology of reproduction* **85**, 871-883.

Mark-Kappeler, C. J., Sen, N., Keating, A. F., Sipes, I. G., and Hoyer, P. B. (2010). Distribution and responsiveness of rat anti-MÃ¼llerian hormone during ovarian development and VCD-induced ovotoxicity. *Toxicology and applied pharmacology* **249**, 1-7.

Mark-Kappeler, C. J., Sen, N., Lukefahr, A., McKee, L., Sipes, I. G., Konhilas, J., and Hoyer, P. B. (2011b). Inhibition of ovarian KIT phosphorylation by the ovotoxicant 4-vinylcyclohexene diepoxide in rats. *Biology of reproduction* **85**, 755-762.

Matikainen, T., Perez, G. I., Jurisicova, A., Pru, J. K., Schlezinger, J. J., Ryu, H. Y., Laine, J., Sakai, T., Korsmeyer, S. J., and Casper, R. F. (2001). Aromatic hydrocarbon receptor-driven Bax gene expression is required for premature ovarian failure caused by biohazardous environmental chemicals. *Nature genetics* **28**, 355-360.

Mattison, D. R. (1980). Morphology of oocyte and follicle destruction by polycyclic aromatic hydrocarbons in mice. *Toxicology and applied pharmacology* **53**, 249-259.

Mattison, D. R., Shiromizu, K., and Nightingale, M. S. (1983). Oocyte destruction by polycyclic aromatic hydrocarbons. *American journal of industrial medicine* **4**, 191-202.

McNatty, K. P., Fidler, A. E., Juengel, J. L., Quirke, L. D., Smith, P. R., Heath, D. A., Lundy, T., O'Connell, A., and Tisdall, D. J. (2000). Growth and paracrine factors regulating follicular formation and cellular function. *Molecular and Cellular Endocrinology* **163**, 11-20.

Miao, Y. L., Kikuchi, K., Sun, Q. Y., and Schatten, H. (2009). Oocyte aging: cellular and molecular changes, developmental potential and reversal possibility. *Human reproduction update* **15**, 573-585.

Neal, M. S., Zhu, J., and Foster, W. G. (2008). Quantification of benzo [a] pyrene and other PAHs in the serum and follicular fluid of smokers versus non-smokers. *Reproductive toxicology* **25**, 100-106.

Paterson, M., Koothan, P. T., Morris, K. D., O'Byrne, K. T., Braude, P., Williams, A., and Aitken, R. J. (1992). Analysis of the contraceptive potential of antibodies against native and deglycosylated porcine ZP3 in vivo and in vitro. *Biology of reproduction* **46**, 523-534.

Paulose, T., Hernandez-Ochoa, I., Basavarajappa, M. S., Peretz, J., and Flaws, J. A. (2011). Increased sensitivity of estrogen receptor alpha overexpressing antral follicles to methoxychlor and its metabolites. *Toxicological Sciences* **120**, 447-459.

Picut, C. A., Swanson, C. L., Scully, K. L., Roseman, V. C., Parker, R. F., and Remick, A. K. (2008). Ovarian follicle counts using proliferating cell nuclear antigen (PCNA) and semi-automated image analysis in rats. *Toxicologic pathology* **36**, 674-679.

Pike, C. J., Carroll, J. C., Rosario, E. R., and Barron, A. M. (2009). Protective actions of sex steroid hormones in Alzheimer's disease. *Frontiers in Neuroendocrinology* **30**, 239-258.

Rajapaksa, K. S., Sipes, I. G., and Hoyer, P. B. (2007). Involvement of microsomal epoxide hydrolase enzyme in ovotoxicity caused by 7, 12-dimethylbenz [a] anthracene. *Toxicological sciences* **96**, 327-334.

Reddy, P., Zheng, W., and Liu, K. (2010). Mechanisms maintaining the dormancy and survival of mammalian primordial follicles. *Trends in Endocrinology & Metabolism* **21**, 96-103.

Rosano, G. M. C., Vitale, C., Marazzi, G., and Volterrani, M. (2007). Menopause and cardiovascular disease: the evidence. *Climacteric* **10**, 19-24.

Schwarz, D., Kisselev, P., Cascorbi, I., Schunck, W. H., and Roots, I. (2001). Differential metabolism of benzo [a] pyrene and benzo [a] pyrene-7, 8-dihydrodiol by human CYP1A1 variants. *Carcinogenesis* **22**, 453-459.

Shiromizu, K., and Mattison, D. R. (1985). Murine oocyte destruction following intraovarian treatment with 3-• methylcholanthrene or 7, 12-dimethylbenz (a) anthracene: Protection by alpha-naphthoflavone. *Teratogenesis, carcinogenesis, and mutagenesis* **5**, 463-472.

Shuster, L. T., Rhodes, D. J., Gostout, B. S., Grossardt, B. R., and Rocca, W. A. (2010). Premature menopause or early menopause: long-term health consequences. *Maturitas* **65**, 161-166.

Skinner, S. M., Mills, T., Kirchick, H. J., and Dunbar, B. S. (1984). Immunization with zona pellucida proteins results in abnormal ovarian follicular differentiation and inhibition of gonadotropin-induced steroid secretion. *Endocrinology* **115**, 2418-2432.

Sobinoff, A. P., Bernstein, I. R., and McLaughlin, E. A. (2012a). All Your Eggs in One Basket; Mechanisms of Xenobiotic Induced Female Reproductive Senescence. In Senescence (T. Nagata, ed., pp. 559-584. InTech Press Rijeka, Croatia.

Sobinoff, A. P., Mahony, M., Nixon, B., Roman, S. D., and McLaughlin, E. A. (2011). Understanding the Villain: DMBA-Induced Preantral Ovotoxicity Involves Selective Follicular Destruction and Primordial Follicle Activation through PI3K/Akt and mTOR Signaling. *Toxicological Sciences* **123**, 563-575.

Sobinoff, A. P., Mahony, M., Nixon, B., Roman, S. D., and McLaughlin, E. A. (2012b). Staying alive: PI3K pathway promotes primordial follicle activation and survival in response to 3-MC induced ovotoxicity. *Toxicological Sciences*, doi:10.1093/toxsci/kfs137.

Sobinoff, A. P., Pye, V., Nixon, B., Roman, S. D., and McLaughlin, E. A. (2010). Adding Insult to Injury: Effects of Xenobiotic-Induced Preantral Ovotoxicity on Ovarian Development and Oocyte Fusibility. *Toxicological Sciences* **118**, 653-666.

Sobinoff, A. P., Pye, V., Nixon, B., Roman, S. D., and McLaughlin, E. A. (2012c). Jumping the gun: Smoking constituent BaP causes premature primordial follicle activation and impairs oocyte fusibility through oxidative stress. *Toxicology and Applied Pharmacology* **260**, 70-80.

Springer, L. N., McAsey, M. E., Flaws, J. A., Tilly, J. L., Sipes, I. G., and Hoyer, P. B. (1996). Involvement of apoptosis in 4-vinylcyclohexene diepoxide-induced ovotoxicity in rats. *Toxicology and applied pharmacology* **139**, 394-401.

Sullivan, J. L., and Newton, R. B. (1988). Serum antioxidant activity in neonates. *Archives of disease in childhood* **63**, 748-750.

Tarin, J. J. (1996). Potential effects of age-associated oxidative stress on mammalian oocytes/embryos. *Molecular human reproduction* **2**, 717-724.

Trevor, M., Ohnishi, S. T., Ohnishi, T., and Harvey, R. G. (1996). Generation of reactive oxygen species during the enzymatic oxidation of polycyclic aromatic hydrocarbon trans-dihydrodiols catalyzed by dihydrodiol dehydrogenase. *Chemical research in toxicology* **9**, 84-92.

Tsai-Turton, M., Nakamura, B. N., and Luderer, U. (2007). Induction of apoptosis by 9, 10-dimethyl-1, 2-benzanthracene in cultured preovulatory rat follicles is preceded by a rise in reactive oxygen species and is prevented by glutathione. *Biology of Reproduction* **77**, 442-451.

Wells, P. G., McCallum, G. P., Chen, C. S., Henderson, J. T., Lee, C. J. J., Perstin, J., Preston, T. J., Wiley, M. J., and Wong, A. W. (2009). Oxidative stress in developmental origins of disease: teratogenesis, neurodevelopmental deficits, and cancer. *Toxicological sciences* **108**, 4-18.

Windham, G. C., Mitchell, P., Anderson, M., and Lasley, B. L. (2005). Cigarette smoking and effects on hormone function in premenopausal women. *Environmental health perspectives* **113**, 1285-1290.

Appendix A: Additional Publications with Relevance to this Thesis

Appendix A.1: Contraception targets in mammalian ovarian development

Contraception Targets in Mammalian Ovarian Development

Eileen A. McLaughlin and Alexander P. Sobinoff

Contents

Abstract In the human ovary, early in pre-natal life, oocytes are surrounded by pre-granulosa follicular cells to form primordial follicles. These primordial oocytes remain dormant, often for decades, until recruited into the growing pool throughout a woman's adult reproductive years. Activation of follicle growth and subsequent development of growing oocytes in pre-antral follicles are major biological checkpoints that determine an individual females reproductive potential. In the past decade, great strides have been made in the elucidation of the molecular and cellular mechanisms underpinning maintenance of the quiescent primordial follicle

E.A. McLaughlin (✉)
Reproductive Science Group, School of Environmental & Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia
ARC Centre of Excellence in Biotechnology & Development, University of Newcastle, Callaghan, NSW 2308, Australia
e-mail: eileen.mclaughlin@newcastle.edu.au
A.P. Sobinoff
Reproductive Science Group, School of Environmental & Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia

U.-F. Habenicht and R.J. Aitken (eds.), *Fertility Control*,
Handbook of Experimental Pharmacology 198,
DOI 10.1007/978-3-642-02062-9_4, © Springer-Verlag Berlin Heidelberg 2010

pool and initiation and development of follicle growth. Gaining an in-depth knowledge of the intracellular signalling systems that control oocyte preservation and follicle activation has significant implications for improving female reproductive productivity and alleviating infertility. It also has application in domestic animal husbandry, feral animal population control and contraception in women.

Keywords Fertility control · Granulosa cells · Oocyte · Primary follicle · Primordial follicle

1 Introduction

In the mammalian ovary, early in pre-natal life, oocytes are surrounded by pre-granulosa follicular cells to form primordial follicles. These primordial oocytes remain dormant, often for decades, until recruited into the growing pool throughout a woman's adult reproductive years (Choi and Rajkovic 2006; Reynaud and Driancourt 2000). Once in the primordial follicle, there are only two fates awaiting the oocyte; either the germ cell will be directed to grow and eventually be ovulated or more likely, the oocyte will become atretic. In most mammals, greater than 99% of the oocytes are lost – with only a tiny proportion selected for ovulation. The growing follicles doomed to atresia are easily recognisable within the ovary as they display several markers of apoptotic cell death (Krysko et al. 2008). In contrast, the mechanisms that underpin the maintenance, selection and maturation of the very small population of good-quality and presumably functional oocytes in primordial follicles are only just becoming clear.

Bi-directional signalling between the oocyte and the surrounding somatic cells is considered fundamental in the delicate balance of positive and negative forces controlling the maintenance and activation of the primordial follicle pool (Hutt et al. 2006a; McLaughlin and McIver 2009; Skinner 2005). Both the maintenance of healthy follicles and the highly regulated and selective release of primordial oocytes into the growing pool (Jin et al. 2005) involves cross-talk between an every growing list of cytokines and growth factors (Dissen et al. 2009; Picton et al. 2008; Trombly et al. 2009). Excitingly in the past few years, great strides have been made in characterising the intracellular signalling pathways activated during pre-antral follicle development (John et al. 2007; Reddy et al. 2008) providing fundamental knowledge and insight into the molecular systems responsible for ensuring production of functional oocytes for fertilisation (McLaughlin and McIver 2009).

Throughout the globe, many couples are unable to control their fertility, with an estimated 200 million plus women in the developing world either not using any form of contraception or relying on traditional methods only (Rowlands 2009). In contrast, in the Western world, both access and use of contraception are high; however, the vast majority of women still rely on the oral contraceptive pill or on barrier methods, principally the condom (Rowlands 2009). Ostensibly, there have been many advances in the "pill" over the past 50 years including improved

formulations and the introduction of a range of local vaginal and intrauterine, injectable or sub/transdermal hormonal-based contraceptives. Barrier methods with complementary spermicide/microbicide activity are also under development – particularly as adjuncts for the control of sexually transmitted diseases, such as HIV/AIDS (Rowlands 2009). Since no novel methods of contraception have been introduced since the 1960s, recent insights into the basic cellular mechanisms underpinning follicle maintenance and oocyte development will inform biotechnology strategies for the manipulation of reproduction in humans.

2 Ovarian Folliculogenesis and Exhaustion of the Primordial Follicle Pool

In mammals, primordial germ cells (PGCs) migrate early in embryonic development to colonise the naive gonad where they differentiate to become oogonia. Folliculogenesis begins with recruitment of somatic pre-granulosa cells to the oocyte to form the primordial follicle (Fig. 1) (Dickinson et al. 2010). In rodents and lagomorphs, synchronous primordial follicle formation occurs during the first

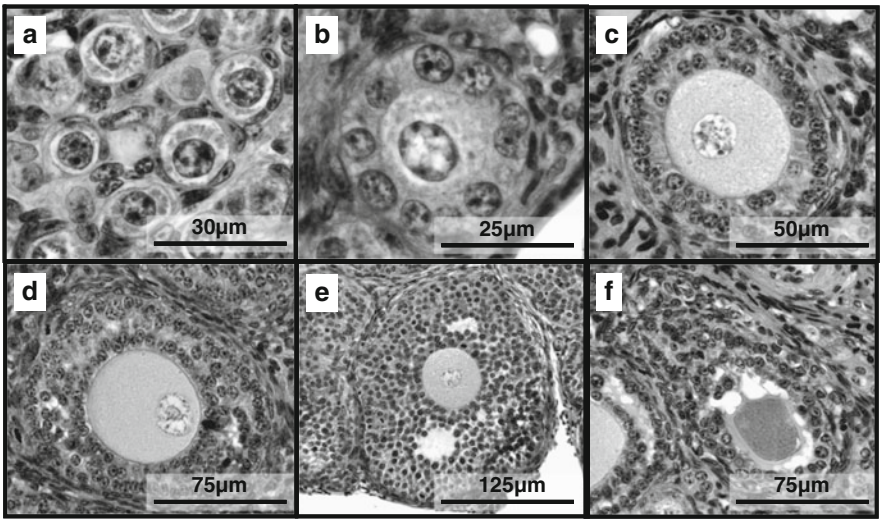
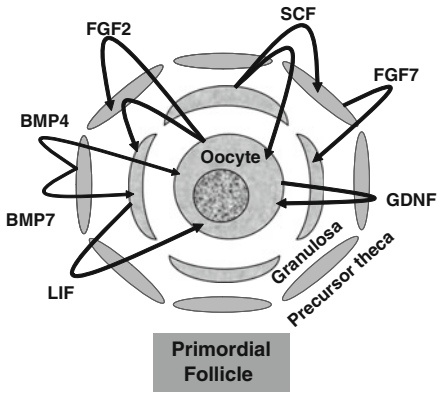


Fig. 1 Photomicrographs depicting the architecture and classification of ovarian follicles during folliculogenesis (**a**) primordial follicles; quiescent oocytes surrounded by a single layer of squamous granulosa cells, (**b**) primary follicle; primary oocytes characterised by a single layer of cuboidal granulosa cells, (**c**) secondary follicle; enlarged oocytes surrounded by a second layer of granulosa cells and marked by the acquisition of a thecal cell layer surrounding the follicle, (**d**) pre-antral follicle; large oocytes surrounded by multiple layers of granulosa cells, (**e**) antral follicle; mature follicle characterised by the presence of a fluid filled cavity within the granulosa cell layer known as an antrum, (**f**) atretic follicle; degenerated follicles found at all stages of follicular development characterised by "detached" granulosa cells and apoptotic oocytes

few days and weeks of early post natal life (Hutt and Albertini 2006; Pedersen and Peters 1968). In contrast, in livestock and primates including the human, follicle formation and activation occur asynchronously during foetal life (Lintern-Moore et al. 1974; Wandji et al. 1996), resulting in ovaries at birth containing both growing pre-antral and early antral follicles in the neonatal ovary (Picton et al. 1998).

Endocrine mechanisms are thought to trigger primordial follicle formation and these are mediated by the maternal hormonal milieu. High levels of maternal oestrogen in the foetal ovary act to maintain intact germline nests and the subsequent decrease of oestrogen and progesterone allows follicle formation (Chen et al. 2007, 2009; Nilsson et al. 2006; Nilsson and Skinner 2009; Pepling et al. 2009). Similarly, a progesterone and oestrogen endocrine-based mechanism of primordial follicle activation has been postulated as both hormones act in in vitro culture experiments to decrease primordial follicle recruitment during the first wave of folliculogenesis in rodents (Kezele and Skinner 2003). Multiple perinatal mechanisms establish the size of the primordial follicle reserve with follicle loss resulting from apoptotic germ cell loss, substantial autophagy and ovarian morphogenesis comprising active extrusion of non-apoptotic germ cells, all resulting in substantial depletion of the follicle population (Rodrigues et al. 2009). As progesterone and oestrogen levels have been noted to drop in bovine and primate foetal ovaries during mid to late gestation coincident with follicle assembly and growth initiation, this further implicates a steroid-based negative regulatory mechanism (Kezele and Skinner 2003; Nilsson and Skinner 2009; Yang and Fortune 2008). Interestingly, earlier studies indicate that there are increased populations of pre-antral and antral follicles in juvenile oestrogen-deficient aromatase knockout (ArKO) mice, also supporting the notion that primordial follicle activation is dependent on a decline in oestrogen levels (Britt et al. 2004). Furthermore, diethylstilbestrol inhibits follicle formation and development in neonatal mouse ovaries in vitro, by acting through oestrogen receptor alpha (EPα) (Kim et al. 2009a, b). Latterly, the androgen, testosterone, has been demonstrated to increase follicle activation in vitro (Yang et al. 2010) and extrapolating from this observation is the hypothesis that excess intra-ovarian androgen is linked to polycystic ovary syndrome (PCOS) (Yang et al. 2010).

In the primordial follicle, and throughout most of its subsequent growth and development, the oocyte is arrested in prophase I of the first meiotic division and only re-enters meiosis or germinal vesicle breakdown upon ovulation (Fig. 1) (Pedersen 1969, 1970). The resting primordial follicles in the ovarian pool are sequentially stimulated to activate and grow, at which point a majority become atretic (Rodrigues et al. 2008; Tingen et al. 2009), with a small minority developing through pre-antral and antral stages into mature Graffian follicles (McLaughlin and McIver 2009; Skinner 2005) (see Fig. 1). Following activation, the rapidly enlarging oocyte synthesises an acellular extracellular matrix, the zona pellucida, and this process is supported by proliferating granulosa cells (Fig. 1). Finally, during antral follicle development, the oocytes become meiotically competent (Zheng and Dean 2007). A surge of luteinising hormone initiated just prior to ovulation resulting in nuclear maturation, completion of the first meiotic division and first polar body extrusion, then re-arrest in meiosis II at metaphase II (Hutt and Albertini 2007) (Fig. 1).



Factor	Effect	Reference
SCF	Stimulates follicular recruitment and promotes precursor theca function	Jin et al., 2005; Hutt et al., 2006
LIF	Stimulates follicular recruitment	Nilsson et al., 2002
FGF2	Promotes SCF expression and cell growth	Nilsson et al., 2001; Nilsson and skinner, 2004
FGF7	Promotes SCF expression	Kezele et al., 2005
GDNF	Autocrine signalling promotes follicular recruitment	Dole et al., 2008
BMP4, BMP7	Stimulates follicular recruitment and survival	Nilsson and Skinner, 2003; Lee et al., 2001; Lee et al 2004

Fig. 2 Ovarian cytokine and growth factor signalling factors involved in primordial follicle recruitment. Abbreviations: *SCF* stem cell factor; leukaemia inhibitory factor; *FGF* fibroblast growth factor; *GDNF* glial-derived neurotrophic factor; *BMP* bone morphogenic factor

In female mammals, the primary determinant of successful reproductive performance is the initial size and then the controlled release of primordial follicles from the resting pool (Maheshwari and Fowler 2008). Studies of total non-growing ovarian follicle populations have determined that the rate of decline in quiescent functional follicles in the adult ovary increases with age (Hansen et al. 2008).

So how does the ovary regulate primordial follicle population dynamics? Recently, quantification of the relative spatial positions and inter-follicular distances between the quiescent primordial follicles and growing pre-antral follicles in neonatal mouse ovaries indicated that follicles were significantly less likely to have started growing if they had one or more primordial follicles closely adjacent (Da Silva-Buttkus et al. 2009). This observation is consistent with the notion that primordial follicles may, as has been previously hypothesised (McLaughlin and McIver 2009), produce diffusible factor(s) that inhibit neighbouring primordial follicles from activating and initiating development (Adhikari and Liu 2009). Thus, the use of positive and negative paracrine signalling mechanisms (Fig. 2) may allow the ovary to both maintain an ovarian pool of follicles throughout the reproductive lifespan, while providing a highly selected supply of functional oocytes for ovulation (Adhikari and Liu 2009; Edson et al. 2009; McLaughlin and McIver 2009).

3 Early Folliculogenesis: Roles of Cytokines, Chemokines, Hormones and Growth Factors

Folliculogenesis in the mammalian ovary has been well characterised into major morphologically distinct entities based on the size of the oocyte and

number of granulosa cells (see Fig. 1) surrounding the oocyte (Pedersen and Peters 1968). More generally these stages are classified as either pre-antral and gonadotrophin independent, comprising the primordial, primary and secondary follicles or the antral, gonadotrophin-dependent phase consisting of the antral, pre-ovulatory, ovulatory follicles and the corpus luteum (see Fig. 1) (Pedersen and Peters 1968; 1971). Studies of naturally occurring mutant mice, particularly mice with Kit mutations (Geissler et al. 1981), were able to determine some of the signalling cascades inherent to normal folliculogenesis (Hutt et al. 2006b). Subsequently, large scale genomic and more recently proteomic approaches have identified a number of paracrine and autocrine signalling pathways associated with primordial follicle and pre-antral follicle development (Arraztoa et al. 2005; Holt et al. 2006; Kezele et al. 2005b; Serafica et al. 2005; Wang et al. 2009).

Key findings from these studies have included the identification of ligands and their receptors localised in primordial follicles, which are implicated as key regulators of the primordial to primary and secondary follicle transition as well as atresia and maintenance of quiescence (see Figs. 2 and 4).

Intracellular signalling pathways initiated, via a wide range growth factors and pleiotrophic cytokines, are known to activate mammalian primordial follicles in mammalian ovarian explant culture systems. These include basic fibroblast growth factor (FGF2) (Garor et al. 2009; Nilsson et al. 2001), vascular endothelial growth factor A (VEGFA) (Artac et al. 2009; McFee et al. 2009), platelet-derived growth factor (PDGF) (Nilsson et al. 2006), kit ligand/stem cell factor (KIT-L/SCF) (Hutt et al. 2006b), leukaemia inhibitory factor (LIF) (Nilsson et al. 2002), keratinocyte growth factor (KGF) (Kezele et al. 2005a), bone morphogenic proteins (BMP 4 and 7) (Craig et al. 2007; Lee et al. 2001, 2004; Nilsson and Skinner 2003), glial-derived neurotrophic factor (GDNF) (Dole et al. 2008) and the neurotrophins (NGF, NT4, BDNF, NT3) (Dissen et al. 2002; Dole et al. 2008; Nilsson et al. 2009; Paredes et al. 2004; Romero et al. 2002; Spears et al. 2003) (see Fig. 2).

Repressors of follicle activation include anti-Müllerian hormone (AMH) which, when added to ovarian explant cultures, inhibits the primordial to primary follicle transition in rodents (Durlinger et al. 2002a). However, no mouse model exists that supports this claim as over-expression of AMH in vivo results in germ cell loss and ovarian degeneration (Behringer 1995; Lyet et al. 1996) probably via activation of an AMHRII-mediated pathway (Mishina et al. 1999). Interestingly, prolonged exposure to AMH initiates follicle development in human ovarian explant cultures (Schmidt et al. 2005). Recently, the chemoattractive chemokine SDF-1 (also known as CXCL-12) was identified as second inhibitor of primordial follicle activation in vitro (Holt et al. 2006). Manipulating the primordial follicle pool remains an attractive method of controlling female fertility though as yet no unique and most importantly reversible contraceptive agent targeting primordial follicle activation has been identified.

4 Multiple Activator and Repressor Pathways Converge to Regulate Activation of the Primordial Follicle

Numerous studies point to multiple stimulatory and negative pathways converging to regulate the activation of the primordial follicle. In in vitro culture systems, large numbers of primordial follicles spontaneously enter the growing pool – and treatment with exogenous cytokines, chemokines, hormones and growth factors increases (or decreases) the proportion of activated follicles. Notably treatment with antagonists or function blocking antibodies will also suppress but not abolish primordial follicle activation indicating multiple possible redundant endogenous mechanisms (Holt et al. 2006; Hutt et al. 2006b; Kezele et al. 2002; Nilsson et al. 2007).

Studies of null or mutated mice indicate that many of the ligands or their receptors implicated in follicle activation are not essential (Dono et al. 1998; Stewart et al. 1992). A recent example is the knock-in mutation (Kit^{Y19F}) mice, which despite a complete abrogation of the PI3K pathway have normal folliculogenesis, ovarian morphology and are fertile (John et al. 2009).

In conclusion, multiple cytokine and growth factor-activated pathways must undertake cross talk to produce an intracellular balance of positive and negative signals, thus ensuring the long-term stability of quiescent primordial follicles, with the release of selected oocytes from repression into the growing population from this precious and finite resource. Targeting the intracellular pathways activated by these pleiotrophic cytokines/growth factors is an attractive prospect as a mechanism to influence follicle growth. Great strides have been made in the past 5 years in the characterisation of these pathways and the development of primordial follicle-specific contraceptive pharmacological agents is now a very real possibility.

5 Intracellular Signalling in Oocytes and Pregranulosa Cells in Primordial Follicles

A member of the forkhead transcription family *FoxO3a* is a central player in the pathway(s) implicated in primordial follicle activation (Fig. 3). FoxO3a is a well-characterised regulator of embryogenesis, tumorigenesis and the maintenance of differentiated cell states through direction of key cellular processes such as stress responses, cell cycle arrest and programmed cell death (Hosaka et al. 2004; Kaufmann and Knochel 1996).

FoxO3a null mice suffer from a lack of primordial follicles in early neonatal life coupled with increased numbers of growing follicles and a subsequent increase in oocyte degeneration of newly growing follicles (Castrillon et al. 2003; Hosaka et al. 2004). Localised initially to granulosa cells (Richards et al. 2002), FoxO3a was thought to mediate its suppressive effects on primordial oocyte recruitment by increasing the expression in the oocyte of a cell cycle inhibitor, p27kip1 (or Cdkn1b), whilst concurrently decreasing cyclin D1 and D2 expression, thereby arresting the cell cycle (Brenkman and Burgering 2003) (Fig. 3). However,

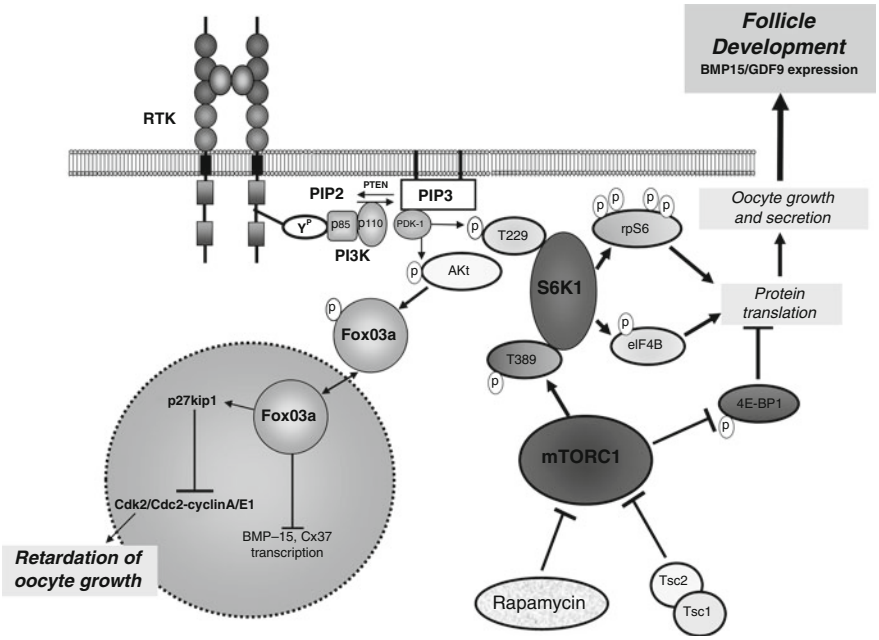


Fig. 3 The oocyte PTEN-PI3K – FoxO3a and mTORC pathways govern follicle activation through control of initiation of oocyte growth and maintenance of oocyte quiescence. Abbreviations: *PIP2* Phosphatidyl inositol bisphosphate; *PI3K* Phosphoinositide 3-kinase; *PIP3* Phosphatidylinositol (3,4,5)-trisphosphate; *PDK1* 3-Phosphoinositide-dependent protein kinase, S6K1, p70 S6 kinase; *PTEN* phosphatase and tensin homolog; *Akt* Protein Kinase B; *rpS6* Ribosomal protein S6; *FoxO3a* Forkhead box O3A; *GSK3* Glycogen synthase kinase 3; *p27kip1* Cyclin-dependent kinase inhibitor 1B (CDKN1); *Cdk2* Cyclin dependent kinase 2; *BMP15* Bone Morphogenic Protein 15; *Cx37* Connexin 37; *GDF9* Growth Differentiation factor 9; *mTORC1* mammalian Target of Rapamycin Complex 1; *eIF4B* eukaryotic initiation factor 4B; *TSC1/2* tuberous sclerosis complex 1/2; *4E-BP1* Eukaryotic translation initiation factor 4E binding protein 1

characterisation of the p27kip1 deficient mouse revealed accelerated postnatal follicle assembly and a vast increase in the founding primordial follicle population, which then underwent premature activation (Rajareddy et al. 2007). Consequently, it was established that p27kip1 controls oocyte development by suppressing the functions of Cdk2/Cdc2-Cyclin A/E1 in the diplotene arrested oocytes (Rajareddy et al. 2007), while simultaneously activating a caspase-mediated apoptotic cascade, thus also inducing follicle atresia (Rajareddy et al. 2007).

6 Signal Transduction: The Phosphatidylinositol 3-Kinase (PI3K) and the mTOR Pathways

Akt (Protein Kinase B) signalling was predicted to be activated via extracellular receptor tyrosine kinase (RTK) signalling pathways that regulate the phosphorylation control of FoxO3a inactivation via nuclear exclusion (Junger et al. 2003).

The use of multiple germ cell-specific knockout mice indicates that extracellular signals transduced through the PI3K (phosphatidylinositol 3-kinase) pathway (Reddy et al. 2005) are fundamentally important for the regulation of early follicular development (Liu et al. 2006) (Fig. 3). The majority of the constituents of one PI3K pathway, including GSK-3α and GSK-3β, Akt, Foxo3a, (Liu et al. 2007b), FoxO3a and p27kip1 (Rajareddy et al. 2007) have been demonstrated to be present in growing mouse oocytes. It was proposed that stimulation of the PIK3 pathway through an RTK (possibly c-kit though not exclusively) (see John et al. 2009) results in the phosphorylation and functional suppression of FoxO3a and thus the release of quiescent oocytes into a state of active growth and development (Liu et al. 2007a). This hypothesis was supported by evidence that FoxO3a is capable of both suppressing BMP15, connexin 37 and connexin 43 production in mouse oocytes (Fig. 3), important factors in oocyte-granulosa and inter-granulosa cell communications, and also to up-regulate expression of p27 in the oocyte nucleus, ultimately resulting in the suppression of oocyte growth and follicular activation (Liu et al. 2006).

Phenotypic analysis of a null mouse with an oocyte-specific conditional knock-out of Pten (phosphatase and tensin homolog deleted on chromosome 10), a major negative regulator of PI3K (phosphatidylinositol 3-kinase) revealed the entire primordial follicle pool becomes activated and all primordial follicles become depleted in early adulthood, causing premature ovarian failure (Fig. 3). The authors concluded that the oocyte Pten-PI3K pathway governs follicle activation through control of the initiation of oocyte growth (Reddy et al. 2008) (see Fig. 3). This was subsequently confirmed with the development of an oocyte-specific inducible [Vasa-Cre(ERT2)] conditional knock-out mouse (John et al. 2008). Using this model, targeted ablation of Pten was shown to activate the PI3K/Akt pathway leading to hyperphosphorylation of Foxo3 and primordial follicle activation. Moreover, Foxo 3 was shown to control primordial follicle activation via a nucleocytoplasmic shuttling mechanism; the phosphorylation of this transcription factor catalysing its movement from the nucleus to the cytoplasm.

More recent studies of Akt1 null females indicate that they have both reduced fertility and abnormal oestrous cyclicity (Brown et al. 2009). In early postnatal life, Akt1 null ovaries display abnormal folliculogenesis and this is followed in early adulthood by a significant decrease in the primordial follicle population (Brown et al. 2009), reinforcing the notion that the PI3K/Akt pathway is critical for primordial follicle development.

Subsequent to their studies of the Pten null mouse, another member of the PI3K pathway, 3-phosphoinositide-dependent protein kinase-1 (Pdpk1), was implicated in follicle activation (Fig. 3). In stage-specific Pdpk1 null mice, the majority of primordial follicles were depleted prematurely, causing ovarian failure by early adulthood (Jagarlamudi et al. 2009; Reddy et al. 2009). This outcome was linked to the suppression of Pdpk1– p70 S6 kinase 1 (S6K1)-ribosomal protein S6 (rpS6) signalling (Jagarlamudi et al. 2009; Reddy et al. 2009), thus continuing to implicate the PI3K/Pten/Pdpk1 signalling pathway as central to the molecular oocyte network that controls the primordial follicle population. Thus, reproductive ageing in

females appears fundamentally linked to the dysregulation of this signalling pathway in oocytes resulting in subfertility and premature ovarian failure.

A second pathway appears to function synergistically with the PI3K–Pten network described above. Oocyte-specific deletion of a negative regulator of Target of Rapamycin Complex 1 (mtorc1), tumour suppressor tuberous sclerosis complex 1 (Tsc1), results in the premature activation of the entire pool of primordial follicles and follicular depletion in early adulthood, causing premature ovarian failure (Adhikari et al. 2010). Not surprisingly, oocyte-specific tumour suppressor tuberous sclerosis complex 2 (Tsc2), which also negatively regulates (mtorc1), also functions to maintain the primordial follicle population in quiescence (Adhikari et al. 2009). As described for Tsc1 null mice, the absence of the Tsc2 gene in oocytes results in a phenotype in which the primordial follicles are prematurely activated and depletion of follicles in early adulthood, causing premature ovarian failure (Fig. 3). These findings' results suggest that the Tsc1–Tsc2 complex is required to establish the quiescent state of primordial follicles via suppression of mtorc1 activity and that activation of the primordial follicle is dependent on mtorc1 activity in oocytes (Adhikari et al. 2009).

Supporting somatic cell lineages also play a major role in controlling and nurturing primordial follicle activation and development. A key example is the forkhead transcription factor Foxl2, as mutations in the FOXL2 gene are associated with ovarian failure in both humans and mice (Duffin et al. 2009; Uda et al. 2004). Foxl2-deficient mice display major defects in primordial follicle activation with consequent follicle loss. In addition, roles in gonadal development and sex determination have also been suggested (Uda et al. 2004). Features of Foxl2 null animals point towards a new mechanism of premature ovarian failure, with all major somatic cell lineages failing to develop around growing oocytes from the time of primordial follicle formation (Uda et al. 2004).

7 Promoting and Regulating Early Follicle Growth and Development

Many members of the TGFβ superfamily act as paracrine growth factors and are expressed by both ovarian somatic cells and oocytes in a developmental-stage specific manner. A well characterised marker of ovarian follicular reserve is AMH – originally identified in Sertoli cells of the foetal testis and known to promote the regression of the Müllerian ducts during differentiation of the male reproductive tract (Munsterberg and Lovell-Badge 1991). AMH is also expressed in ovarian granulosa cells (Durlinger et al. 1999) and as outlined above both acts as an inhibitor of the initiation of primordial follicle growth and decreases the sensitivity of follicles to the FSH-dependent selection for dominance in both mice (Visser and Themmen 2005) and humans (Dumesic et al. 2009).

During folliculogenesis, AMH expression is initiated in the granulosa cells of primary follicles, peaks in granulosa cells of pre-antral and small antral follicles and

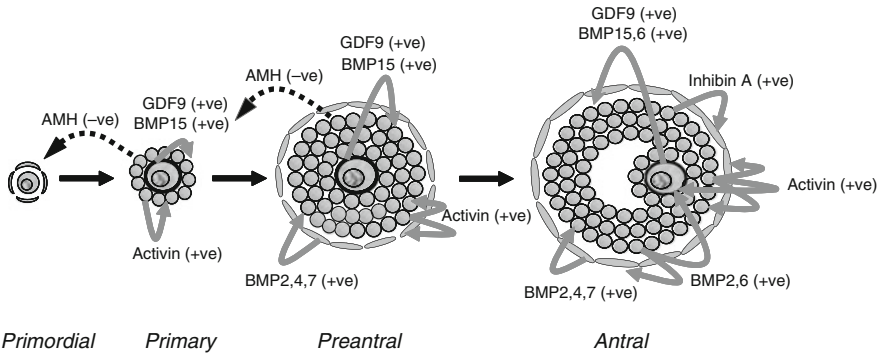


Fig. 4 Potential signalling interactions (+ve, stimulatory; −ve inhibitory) of TGF-β superfamily members involved in the primary to early antral follicle stages. Abbreviations: *AMH* anti Müllerian hormone; *GDF* growth differentiation factor; *BMP* bone morphogenic protein

gradually diminishes in pre-ovulatory follicles (Dumesic et al. 2009; Durlinger et al. 2002b; Visser and Themmen 2005). This continued expression of AMH until the antral stage indicates a continual function in folliculogenesis beyond inhibiting the initiation of primordial follicle growth (Fig. 4). AMH inhibits FSH-stimulated follicle growth in both the mouse and human, suggesting that AMH is one of the factors determining the sensitivity of ovarian follicles for FSH (Durlinger et al. 2001) and is therefore a dominant regulator of early follicle growth (Dumesic et al. 2009).

In mammals, FSH also acts as the predominant survival factor for selected antral follicles, preventing the spontaneous onset of follicular apoptosis. Within this subpopulation, the follicles with the highest FSH sensitivity become dominant and continue to develop into Graffian follicles. On the other hand, AMH inhibition of FSH follicle sensitivity may play a role in antral follicle selection (McGee and Hsueh 2000; Durlinger et al. 2001). On binding to AMH receptors located on the granulosa cells of small pre-antral follicles (Fig. 4), AMH signalling activates SMAD transcriptional regulators which reduce LH receptor expression, leading to a decrease in aromatase (Diclemente et al. 1994) and therefore oestradiol levels (Andersen and Byskov 2006) and reduced FSH sensitivity (Kevenaar et al. 2007a, b).

Recent studies in normo-ovulatory women have indicated that serum AMH levels decrease with age in pre-menopausal women (de Vet et al. 2002), and that there is a direct correlation between serum AMH levels and antral follicle number (van Rooij et al. 2002), thus reflecting the size of the primordial follicle pool (Fig. 4). Therefore, AMH levels can be used to indicate ovarian follicle reserve (Visser et al. 2006) and to determine treatment strategies for women undergoing assisted conception (Macklon et al. 2006; Nelson et al. 2007, 2009).

Given that AMH plays important roles in both primordial follicle activation and antral follicle selection, it is a tempting target for a contraceptive agent. One possible avenue involves the use of recombinant AMH, or AMH agonists and/or antagonists for the long-term control of female fertility. In the case of contraception, the use of recombinant AMH and/or agonists that mimic the endogenous

growth factor could be used to halt primordial follicle recruitment, augmenting or providing an alternative form of hormonal contraceptive with the added benefit of possibly preserving the primordial follicle pool and therefore prolonging the reproductive life cycle. Indeed, advancements in recombinant technology have allowed the production of bio-activated AMH, which could possibly be used in such a role (Weenen et al. 2004).

GDF9, an oocyte-specific member (McGrath et al. 1995) of the growth and differentiation subfamily of TGFβ growth factors and it's receptor, bone morphogenic receptor type II, are expressed in rodent and human primary follicle stage oocytes and granulosa cells (Aaltonen et al. 1999; Hayashi et al. 1999), suggesting a role in paracrine signalling within the follicular microenvironment (Vitt et al. 2002). GDF9 has been shown to be essential for the development of primary follicles (Juengel et al. 2004), plays a crucial role in somatic ovarian cell development (Hreinsson et al. 2002) and is a key regulator of normal cumulus cell function (Gilchrist et al. 2008; Su et al. 2004) (Fig. 4).

GDF9 null mice have abnormal primary follicles which fail to develop beyond the primary stage, are unable to form a theca, and have impaired meiotic competence (Dong et al. 1996; Yan et al. 2001). In addition, in vitro cultures of ovarian tissue supplemented by GDF9 in both rats and humans enhance the progression of early to late stage primary follicles (Hreinsson et al. 2002; Nilsson and Skinner 2002). Interestingly, the abnormal follicles seen in GDF9 null mice consist of enlarged oocytes surrounded by a single layer of cuboidal granulosa cells. While these granulosa cells are typical of primary follicles, ultrastructural analysis of oocytes obtained from GDF9 null mice revealed that these oocytes had progressed to advanced stages of differentiation and were capable of resuming meiosis after in vitro maturation (Carabatsos et al. 1998). GDF9 signalling is also required for pre-antral follicle growth and ovulation (Elvin et al. 1999b) (Fig. 4), and GDF9 expression has been detected in the oocytes of both murine and human antral follicles (Elvin et al. 1999b; Gilchrist et al. 2004; Hreinsson et al. 2002). Culture of rat granulosa cells isolated from antral follicles has shown that GDF9 stimulates proliferation, but also suppresses FSH-induced granulosa cell differentiation, as indicated by lower progesterone and oestradiol levels with attenuated LH receptor formation (Vitt et al. 2000; Yamamoto et al. 2002). These results suggest that GDF9 may regulate antral follicle development by ensuring continued granulosa cell proliferation and preventing premature luteinisation (Fig. 4).

In addition to its proposed role in ensuring antral follicle development, GDF9 has also been implicated in ensuring pre-antral follicular survival by suppressing granulosa cell apoptosis and inducing thecal cell androgen production. In a recent study by Orisaka et al. down regulation of GDF9 expression in cultured rat granulosa cells led to a subsequent increase in caspase-3 activation and granulosa apoptosis (Orisaka et al. 2006). GDF9 was also capable of preventing ceramide induced apoptosis in granulosa cells cultured from pre-antral follicles, but had no affect on pre-ovulatory follicles (Orisaka et al. 2006). Studies involving double mutant GDF9 and inhibin-α knockouts have also shown GDF9 is required to induce theca cell differentiation, as indicated by the formation of theca-like cells in

developing follicles lacking thecal cell-specific markers Cyp17a1 and LH receptor (Wu et al. 2004). This observation is supported by a number of in vitro studies, which have demonstrated that GDF9 increases androgen production, *Cyp17a1* and *c-kit* expression in rat pre-antral follicles (Elvin et al. 1999b; Orisaka et al. 2009; Solovyeva et al. 2000).

Further studies have shown GDF9 signalling is also essential for cumulus development and metabolism (Elvin et al. 1999a; Su et al. 2008, 2009). During ovulation, GDF9 induces cumulus cell expansion, a process associated with the intricate association of cumulus cells with the oocyte throughout the ovulatory process and subsequent fertilisation. This process is essential, as it protects the oocytes during follicular extrusion,and assists fertilisation. Treatment of isolated granulosa cells in vitro with recombinant GDF9 has been shown to influence the expression of a suite of genes involved in cumulus cell expansion (*HAS2*, *COX-2*, *StAR*, *uPA* and *LHR*) and induce the same process in oocytectomised cumulus cell-oocyte complexes in vitro (Elvin et al. 1999b). These results are also supported by RNAi studies which show that selective knockdown of GDF9 in mature mouse oocytes reduces cumulus expansion in vitro (Gui and Joyce 2005).

GDF9 is also a viable target for a contraceptive agent due to its essential requirement for oocyte maturation. Immunocontraceptive studies in sheep have found that antisera generated against peptides corresponding to the first 1–15 amino acid residues on the N-terminus of GDF9 cause anovulation in ewes following primary and single booster vaccinations (McNatty et al. 2007). This raises the possibility of inhibitory/antisense compounds which target the N-terminus of GDF9 being used as potential human contraceptives in the near future.

Bone Morphogenic Proteins (BMPs) are the largest group of multifunctional growth factor cytokines belonging to the TGFβ superfamily. BMPs are expressed in numerous cell types and tissues and are involved in a wide variety of biological processes including mesoderm patterning, neurogenesis, bone formation and angiogenesis (David et al. 2009; Furtado et al. 2008; Morikawa et al. 2009; Xiao et al. 2007). First reported in mammalian ovary development in 1999, a whole host of BMPs have subsequently been identified in the oocyte, granulosa and thecal cells of the ovarian follicle (Knight and Glister 2006; Shimasaki et al. 2003).

BMP15 expression remains constant throughout folliculogenesis, being detected initially in oocytes and granulosa cells in the primordial follicle stage in the human ovary (Aaltonen et al. 1999; Margulis et al. 2008; Teixeira Filho et al. 2002), in primary follicle stage oocytes in the mouse (Dube et al. 1998) and in primordial follicle stage oocytes in the sheep (McNatty et al. 2001) suggesting a species-specific role for BMP15 (Fig. 4).

Knockout studies conducted in the mouse have shown females lacking a functional BMP15 gene are sub-fertile, due to impaired ovulation and fertilisation (Yan et al. 2001). Sheep with homologous point mutations corresponding to the chromosomal location of BMP15 are infertile, with follicular development beyond the primary stage being impaired (Galloway et al. 2000; Hanrahan et al. 2004). In humans, studies have led to the discovery of various missense mutations and polymorphisms in the BMP15 gene, which have all been associated with primary

and secondary amenorrhea (Di Pasquale et al. 2004, 2006). Interestingly, these mutations were described as having a similar phenotype to sheep with BMP15 homologous point mutations, with impaired follicular development beyond the primary stage (Di Pasquale et al. 2004; Galloway et al. 2000). When combined, these studies suggest that BMP15 may play a "curtailing" role in the transition of primary to secondary follicles during human folliculogenesis (Fig. 4).

In terms of BMP15's mechanism of action, follow-up in vitro experiments on isolated human granulosa cells have shown that treatment with recombinant BMP-15 in culture stimulates granulosa cell growth, while treatment with recombinant "mutant" BMP15 had no effect (Di Pasquale et al. 2004). This advocates that BMP-15 may exert its effect on follicular development by stimulating granulosa cell proliferation at the primary stage. Interestingly, co-culture with both recombinant wild type and mutant BMP15 had no effect on granulosa growth, suggesting an antagonistic effect (Di Pasquale et al. 2004) (Fig. 4).

As well as its role in the transition of primary to secondary follicles, BMP15 has also been implicated in the suppression of FSH-induced progesterone synthesis in rat and ruminants, and the stimulation of cumulus cell expansion and metabolism in the mouse (McNatty et al. 2005; Yoshino et al. 2006; Sugiura et al. 2007). Although BMP15 has been found to play varying roles beyond primary follicle growth, given the species-specific nature of BMP15's function, and the lack of analogous studies, it is unknown whether human BMP15 mimics any of these reported functions.

A recent study into the expression of BMP15 in human oocyte and cumulus granulosa cells has mapped BMP15 expression in pre-ovulatory stage oocytes and pre-ovulatory/ovulatory stage cumulus cells (Chen et al. 2009). BMP15 expression was found to increase significantly during late stage pre-ovulatory oocytes, suggesting a role for BMP15 in the final stages of oogenesis (Fig. 4). Additionally, the level of BMP15 significantly decreased in cumulus cells surrounding ovulatory oocytes compared with those surrounding pre-ovulatory oocytes. This decreased level of BMP15 in cumulus cells after oocyte maturation, coupled with the fact that BMP15 suppresses progesterone synthesis in mammalian models and is involved in human granulosa cell growth, suggests that this protein has the ability to act as an inhibitor of the premature luteinisation of cumulus cells (Chen et al. 2009; Di Pasquale et al. 2004; Gilchrist et al. 2008; McNatty et al. 2005; Otsuka et al. 2001).

BMP15 is an autosomal homologue of GDF9, both of which are expressed from a very early stage of follicular growth and play key roles in promoting follicular growth beyond the primary stage (Di Pasquale et al. 2004; Dong et al. 1996; Galloway et al. 2000) (Fig. 4). BMP15 and GDF9 have also been shown to act synergistically in mice during development of the oocyte-cumulus cell complex (Yan et al. 2001). In terms of fertility regulation, BMP15 immunisation studies in sheep have found that both active and passive immunisations are able to influence the biological activity of BMP15 in ewes (Juengel et al. 2002). Furthermore, recent immunocontraceptive studies in sheep have found that antisera generated against peptides corresponding to the first 1–15 amino acid residues on the N-terminus of BMP15 cause anovulation in ewes following primary and single booster vaccinations (McNatty et al. 2007). This raises the possibility of inhibitory/antisense

compounds which target the N-terminus of BMP15 being used as potential contraceptives in the near future. Another novel concept for a BMP15-based contraceptive agent involves the use of recombinant BMP15 mutants as potential antagonists. As described above, in vitro experiments using a mutant recombinant protein ($BMP15^{Y235C}$) showed that the mutant BMP15 was able to antagonise the stimulatory effects wild-type BMP15 on granulosa cell growth (Di Pasquale et al. 2004). Additionally, in the study through which this mutation was identified, the women who are heterozygous carriers of the Y235C mutation are infertile, with an impaired follicular phenotype. Therefore, the Y235C mutation may antagonise wild-type BMP15 in vivo (Di Pasquale et al. 2004) and underpin development of a BMP15 antagonist, or recombinant $BMP15^{Y235C}$, as a possible contraceptive.

In a recent paper by McMahon et al., recombinant human BMP15 and GDF9 were shown to undergo phosphorylation, and that this phosphorylation was required for normal bioactivity (McMahon et al. 2008). This study is novel, in that it is the first to report any member of the TGFβ superfamily as phosphoproteins. More interesting though was the fact that dephosphorylated BMP15 and GDF were capable of antagonising their wild-type counterparts by competitively binding to BMP receptors and failing to induce the BMP/Smad pathway (McMahon et al. 2008). These results raise the interesting possibility of using phosphorylation as a method of BMP15 and GDF9 regulation in the context of fertility control. The use of modified recombinant BMP15 and GDF9 incapable of undergoing phosphorylation could also be theoretically used as potential antagonists, and therefore as possible contraceptives.

8 Conclusions

Recent improvements in our understanding of the intracellular signalling systems, such as the PI3K and Tsc1 pathways, that control maintenance of the primordial follicle population and transduce the as yet elusive extracellular signals necessary for primordial follicle activation, have significant implications for the design of new contraceptive agents for women. As primordial follicle activation requires close communication between oocyte and somatic cells and many cytokine and chemokine factors have a clearly demonstrated role in releasing oocytes into the growing pool, then if the trigger is oocyte generated, an early response must include suppression of FOXO3A and mTORC activity and ultimately regulation of the "folliculogenesis clock" (Matzuk et al. 2002). Once activated to grow, the oocyte orchestrates and coordinates the development of mammalian ovarian follicles, the rate of follicle development being controlled by the oocyte (Eppig et al. 2002). Importantly, we are also beginning to elucidate those pathways activated by members of the TGFβ superfamily that regulate and support oocyte development. Pharmacological inhibition of these signalling pathways may hold the key development of non-steroidal ovarian contraceptives for the twenty-first century.

Acknowledgements The authors gratefully acknowledge the financial assistance to EAM by the Australian Research Council, National Health and Medical Research Council, Hunter Medical Research Institute and the Newcastle Permanent Building Society Charitable Trust. APS is the recipient of an Australian Postgraduate Award PhD scholarship.

References

Aaltonen J et al (1999) Human growth differentiation factor 9 (GDF-9) and its novel homolog GDF-9B are expressed in oocytes during early folliculogenesis. J Clin Endocrinol Metab 84:2744–2750

Adhikari D, Liu K (2009) Molecular mechanisms underlying the activation of mammalian primordial follicles. Endocr Rev 30:438–464

Adhikari D et al (2009) Disruption of Tsc2 in oocytes leads to overactivation of the entire pool of primordial follicles. Mol Hum Reprod 15:765–770

Adhikari D et al (2010) Tsc/mTORC1 signaling in oocytes governs the quiescence and activation of primordial follicles. Hum Mol Genet 19:397–410

Andersen CY, Byskov AG (2006) Estradiol and regulation of anti-mullerian hormone, Inhibin-A, and Inhibin-B secretion: analysis of small antral and preovulatory human follicles' fluid. J Clin Endocrinol Metab 91:4064–4069

Arraztoa JA et al (2005) Identification of genes expressed in primate primordial oocytes. Hum Reprod 20:476–483

Artac RA et al (2009) Neutralization of vascular endothelial growth factor antiangiogenic isoforms is more effective than treatment with proangiogenic isoforms in stimulating vascular development and follicle progression in the perinatal rat ovary. Biol Reprod 81:978–988

Behringer RR (1995) The mullerian inhibitor and mammalian sexual development. Philos Trans R Soc Lond B Biol Sci 350:285–288; discussion 289

Brenkman AB, Burgering BM (2003) FoxO3a eggs on fertility and aging. Trends Mol Med 9:464–467

Britt KL et al (2004) Estrogen actions on follicle formation and early follicle development. Biol Reprod 71:1712–1723

Brown C et al (2009) Subfertility caused by altered follicular development and oocyte growth in female mice lacking PKBalpha/Akt1. Biol Reprod 82(2):246–256

Carabatsos MJ et al (1998) Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice. Dev Biol 204:373–384

Castrillon DH et al (2003) Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science 301:215–218

Chen Y et al (2007) Estradiol, progesterone, and genistein inhibit oocyte nest breakdown and primordial follicle assembly in the neonatal mouse ovary in vitro and in vivo. Endocrinology 148:3580–3590

Chen Y et al (2009) Estrogen can signal through multiple pathways to regulate oocyte cyst breakdown and primordial follicle assembly in the neonatal mouse ovary. J Endocrinol 202:407–417

Choi Y, Rajkovic A (2006) Genetics of early mammalian folliculogenesis. Cell Mol Life Sci 63:579–590

Craig J et al (2007) Gonadotropin and intra-ovarian signals regulating follicle development and atresia: the delicate balance between life and death. Front Biosci 12:3628–3639

Da Silva-Buttkus P et al (2009) Inferring biological mechanisms from spatial analysis: prediction of a local inhibitor in the ovary. Proc Natl Acad Sci USA 106:456–461

David L et al (2009) Emerging role of bone morphogenetic proteins in angiogenesis. Cytokine Growth Factor Rev 20:203–212

de Vet A et al (2002) Antimüllerian hormone serum levels: a putative marker for ovarian aging. Fertil Steril 77:357–362
Di Pasquale E et al (2004) Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. Am J Hum Genet 75:106–111
Di Pasquale E et al (2006) Identification of new variants of human BMP15 gene in a large cohort of women with premature ovarian failure. J Clin Endocrinol Metab 91:1976–1979
Dickinson RE et al (2010) Involvement of the SLIT/ROBO pathway in follicle development in the fetal ovary. Reproduction 139(2):395–407
Diclemente N et al (1994) Inhibitory effect of amh upon the expression of aromatase and lh receptors by cultured granulosa-cells of rat and porcine immature ovaries. Endocrine 2:553–558
Dissen GA et al (2002) Neurotrophic control of ovarian development. Microsc Res Tech 59:509–515
Dissen GA et al (2009) Role of neurotrophic factors in early ovarian development. Semin Reprod Med 27:24–31
Dole G et al (2008) Glial derived neurotrophic factor promotes ovarian primordial follicle development and cell-cell interactions during folliculogenesis. Reproduction 135:671–682
Dong J et al (1996) Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature 383:531–535
Dono R et al (1998) Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice. EMBO J 17:4213–4225
Dube JL et al (1998) The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes. Mol Endocrinol 12:1809–1817
Duffin K et al (2009) The forkhead transcription factor FOXL2 is expressed in somatic cells of the human ovary prior to follicle formation. Mol Hum Reprod 15(12):771–777
Dumesic DA et al (2009) Intrafollicular antimullerian hormone levels predict follicle responsiveness to follicle-stimulating hormone (FSH) in normoandrogenic ovulatory women undergoing gonadotropin releasing-hormone analog/recombinant human FSH therapy for in vitro fertilization and embryo transfer. Fertil Steril 92:217–221
Durlinger ALL et al (1999) Control of primordial follicle recruitment by Anti-Mullerian hormone in the mouse ovary. Endocrinology 140:5789–5796
Durlinger ALL et al (2001) Anti-Mullerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. Endocrinology 142:4891–4899
Durlinger AL et al (2002) Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. Endocrinology 143:1076–1084
Edson MA et al (2009) The Mammalian ovary from genesis to revelation. Endocr Rev 30:624–712
Elvin JA et al (1999a) Paracrine actions of growth differentiation factor-9 in the mammalian ovary. Mol Endocrinol 13:1035–1048
Elvin JA et al (1999b) Molecular characterization of the follicle defects in the growth differentiation factor 9-deficient ovary. Mol Endocrinol 13:1018–1034
Eppig JJ et al (2002) The mammalian oocyte orchestrates the rate of ovarian follicular development. Proc Natl Acad Sci USA 99:2890–2894
Furtado MB et al (2008) BMP/SMAD1 signaling sets a threshold for the left/right pathway in lateral plate mesoderm and limits availability of SMAD4. Genes Dev 22:3037–3049
Galloway SM et al (2000) Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. Nat Genet 25:279–283
Garor R et al (2009) Effects of basic fibroblast growth factor on in vitro development of human ovarian primordial follicles. Fertil Steril 91:1967–1975
Geissler EN et al (1981) Analysis of pleiotropism at the dominant white-spotting (W) locus of the house mouse: a description of ten new W alleles. Genetics 97:337–361
Gilchrist RB et al (2004) Oocyte-somatic cell interactions during follicle development in mammals. Anim Reprod Sci 82–83:431–446

Gilchrist RB et al (2008) Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. Hum Reprod Update 14:159–177
Gui LM, Joyce IM (2005) RNA interference evidence that growth differentiation factor-9 mediates oocyte regulation of cumulus expansion in mice. Biol Reprod 72:195–199
Hanrahan JP et al (2004) Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (Ovis aries). Biol Reprod 70:900–909
Hansen KR et al (2008) A new model of reproductive aging: the decline in ovarian non-growing follicle number from birth to menopause. Hum Reprod 23:699–708
Hayashi M et al (1999) Recombinant growth differentiation factor-9 (GDF-9) enhances growth and differentiation of cultured early ovarian follicles. Endocrinology 140:1236–1244
Holt JE et al (2006) CXCR4/SDF1 interaction inhibits the primordial to primary follicle transition in the neonatal mouse ovary. Dev Biol 293(2):449–460
Hosaka T et al (2004) Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. Proc Natl Acad Sci USA 101:2975–2980
Hreinsson JG et al (2002) Growth differentiation factor-9 promotes the growth, development, and survival of human ovarian follicles in organ culture. J Clin Endocrinol Metab 87:316–321
Hutt KJ, Albertini DF (2006) Clinical applications and limitations of current ovarian stem cell research: a review. J Exp Clin Assist Reprod 3:6
Hutt KJ, Albertini DF (2007) An oocentric view of folliculogenesis and embryogenesis. Reprod Biomed Online 14:758–764
Hutt KJ et al (2006a) Kit ligand and c-Kit have diverse roles during mammalian oogenesis and folliculogenesis. Mol Hum Reprod 12(2):61–69
Hutt KJ et al (2006b) KIT/KIT ligand in mammalian oogenesis and folliculogenesis: roles in rabbit and murine ovarian follicle activation and oocyte growth. Biol Reprod 75:421–433
Jagarlamudi K et al (2009) Oocyte-specific deletion of Pten in mice reveals a stage-specific function of PTEN/PI3K signaling in oocytes in controlling follicular activation. PLoS One 4:e6186
Jin X et al (2005) Signal transduction of stem cell factor in promoting early follicle development. Mol Cell Endocrinol 229:3–10
John GB et al (2007) Specificity of the requirement for Foxo3 in primordial follicle activation. Reproduction 133:855–863
John GB et al (2008) Foxo3 is a PI3K-dependent molecular switch controlling the initiation of oocyte growth. Dev Biol 321:197–204
John GB et al (2009) Kit signaling via PI3K promotes ovarian follicle maturation but is dispensable for primordial follicle activation. Dev Biol 331:292–299
Juengel JL et al (2002) Growth differentiation factor 9 and bone morphogenetic protein 15 are essential for ovarian follicular development in sheep. Biol Reprod 67:1777–1789
Juengel JL et al (2004) Physiology of GDF9 and BMP15 signalling molecules. Anim Reprod Sci 82–83:447–460
Junger MA et al (2003) The Drosophila forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. J Biol 2:20
Kaufmann E, Knochel W (1996) Five years on the wings of fork head. Mech Dev 57:3–20
Kevenaar ME et al (2007a) Anti-Mullerian hormone and anti-Mullerian hormone type II receptor polymorphisms are associated with follicular phase estradiol levels in normo-ovulatory women. Hum Reprod 22:1547–1554
Kevenaar ME et al (2007b) A polymorphism in the AMH type II receptor gene is associated with age at menopause in interaction with parity. Hum Reprod 22:2382–2388
Kezele P, Skinner MK (2003) Regulation of ovarian primordial follicle assembly and development by estrogen and progesterone: endocrine model of follicle assembly. Endocrinology 144:3329–3337
Kezele P et al (2002) Cell-cell interactions in primordial follicle assembly and development. Front Biosci 7:d1990–d1996

Kezele P et al (2005a) Keratinocyte growth factor acts as a mesenchymal factor that promotes ovarian primordial to primary follicle transition. Biol Reprod 73:967–973
Kezele PR et al (2005b) Alterations in the ovarian transcriptome during primordial follicle assembly and development. Biol Reprod 72:241–255
Kim H et al (2009a) Effects of diethylstilbestrol on ovarian follicle development in neonatal mice. Reprod Toxicol 27:55–62
Kim H et al (2009b) Effects of diethylstilbestrol on programmed oocyte death and induction of polyovular follicles in neonatal mouse ovaries. Biol Reprod 81:1002–1009
Knight PG, Glister C (2006) TGF-beta superfamily members and ovarian follicle development. Reproduction 132:191–206
Krysko DV et al (2008) Life and death of female gametes during oogenesis and folliculogenesis. Apoptosis 13:1065–1087
Lee WS et al (2001) Effect of bone morphogenetic protein-7 on folliculogenesis and ovulation in the rat. Biol Reprod 65:994–999
Lee WS et al (2004) Effects of bone morphogenetic protein-7 (BMP-7) on primordial follicular growth in the mouse ovary. Mol Reprod Dev 69:159–163
Lintern-Moore S et al (1974) Follicular development in the infant human ovary. J Reprod Fertil 39:53–64
Liu K et al (2006) Control of mammalian oocyte growth and early follicular development by the oocyte PI3 kinase pathway: new roles for an old timer. Dev Biol 299:1–11
Liu L et al (2007a) Infertility caused by retardation of follicular development in mice with oocyte-specific expression of Foxo3a. Development 134:199–209
Liu L et al (2007b) Phosphorylation and inactivation of glycogen synthase kinase-3 by soluble kit ligand in mouse oocytes during early follicular development. J Mol Endocrinol 38:137–146
Lyet L et al (1996) Anti-mullerian hormone in relation to the growth and differentiation of the gubernacular primordia in mice. J Reprod Fertil 108:281–288
Macklon NS et al (2006) The science behind 25 years of ovarian stimulation for in vitro fertilization. Endocr Rev 27:170–207
Maheshwari A, Fowler PA (2008) Primordial follicular assembly in humans – revisited. Zygote 16:285–296
Margulis S et al (2008) Bone morphogenetic protein 15 expression in human ovaries from fetuses, girls, and women. Fertil Steril 92:1666–1673
Matzuk MM et al (2002) Intercellular communication in the mammalian ovary: oocytes carry the conversation. Science 296:2178–2180
McFee RM et al (2009) Inhibition of vascular endothelial growth factor receptor signal transduction blocks follicle progression but does not necessarily disrupt vascular development in perinatal rat ovaries. Biol Reprod 81:966–977
McGee EA, Hsueh AJ (2000) Initial and cyclic recruitment of ovarian follicles. Endocr Rev 21:200–214
McGrath SA et al (1995) Oocyte-specific expression of growth/differentiation factor-9. Mol Endocrinol 9:131–136
McLaughlin EA, McIver SC (2009) Awakening the oocyte: controlling primordial follicle development. Reproduction 137:1–11
McMahon HE et al (2008) Phosphorylation of bone morphogenetic protein-15 and growth and differentiation factor-9 plays a critical role in determining agonistic or antagonistic functions. Endocrinology 149:812–817
McNatty KP et al (2001) Genetic mutations influencing ovulation rate in sheep. Reprod Fertil Dev 13:549–555
McNatty KP et al (2005) Oocyte-expressed genes affecting ovulation rate. Mol Cell Endocrinol 234:57–66
McNatty KP et al (2007) Control of ovarian follicular development to the gonadotrophin-dependent phase: a 2006 perspective. Soc Reprod Fertil Suppl 64:55–68

Mishina Y et al (1999) High specificity of Mullerian-inhibiting substance signaling in vivo. Endocrinology 140:2084–2088
Morikawa Y et al (2009) BMP signaling regulates sympathetic nervous system development through Smad4-dependent and -independent pathways. Development 136:3575–3584
Munsterberg A, Lovell-Badge R (1991) Expression of the mouse anti-mullerian hormone gene suggests a role in both male and female sexual differentiation. Development 113:613–624
Nelson SM et al (2007) Serum anti-Mullerian hormone and FSH: prediction of live birth and extremes of response in stimulated cycles–implications for individualization of therapy. Hum Reprod 22:2414–2421
Nelson SM et al (2009) Anti-Mullerian hormone-based approach to controlled ovarian stimulation for assisted conception. Hum Reprod 24:867–875
Nilsson EE, Skinner MK (2002) Growth and differentiation factor-9 stimulates progression of early primary but not primordial rat ovarian follicle development. Biol Reprod 67:1018–1024
Nilsson EE, Skinner MK (2003) Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. Biol Reprod 69:1265–1272
Nilsson EE, Skinner MK (2009) Progesterone regulation of primordial follicle assembly in bovine fetal ovaries. Mol Cell Endocrinol 313(1–2):9–16
Nilsson E et al (2001) Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis. Mol Cell Endocrinol 175:123–130
Nilsson EE et al (2002) Leukemia inhibitory factor (LIF) promotes the primordial to primary follicle transition in rat ovaries. Mol Cell Endocrinol 188:65–73
Nilsson EE et al (2006) Platelet-derived growth factor modulates the primordial to primary follicle transition. Reproduction 131:1007–1015
Nilsson E et al (2007) Actions of anti-Mullerian hormone on the ovarian transcriptome to inhibit primordial to primary follicle transition. Reproduction 134:209–221
Nilsson E et al (2009) Neurotrophin NT3 promotes ovarian primordial to primary follicle transition. Reproduction 138:697–707
Orisaka M et al (2006) Growth differentiation factor 9 is antiapoptotic during follicular development from preantral to early antral stage. Mol Endocrinol 20:2456–2468
Orisaka M et al (2009) Growth differentiation factor 9 promotes rat preantral follicle growth by up-regulating follicular androgen biosynthesis. Endocrinology 150:2740–2748
Otsuka F et al (2001) Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression. J Biol Chem 276:11387–11392
Paredes A et al (2004) TrkB receptors are required for follicular growth and oocyte survival in the mammalian ovary. Dev Biol 267:430–449
Pedersen T (1969) Follicle growth in the immature mouse ovary. Acta Endocrinol 62:117–132
Pedersen T (1970) Follicle kinetics in the ovary of the cyclic mouse. Acta Endocrinol 64:304–323
Pedersen T, Peters H (1968) Proposal for a classification of oocytes and follicles in the mouse ovary. J Reprod Fertil 17:555–557
Pedersen T, Peters H (1971) Follicle growth and cell dynamics in the mouse ovary during pregnancy. Fertil Steril 22:42–52
Pepling ME et al (2009) Differences in oocyte development and estradiol sensitivity among mouse strains. Reproduction 139(2):349–357
Picton H et al (1998) The molecular basis of oocyte growth and development. Mol Cell Endocrinol 145:27–37
Picton HM et al (2008) The in vitro growth and maturation of follicles. Reproduction 136:703–715
Rajareddy S et al (2007) p27kip1 (cyclin-dependent kinase inhibitor 1B) controls ovarian development by suppressing follicle endowment and activation and promoting follicle atresia in mice. Mol Endocrinol 21:2189–2202
Reddy P et al (2005) Activation of Akt (PKB) and suppression of FKHRL1 in mouse and rat oocytes by stem cell factor during follicular activation and development. Dev Biol 281:160–170

Reddy P et al (2008) Oocyte-specific deletion of Pten causes premature activation of the primordial follicle pool. Science 319:611–613
Reddy P et al (2009) PDK1 signaling in oocytes controls reproductive aging and lifespan by manipulating the survival of primordial follicles. Hum Mol Genet 18:2813–2824
Reynaud K, Driancourt MA (2000) Oocyte attrition. Mol Cell Endocrinol 163:101–108
Richards JS et al (2002) Novel signaling pathways that control ovarian follicular development, ovulation, and luteinization. Recent Prog Horm Res 57:195–220
Rodrigues P et al (2008) Oogenesis: prospects and challenges for the future. J Cell Physiol 216:355–365
Rodrigues P et al (2009) Multiple mechanisms of germ cell loss in the perinatal mouse ovary. Reproduction 137:709–720
Romero C et al (2002) Nerve growth factor induces the expression of functional FSH receptors in newly formed follicles of the rat ovary. Endocrinology 143:1485–1494
Rowlands S (2009) New technologies in contraception. BJOG 116:230–239
Schmidt KL et al (2005) Anti-Mullerian hormone initiates growth of human primordial follicles in vitro. Mol Cell Endocrinol 234:87–93
Serafica MD et al (2005) Transcripts from a human primordial follicle cDNA library. Hum Reprod 20:2074–2091
Shimasaki S et al (2003) The role of bone morphogenetic proteins in ovarian function. Reprod Suppl 61:323–337
Skinner MK (2005) Regulation of primordial follicle assembly and development. Hum Reprod Update 11:461–471
Solovyeva EV et al (2000) Growth differentiation factor-9 stimulates rat theca-interstitial cell androgen biosynthesis. Biol Reprod 63:1214–1218
Spears N et al (2003) The role of neurotrophin receptors in female germ-cell survival in mouse and human. Development 130:5481–5491
Stewart CL et al (1992) Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. Nature 359:76–79
Su YQ et al (2004) Synergistic roles of BMP15 and GDF9 in the development and function of the oocyte-cumulus cell complex in mice: genetic evidence for an oocyte-granulosa cell regulatory loop. Dev Biol 276:64–73
Su YQ et al (2008) Oocyte regulation of metabolic cooperativity between mouse cumulus cells and oocytes: BMP15 and GDF9 control cholesterol biosynthesis in cumulus cells. Development 135:111–121
Su YQ et al (2009) Mouse oocyte control of granulosa cell development and function: paracrine regulation of cumulus cell metabolism. Semin Reprod Med 27:32–42
Sugiura K et al (2007) Oocyte-derived BMP15 and FGFs cooperate to promote glycolysis in cumulus cells. Development 134:2593–2603
Teixeira Filho FL et al (2002) Aberrant expression of growth differentiation factor-9 in oocytes of women with polycystic ovary syndrome. J Clin Endocrinol Metab 87:1337–1344
Tingen C et al (2009) The primordial pool of follicles and nest breakdown in mammalian ovaries. Mol Hum Reprod 15(12):795–803
Trombly DJ et al (2009) Roles for transforming growth factor beta superfamily proteins in early folliculogenesis. Semin Reprod Med 27:14–23
Uda M et al (2004) Foxl2 disruption causes mouse ovarian failure by pervasive blockage of follicle development. Hum Mol Genet 13:1171–1181
van Rooij IAJ et al (2002) Serum anti-Mullerian hormone levels: a novel measure of ovarian reserve. Hum Reprod 17:3065–3071
Visser JA, Themmen APN (2005) Anti-Müllerian hormone and folliculogenesis. Mol Cell Endocrinol 234:81–86
Visser JA et al (2006) Anti-Mullerian hormone: a new marker for ovarian function. Reproduction 131:1–9

Vitt UA et al (2000) Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone-induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles. Biol Reprod 62:370–377

Vitt UA et al (2002) Bone morphogenetic protein receptor type II is a receptor for growth differentiation factor-9. Biol Reprod 67:473

Wandji SA et al (1996) FSH and growth factors affect the growth and endocrine function in vitro of granulosa cells of bovine preantral follicles. Theriogenology 45:817–832

Wang N et al (2009) Comparative proteome profile of immature rat ovary during primordial follicle assembly and development. Proteomics 9:3425–3434

Weenen C et al (2004) Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. Mol Hum Reprod 10:77–83

Wu X et al (2004) Interrelationship of growth differentiation factor 9 and inhibin in early folliculogenesis and ovarian tumorigenesis in mice. Mol Endocrinol 18:1509–1519

Xiao YT et al (2007) Bone morphogenetic protein. Biochem Biophys Res Commun 362:550–553

Yamamoto N et al (2002) Growth differentiation factor-9 inhibits 3'5'-adenosine monophosphate-stimulated steroidogenesis in human granulosa and theca cells. J Clin Endocrinol Metab 87:2849–2856

Yan C et al (2001) Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. Mol Endocrinol 15:854–866

Yang MY, Fortune JE (2008) The capacity of primordial follicles in fetal bovine ovaries to initiate growth in vitro develops during mid-gestation and is associated with meiotic arrest of oocytes. Biol Reprod 78:1153–1161

Yang JL et al (2010) Testosterone induces redistribution of forkhead box-3a and down-regulation of growth and differentiation factor 9 messenger ribonucleic acid expression at early stage of mouse folliculogenesis. Endocrinology 151(2):774–782

Yoshino O et al (2006) A unique preovulatory expression pattern plays a key role in the physiological functions of BMP-15 in the mouse. Proc Natl Acad Sci USA 103:10678–10683

Zheng P, Dean J (2007) Oocyte-specific genes affect folliculogenesis, fertilization, and early development. Semin Reprod Med 25:243–251

Appendix A.2: Conference publications

A.2.1. Xenobiotics; Influence on ovarian follicular development

A. P. Sobinoff [A], V. Pye [A], B. Nixon [A B], S. D. Roman [A B] and E. A. McLaughlin [A B]

[A] *School of Environmental and Life Sciences, The University of Newcastle, Callaghan, NSW, Australia*

[B] *The ARC Centre of Excellence in Biotechnology and Development, The University of Newcastle, Callaghan, NSW, Australia*

Abstract

The mammalian female reproductive lifespan is largely defined by a finite pool of ovarian follicles established around the time of birth. It is now understood that certain synthetic chemical compounds, known as xenobiotics, can cause premature ovarian senescence through the destruction of small ovarian follicles. Although the ovotoxic effects of these chemicals are well documented, the exact molecular mechanisms behind their action are only just becoming understood. Recent evidence suggests that bioactivation of xenobiotics by Phase I detoxifying enzymes may lead to the generation of free oxygen radicals (ROS), which we suspect may perturb intracellular signalling pathways in primordial follicles. In this study we attempted to identify ovarian follicle signalling pathways activated by xenobiotic exposure using ovotoxic agents which target immature follicles. Neonatal ovaries obtained from 3/4-day old Swiss mice were exposed to either 4-Vinylcyclohexene (25µM), Methoxychlor (25µM) or Menadione (5µM) for 96hrs using our in vitro culture system. Total RNA was then collected and analysed using Affymetrix Mouse Genome 430 2.0 Arrays. Bioinformatic analysis identified between ~500–1000 genes with a two-fold significant difference in gene expression ($p<0.05$) for each xenobiotic compared to the control. Differentially expressed genes were analysed for pathways and molecular functions using Ingenuity Pathways Analysis (Ingenuity Systems). In agreement with the current literature, many of the genes belonged to toxic response pathways, such as; Xenobiotic metabolism (10); p53 (15) and Apoptosis (11) signalling. However, the vast majority of the differentially expressed genes belonged to canonical pathways implicated in follicular development, such as PI3K/AKT (18), Wnt/ b -catenin (21), and JAK/Stat (8) signalling. Further qPCR analysis has confirmed a substantial increase in the transcription factor Sox4 and cell cycle inhibitor Cdkn2a in 4-Vinylcyclohexene and Menadione treated ovaries respectively. These results suggest that

xenobiotics which target primordial follicles may exert part of their ovotoxic effects by perturbing signalling pathways involved in follicular activation and development.

A.2.2. Xenobiotics; Influence on long term oocyte viability

V. Pye[1] , A. P. Sobinoff[1] , B. Nixon[1,2] , S. D. Roman[1,2] , E. A. McLaughlin[1,2]

[1] School of Environmental and Life Sciences, The University of Newcastle, Callaghan, NSW, Australia

[2] The ARC Centre of Excellence in Biotechnology and Development, The University of Newcastle, Callaghan, NSW, Australia

Abstract

Mammalian females are born with a finite number of non-renewing primordial follicles, the majority of which can remain in a quiescent state for many years. Due to their non-renewing nature, these "resting" oocytes are particularly vulnerable to environmental and toxic insults, especially those which are capable of inducing oxidative stress. Recent evidence suggests that certain synthetic chemical compounds, known as xenobiotics, have the potential to generate oxidative stress through the production of free oxygen radicals as a byproduct of the cell's detoxification process. Given the redox sensitive nature of the mammalian oocyte, xenobiotic exposure has been hypothesized to have long term adverse effects on oocyte viability. In this study, we attempted to identify the effects of short term xenobiotic exposure on long term oocyte viability. Female Swiss neonatal mice (day 4) were administered 7 daily consecutive doses of 4-Vinylcyclohexene diepoxide (40mg/kg/daily; 80mg/kg/daily) Methoxychlor (50mg/kg/daily; 100mg/kg/daily) or Menadione (7.5mg/kg/daily; 15mg/kg/daily). Mice were superovulated at 6wks and their oocytes collected for sperm-egg fusion assays. Sperm-egg fusion assays revealed a significant decrease in sperm egg binding/fusion ($p<0.05$) in a dose dependent manner for all three xenobiotic treatments *in vivo*, signifying a decrease in oocyte membrane fluidity. Follow-up lipid peroxidation analysis on xenobiotic cultured oocytes also showed a dose dependent increase in membrane lipid peroxidation in response to xenobiotic exposure. These results suggest that short term xenobiotic exposure can cause long term oocyte dysfunction, possibly interfering with the fluidity and/or elasticity of the oocyte plasma membrane through lipid peroxidation.

A.2.3. Consistent mechanism of primordial follicle activation in neonatal mouse ovotoxicity

A. P. Sobinoff [1] , V. Pye[1] , B. Nixon[1,2] , S. D. Roman[1,2] , E. A. McLaughlin[1,2]

[1] School of Environmental and Life Sciences, The University of Newcastle, Callaghan, NSW, Australia

[2] The ARC Centre of Excellence in Biotechnology and Development, The University of Newcastle, Callaghan, NSW, Australia

Abstract

The mammalian female reproductive lifespan is largely defined by a finite pool of primordial follicles established around the time of birth. Overall, <1% of these follicles are destined for ovulation, with the vast majority being lost during development in a process called atresia. While atresia is a normal physiological process, it is now well documented that it can be triggered through exposure to certain synthetic chemical compounds, known as xenobiotics, causing premature ovarian senescence. In addition to follicular atresia, new evidence suggests that aberrant follicular activation may play a role in the xenobiotic ovotoxicity. In this study we attempted to identify similarities between the mechanisms of ovotoxicity for three ovotoxic agents, 4-Vinylcyclohexene Diepoxide (VCD), Methoxychlor (MXC), and Menadione (MEN), which target immature follicles. Microarray analysis of neonatal mouse ovaries exposed to these xenobiotics *in vitro* revealed a more than two-fold significant difference in gene expression ($p<0.05$) for a number of genes associated with apoptotic cell death and primordial follicle activation. Follow-up qPCR analysis on VCD and MXC cultured ovaries confirmed an increase in expression for Akt1 (5.8, 6 fold), Akt2 (2.9, 1.5 fold), and Ccnd2 (5.3, 6 fold), all three of which are involved in follicular development. Histomorphological and immunohistological analysis supported the microarray data, showing signs of primordial follicle activation and pre-antral follicle atresia *in vitro* and *in vivo*. These results indicate a consistent mechanism of primordial follicle activation in pre-antral ovotoxicity for all three xenobiotics.

A.2.4. Short term xenobiotic exposure compromises long term oocyte viability

A. P. Sobinoff [1] , V. Pye[1] , B. Nixon[1,2] , S. D. Roman[1,2] , E. A. McLaughlin[1,2]

[1] School of Environmental and Life Sciences, The University of Newcastle, Callaghan, NSW, Australia

[2] The ARC Centre of Excellence in Biotechnology and Development, The University of Newcastle, Callaghan, NSW, Australia

Abstract

Mammalian females are born with a finite number of non-renewing primordial follicles, the majority of which remain in a quiescent state for many years. These oocyte containing follicles serve as the primary source of all developing follicles in the ovary, and cannot be regenerated post fetal development. Due to their non-renewing nature, these "resting" oocytes are particularly vulnerable to xenobiotic insult, especially those which are capable of inducing oxidative stress. Recent evidence suggests that certain synthetic chemical compounds, known as xenobiotics, have the potential to generate oxidative stress through the production of free oxygen radicals as a byproduct of the cell's detoxification process. Given the redox sensitive nature of the mammalian oocyte, xenobiotic exposure has been hypothesized to have long term adverse effects on oocyte viability. In this study, we attempted to identify the effects of short term xenobiotic exposure on long term oocyte viability. Female Swiss neonatal mice (day 4) were administered 7 daily consecutive doses of 4-Vinylcyclohexene diepoxide (40mg/kg/daily; 80mg/kg/daily) Methoxychlor (50mg/kg/daily; 100mg/kg/daily) or Menadione (7.5mg/kg/daily; 15mg/kg/daily). Mice were superovulated at 6wks and their oocytes collected for sperm-egg fusion assays. Sperm-egg fusion assays revealed a significant decrease in sperm egg binding/fusion ($p<0.05$) in a dose dependent manner for all three xenobiotic treatments *in vivo*, signifying a decrease in oocyte membrane fluidity. Follow-up lipid peroxidation analysis on xenobiotic cultured oocytes also showed a dose dependent increase in membrane lipid peroxidation in response to xenobiotic exposure. These results provide some of the first evidence of short term xenobiotic exposure causing long term oocyte dysfunction, possibly interfering with the fluidity and/or elasticity of the oocyte plasma membrane through lipid peroxidation.

A.2.5. Evidence of selective follicular destruction and primordial follicle activation in DMBA induced ovotoxicity

A. P. Sobinoff [1] , B. Nixon [1,2] , S. D. Roman [1,2] , E. A. McLaughlin [1,2]

[1] School of Environmental and Life Sciences, The University of Newcastle, Callaghan, NSW, Australia

[2] The ARC Centre of Excellence in Biotechnology and Development, The University of Newcastle, Callaghan, NSW, Australia

Abstract

7,12-dimethylbenz-[a]anthracene (DMBA) is an environmental xenobiotic which has a potent ovotoxic affect on rat and mouse ovaries, causing complete follicular depletion resulting in pre-mature ovarian failure (POF). In order to define the molecular mechanisms behind DMBA induced ovotoxicity, we cultured neonatal mouse ovaries in DMBA (50nM) for 4 days and examined its affects on the ovarian transcriptome. Microarray analysis revealed 98 genes were significantly up-regulated >2-fold ($p<0.05$) in response to DMBA exposure, with bioinformatics analysis linking these genes to ovarian signalling pathways associated with follicular growth (mTOR, ILK, VEGF signalling) and atresia (p53 signalling, protein ubiquitination). Validation of our microarray data via qPCR confirmed the up-regulation of several genes, including *Cdkn1a* (3.77 fold), *Ddx5* (1.71 fold), *Hspa8* (1.93 fold), *Dnaja6* (2.85 fold), and *Ccnd1* (5.82 fold). Histomorphological and immunohistological analysis supported the microarray data, showing signs of primordial follicle activation and pre-antral follicle atresia *in vitro* and *in vivo*. Further immunohistological analysis identified a significant increase ($p<0.05$) in Akt1 phosphorylation, mTOR activation, and FOXO3a repression in DMBA treated primordial follicle oocytes, events known to be intimately associated with primordial follicle activation. Our results reveal a novel mechanism of DMBA induced pre-antral ovotoxicity involving selective immature follicle destruction and primordial follicle activation, possibly involving downstream members of the PI3K/Akt and mTOR signalling pathways.

A.2.6. Neonatal xenobiotic exposure compromises adult oocyte viability

A. P. Sobinoff [1] , V. Pye[1] , B. Nixon[1,2] , S. D. Roman[1,2] , E. A. McLaughlin[1,2]

[1] School of Environmental and Life Sciences, The University of Newcastle, Callaghan, NSW, Australia

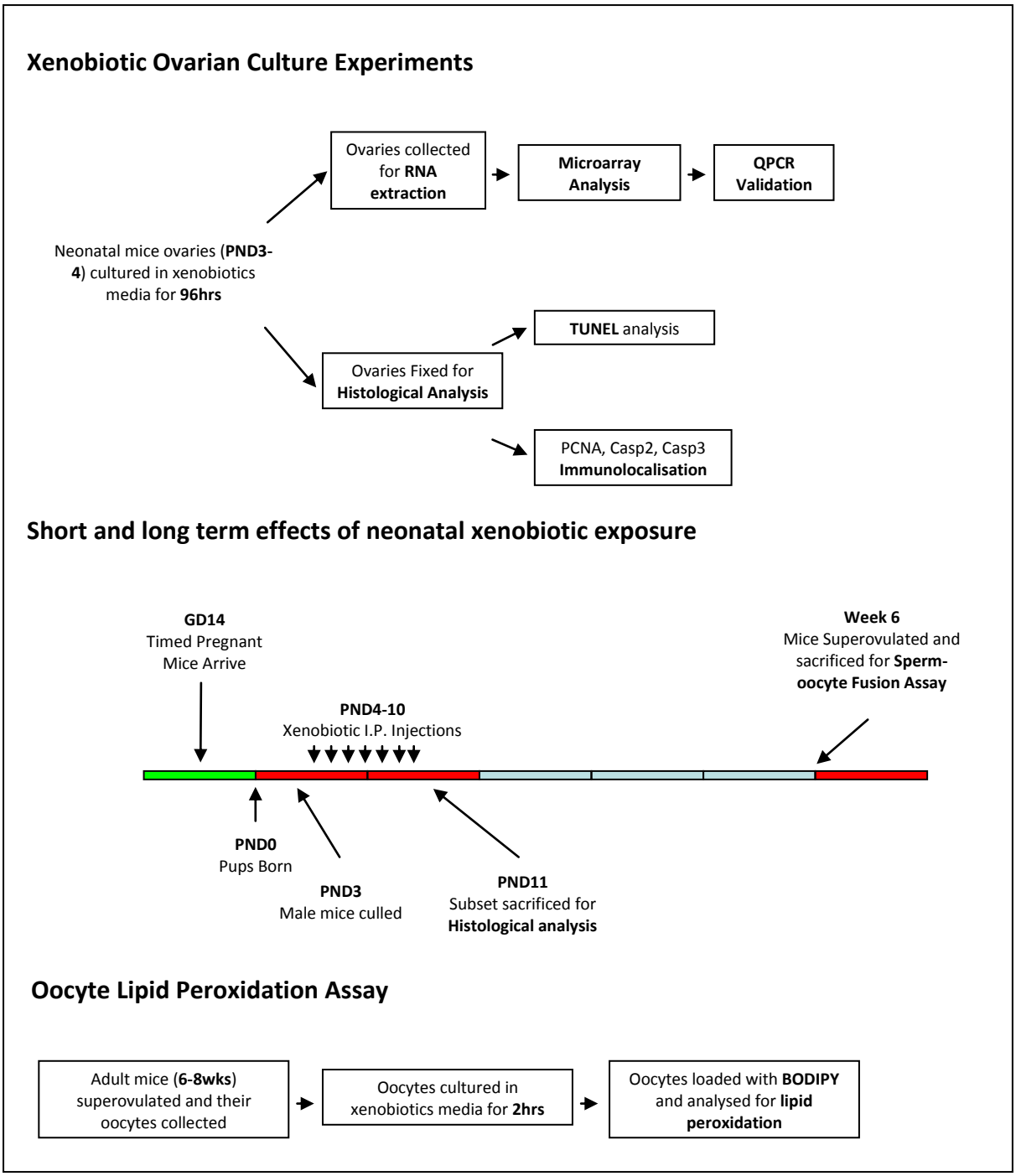
[2] The ARC Centre of Excellence in Biotechnology and Development, The University of Newcastle, Callaghan, NSW, Australia

Abstract

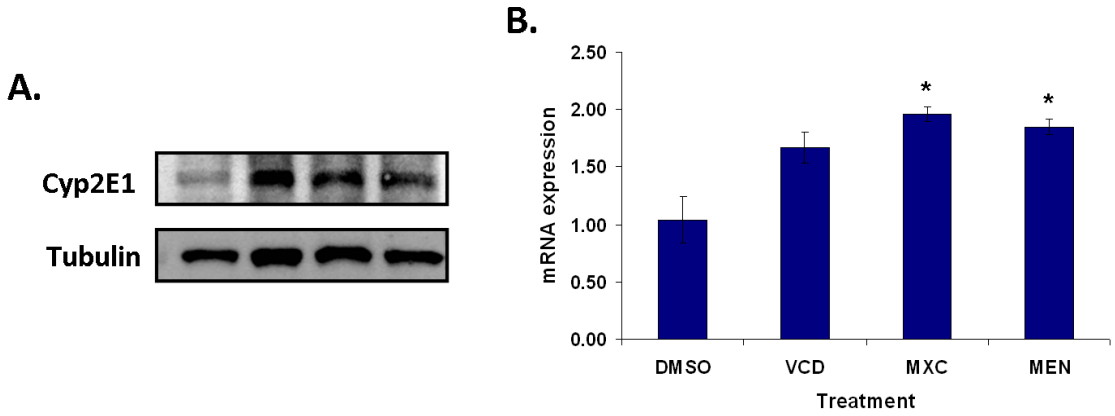
The irreplaceable primordial follicle represents the basic unit of female fertility, serving as the primary source of all developing oocytes in the ovary. Unfortunately, these non-renewing oocytes are particularly vulnerable to xenobiotic insult, with recent evidence suggesting exposure results in oxidative stress. Given the redox sensitive nature of the mammalian oocyte, xenobiotic exposure has been hypothesized to have long term adverse effects on oocyte viability. In this study, we attempted to identify the effects of short term xenobiotic exposure on long term oocyte viability. Female Swiss neonatal mice (day 4) were administered 7 daily consecutive doses of 4-Vinylcyclohexene diepoxide (40mg/kg/daily; 80mg/kg/daily) Methoxychlor (50mg/kg/daily; 100mg/kg/daily) or Menadione (7.5mg/kg/daily; 15mg/kg/daily). Mice were superovulated at 6wks and their oocytes collected for analysis. Sperm-egg fusion assays revealed a significant decrease in sperm egg binding/fusion ($p<0.05$) in a dose dependent manner for all three xenobiotic treatments *in vivo*, signifying a decrease in oocyte membrane fluidity. Lipid peroxidation analysis confirmed this observation, demonstrating a dose dependent increase in membrane lipid peroxidation in response to xenobiotic exposure. Finally, we detected significantly increased levels of mitochondrial ROS ($p<0.05$) in all three xenobiotic treatments *in vivo*. These results suggest that short term xenobiotic exposure can cause long term oocyte dysfunction, possibly through increased mitochondrial ROS production interfering with the fluidity and/or elasticity of the oocyte plasma membrane via lipid peroxidation.

Appendix B: Supplementary data for publications

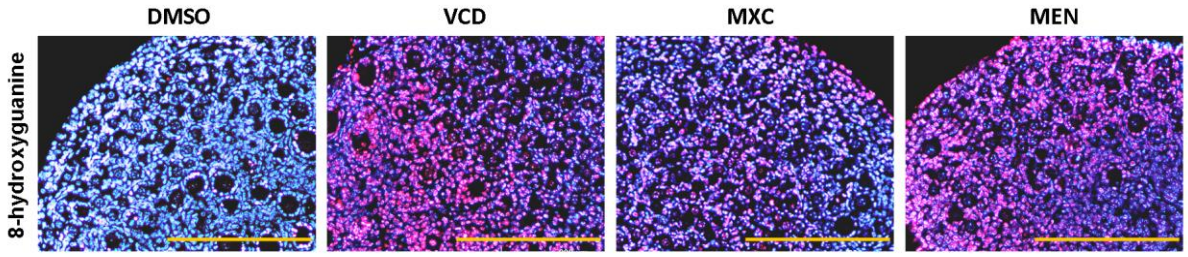
B.1. Adding Insult to Injury; Effects of Xenobiotic-Induced Preantral Ovotoxicity on Ovarian Development and Oocyte Fusibility



sFigure 1. Flow chart of experiments



sFigure 2. Ovarian cyp2E1 expression in xenobiotic cultured neonatal ovaries. (A) Ovarian Cyp2E1 protein expression. Ovaries excised from neonatal mice (4 days old) were cultured in xenobiotic treated medium for 96 hours and processed for immunoblotting with polyclonal antibodies against cyp2E1 as described in the materials and methods. (B) Ovarian Cyp2E1 mRNA expression. Total RNA was isolated from xenobiotic cultured ovaries, reverse transcribed, and qPCR performed with primers specific for Cyp2E1 cDNA as described in the materials and methods. Values are mean ± SE. The symbols * represents $p<0.05$ in comparison with DMSO control values.



sFigure 3. Fluorescent immunolocalisation of the hydroxyl radical induced DNA molecular lesion 8-hydroxyguanine in xenobiotic cultured ovaries. Ovaries excised from neonatal mice (4 days old) were cultured in xenobiotic treated medium for 96 hours and processed for immunohistochemistry as outlined in materials and methods. Blue staining (DAPI) represents nuclear staining in all cells; red staining (Cy-5) represents specific staining for 8-hydroxyguanine. Scale bar is equal to 200µm.

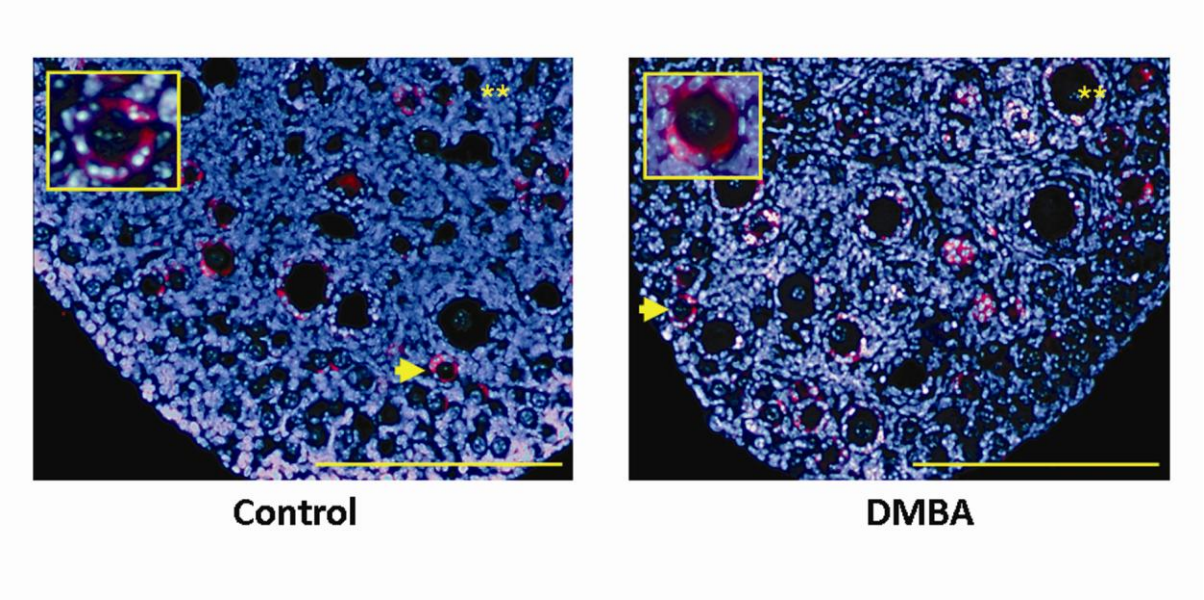
sTable 1. Comparison of genes with significantly altered gene expression commonly regulated between the three xenobiotic treated groups (Available on disc; Supplementary Tables B1.xls).

sTable 2. Top canonical pathways that were significantly up-regulated by xenobiotic cultured neonatal ovaries as identified by Ingenuity® Pathway Analysis (IPA). The significance of the association between up-regulated genes and the canonical pathway was evaluated using a

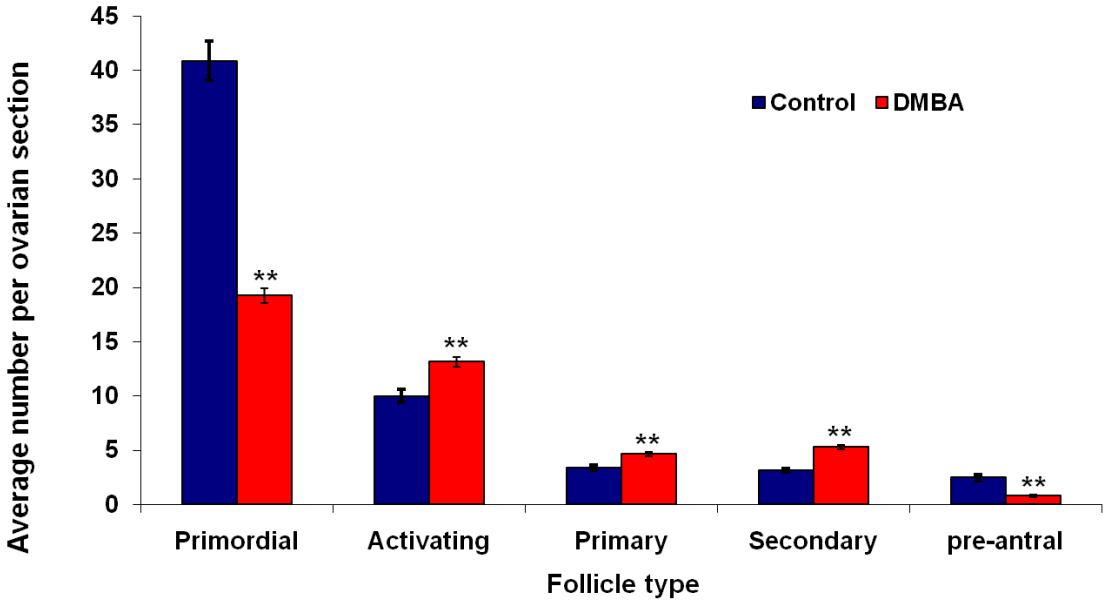
right-tailed Fisher's exact test to calculate a p-value determining the probability that the association is explained by chance alone. Ratios referring to the proportion of up-regulated genes from a pathway related to the total number of molecules that make up that particular pathway are also displayed (Available on disc; Supplementary Tables B1.xls).

sTable3. Primer sequences used in qPCR (Available on disc; Supplementary Tables B1.xls).

B.2. Understanding the Villain: DMBA-Induced Preantral Ovotoxicity Involves Selective Follicular Destruction and Primordial Follicle Activation through PI3K/Akt and mTOR Signaling



sFigure 1. Fluorescent immunolocalisation of AMH in DMBA cultured ovaries. Ovaries excised from neonatal mice (4 days old) were cultured in DMBA treated medium for 96 hours and processed for immunohistochemistry as outlined in materials and methods. Blue staining (DAPI) represents nuclear staining in all cells; red staining (Cy-5) represents specific staining for AMH. Scale bar is equal to 200µm.



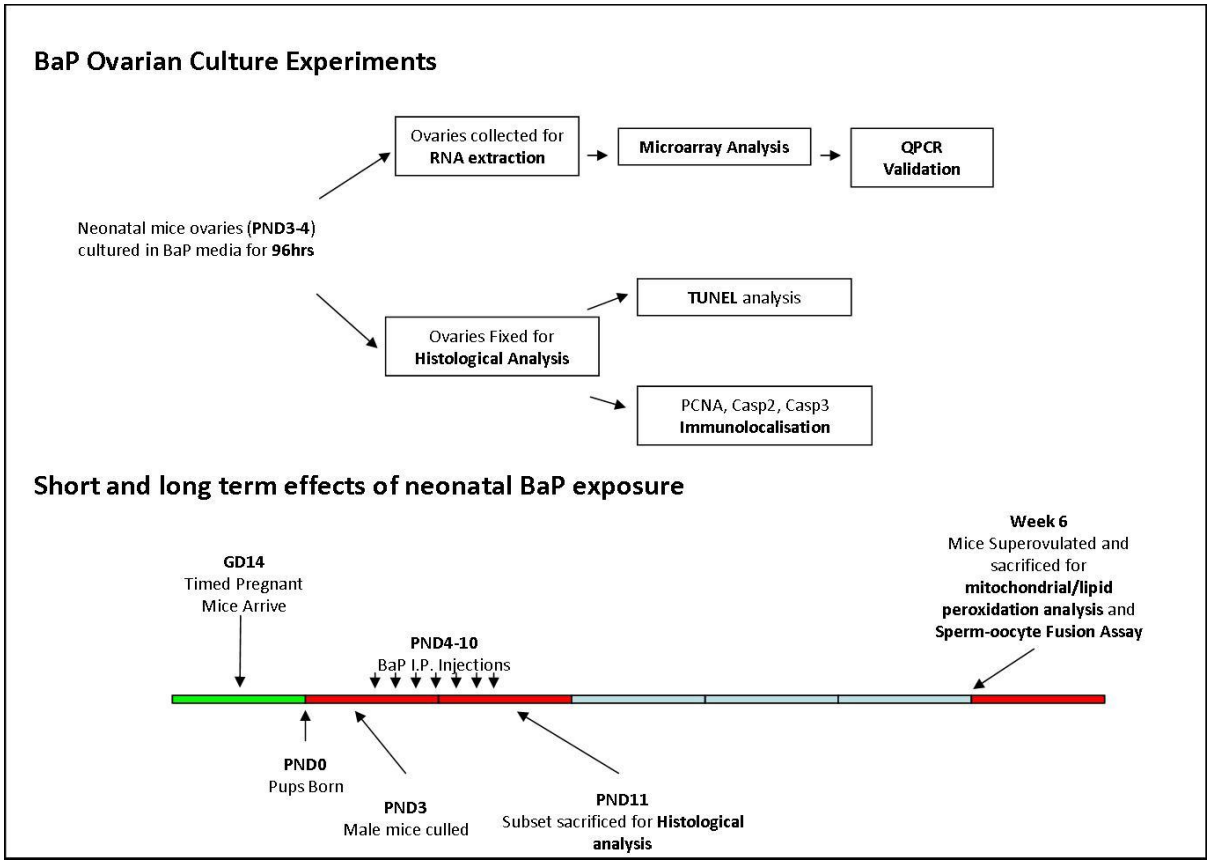
sFigure 2. Effect of xenobiotic exposure on ovarian follicle composition and number *in vivo*. Neonatal mice (4 days old) were dosed with DMBA over a seven day period as described in materials and methods. Ovarian sections were stained with hematoxylin and eosin and healthy oocyte containing follicles were classified and counted under a microscope. Values are mean ± SE, n=3-5 ovaries. The symbol ** represents $p<0.01$ in comparison with control values.

sTable 1. Genes with significantly altered gene expression in DMBA cultured ovaries classified according to molecular and cellular functions (Available on disc; Supplementary Tables B2.xls).

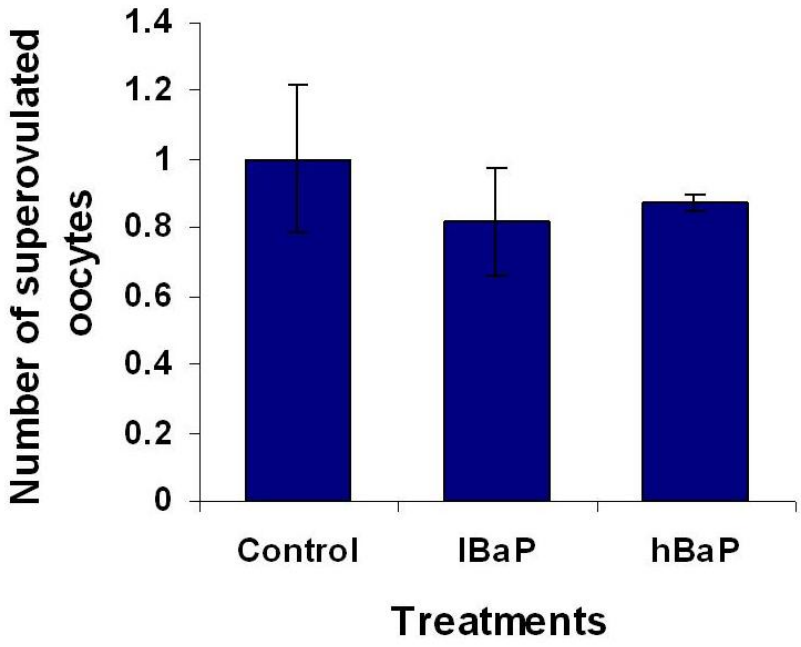
sTable 2. Top canonical pathways that were significantly up-regulated by DMBA cultured neonatal ovaries as identified by Ingenuity® Pathway Analysis (IPA). The significance of the association between up-regulated genes and the canonical pathway was evaluated using a right-tailed Fisher's exact test to calculate a p-value determining the probability that the association is explained by chance alone. Ratios referring to the proportion of up-regulated genes from a pathway related to the total number of molecules that make up that particular pathway are also displayed (Available on disc; Supplementary Tables B2.xls).

sTable3. Primer sequences used in qPCR (Available on disc; Supplementary Tables B2.xls).

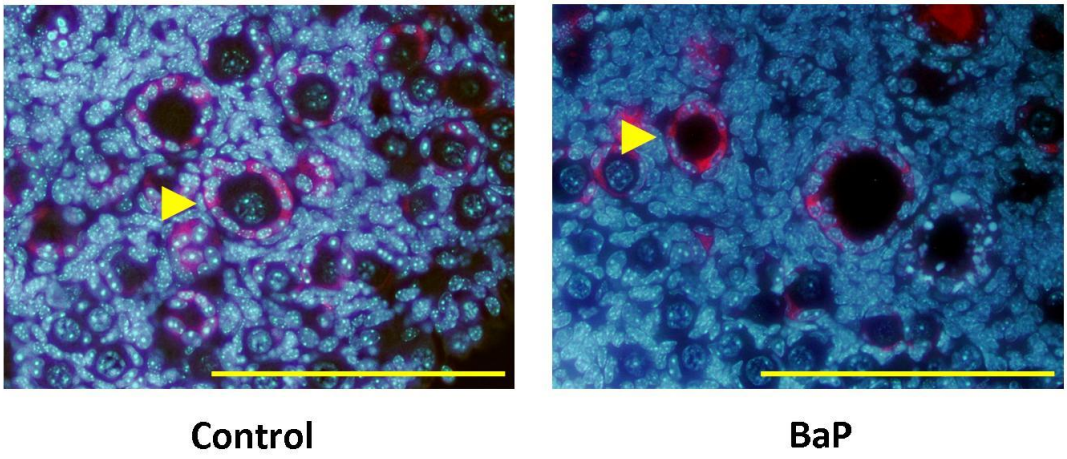
B.3. Jumping the gun: Smoking constituent BaP causes premature primordial follicle activation and impairs oocyte fusibility through oxidative stress



sFigure 1. Experimental design.



sFigure 2. Effect of neonatal in vivo BaP exposure on the production of oocytes post superovulation



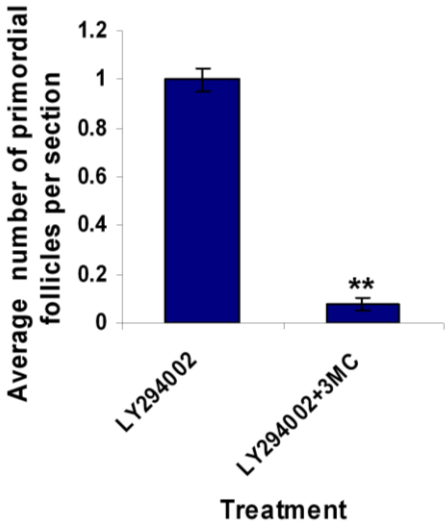
sFigure 3. Fluorescent immunolocalisation of AMH in BaP cultured ovaries. Ovaries excised from neonatal mice (4 days old) were cultured in BaP treated medium for 96 hours and processed for immunohistochemistry as outlined in materials and methods. Blue staining (DAPI) represents nuclear staining in all cells; red staining (Cy-5) represents specific staining for AMH. arrow head=primary follicle; Scale bar is equal to 100µm.

sTable 1. Genes with significantly altered gene expression in BaP cultured ovaries classified according to molecular and cellular functions (Available on disc; Supplementary Tables B3.xls).

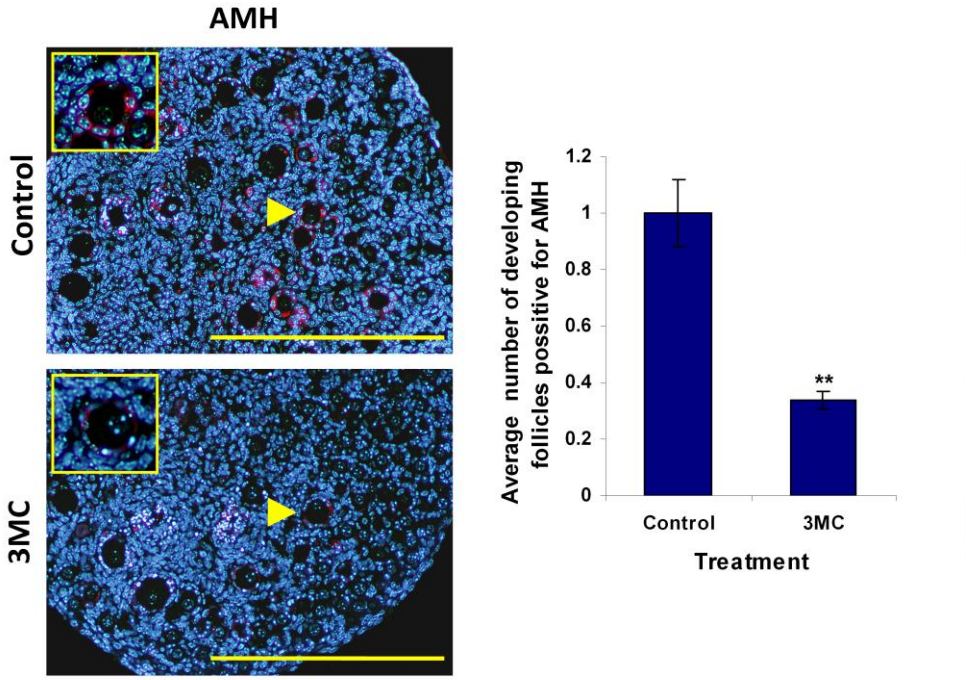
sTable 2. Top canonical pathways that were significantly up-regulated by BaP cultured neonatal ovaries as identified by Ingenuity® Pathway Analysis (IPA). The significance of the association between up-regulated genes and the canonical pathway was evaluated using a right-tailed Fisher's exact test to calculate a p-value determining the probability that the association is explained by chance alone. Ratios referring to the proportion of up-regulated genes from a pathway related to the total number of molecules that make up that particular pathway are also displayed (Available on disc; Supplementary Tables B3.xls).

sTable3. Primer sequences used in qPCR (Available on disc; Supplementary Tables B3.xls).

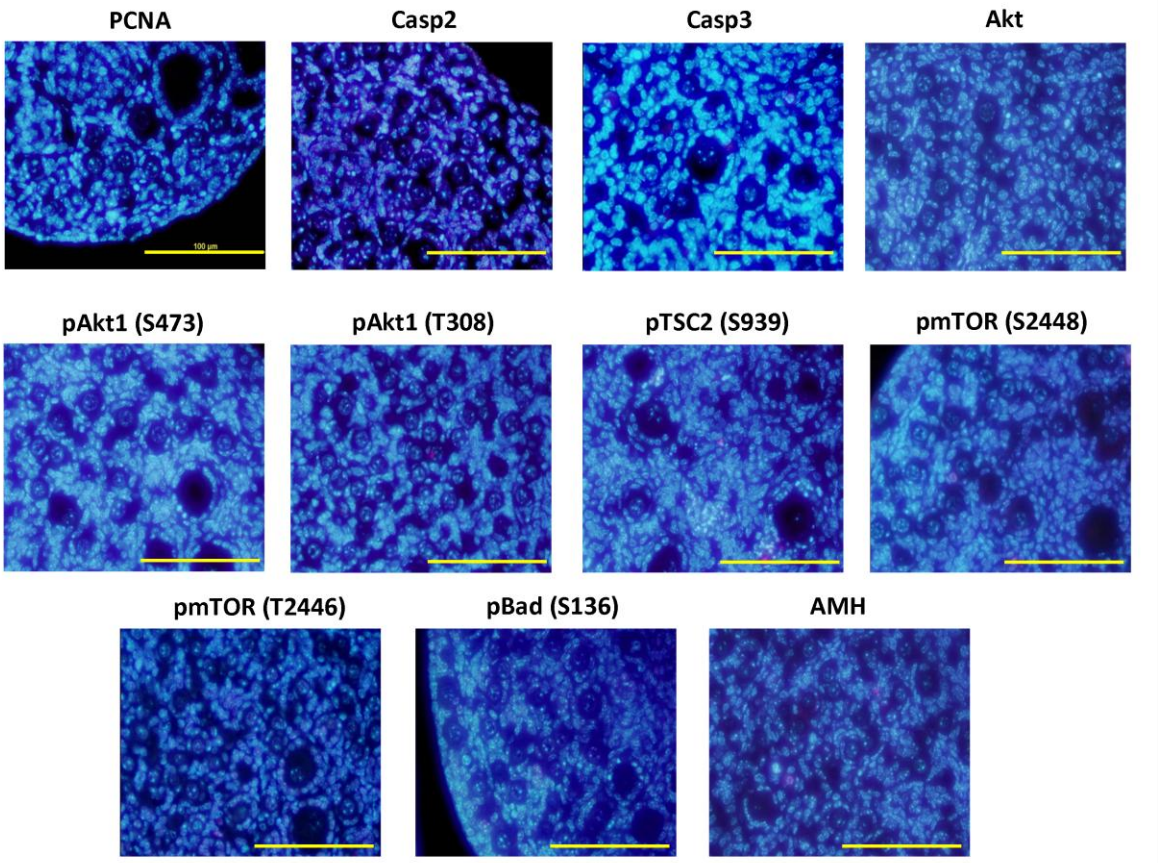
B.4. Staying alive: PI3K pathway promotes primordial follicle activation and survival in response to 3-MC induced ovotoxicity



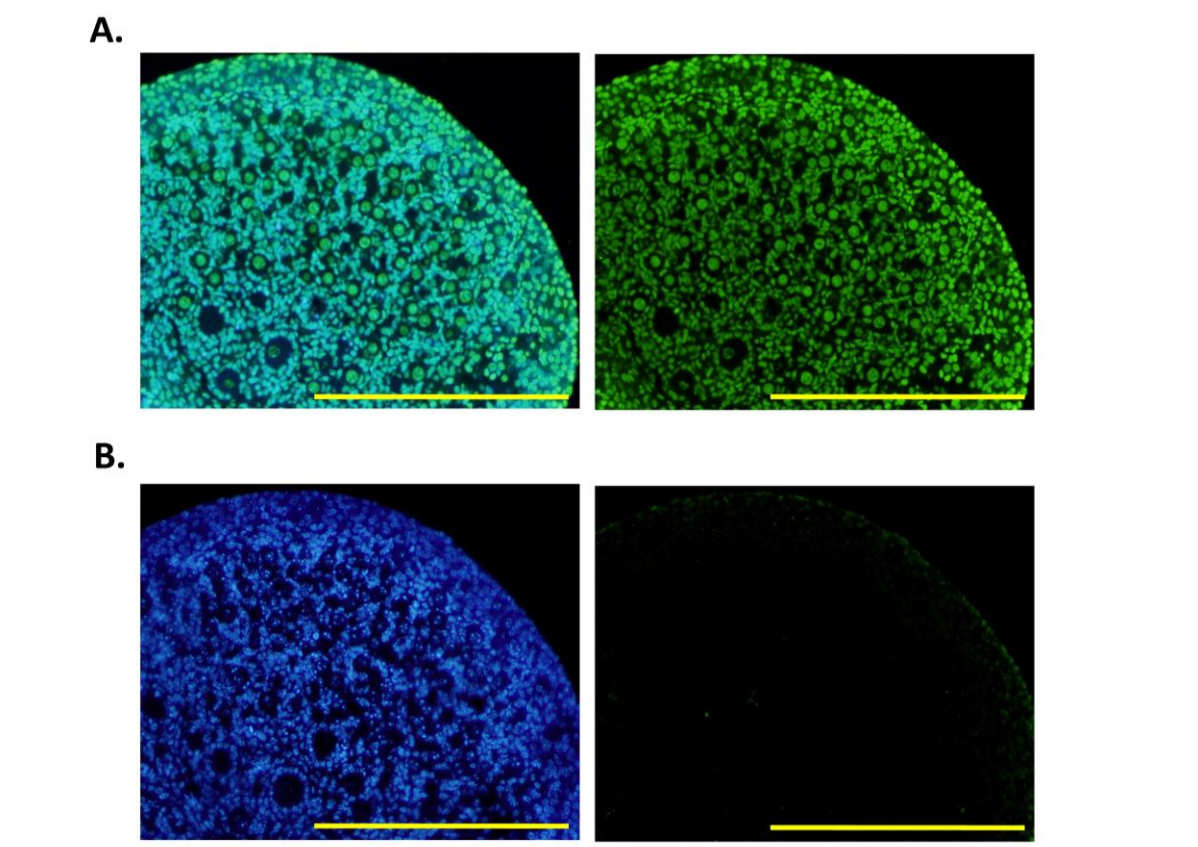
sFigure 1. PI3K inhibition causes excessive primordial follicle depletion in 3MC-treated ovaries *in vitro*. Ovaries excised from neonatal mice (4 days old) were cultured in 3MC-treated medium + LY294002 for 96 hours as described in materials and methods. Ovarian sections were stained with hematoxylin and eosin and healthy primordial follicles were counted under a microscope. Values are mean ± SEM, n=3-5 ovaries from 3-5 mice. The symbol ** represents $p<0.01$ in comparison with control values.



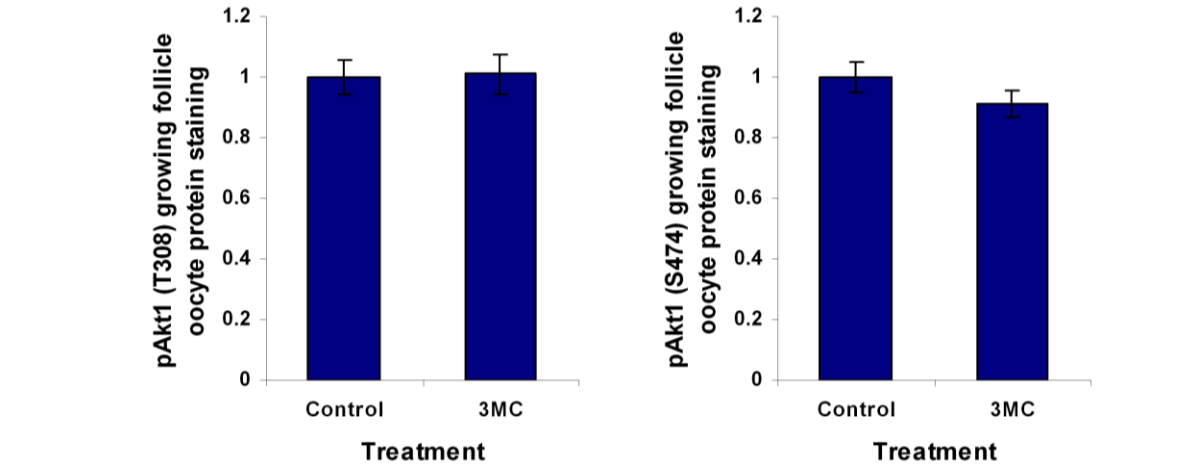
sFigure 2. Fluorescent immunolocalisation of AMH in 3MC-cultured ovaries. Ovaries excised from neonatal mice (4 days old) were cultured in 3MC-treated medium for 96 hours and processed for immunohistochemistry as outlined in materials and methods. Blue staining (DAPI) represents nuclear staining in all cells; red staining (Cy-5) represents specific staining for AMH. Scale bar is equal to 200µm.



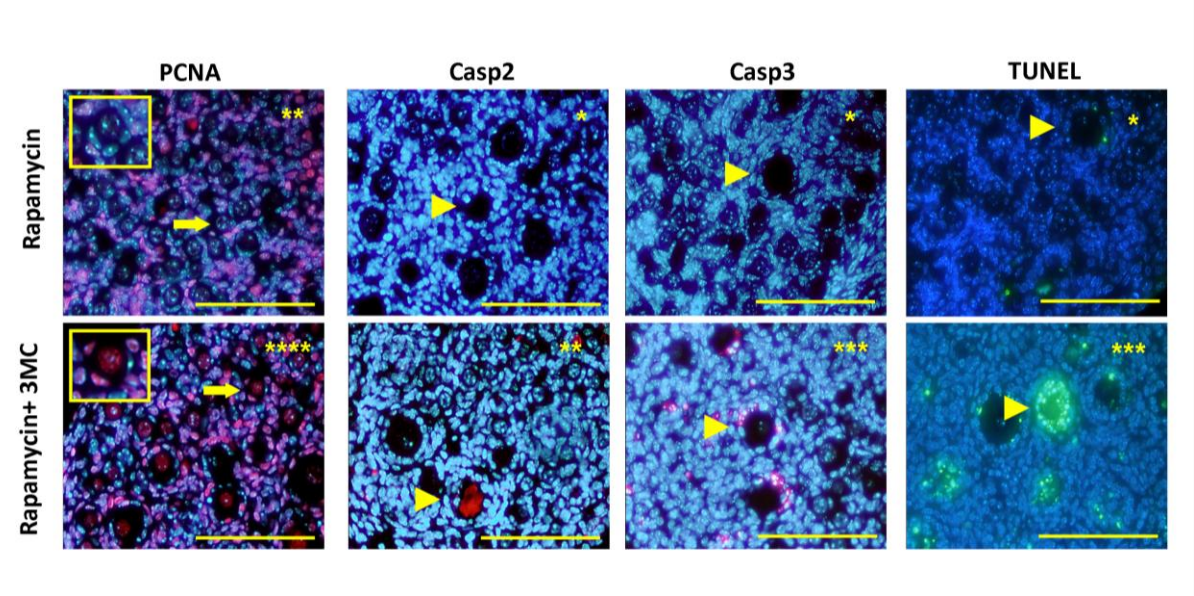
sFigure 3. Representative images of negative control experiments with the omission of the primorday antibody performed alongside immunolocalisation studies. Blue staining (DAPI) represents nuclear staining in all cells; red staining (Cy-5) represents specific staining for the described protein. Scale bar is equal to 100µm.



sFigure 4. Representative images of positive and negative control experiments performed alongside TUNEL analysis. Blue staining (DAPI) represents nuclear staining in all cells; green staining (Fluorescein) represents specific staining for DNA strand breaks (TUNEL). Scale bar is equal to 200µm.



sFigure 5. Quantification of pAkt1 (S473) and pAkt1 (T308) oocyte nuclear staining in 3MC-cultured developing follicles. Ovaries excised from neonatal mice (4 days old) were cultured in 3MC-treated medium for 96 hours and processed for immunohistochemistry as outlined in materials and methods. The results presented here are representative of n=3 experiments.



sFigure 6. mTOR inhibition does not repress primordial follicle activation *in vitro*. (A) Fluorescent immunohistological and TUNEL staining as visualised via epifluorescent microscopy. Ovaries excised from neonatal mice (4 days old) were cultured in 3MC-treated medium + Rapamycin for 96 hours and processed for immunohistochemistry and TUNEL analysis as described in the materials and methods. Ovarian sections were probed 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 labelled follicles per section is represented by the following scale present in the top right hand corner; *=<25%, **=25-50%, ***=51-75%, ****=76-100%. Thin arrow=primordial follicle highlighted in insert at higher magnification; arrow head=primary follicle; scale bar is equal to 100 µm.

sTable 1. Genes with significantly altered gene expression in 3MC-cultured ovaries classified according to molecular and cellular functions (Available on disc; Supplementary Tables B4.xls).

sTable 2. Top canonical pathways that were significantly up-regulated by 3MC-cultured neonatal ovaries as identified by Ingenuity® Pathway Analysis (IPA). The significance of the association between up-regulated genes and the canonical pathway was evaluated using a right-tailed Fisher's exact test to calculate a p-value determining the probability that the association is explained by chance alone. Ratios referring to the proportion of up-regulated genes from a pathway related to the total number of molecules that make up that particular pathway are also displayed (Available on disc; Supplementary Tables B4.xls).

sTable3. Primer sequences used in qPCR (Available on disc; Supplementary Tables B4.xls).