The Impact of Maternal Ageing and Chemotoxicants on Female Fertility

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B. Biomedical Science (Hons Class I)

Thesis submitted to the Faculty of Science and Information Technology, The University of Newcastle, Australia in fulfilment of the requirement of the degree of the Doctor of Philosophy

Friday 7th October 2016
Declaration

I hereby declare that all material found within this thesis to the best of my knowledge has not been published or written by another person, except where due reference is given. In addition, no part of this dissertation has been previously submitted to The University of Newcastle or any other tertiary institution in order to obtain a degree or diploma. I also hereby consent to copy/copies of this thesis to be made available for both loan and photocopying from the University Library subject provisions set forth in the Copyright Act 1968.

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I hereby certify that the work embodied in this thesis contains a published paper/s/scholarly work of which I am a joint author. I have included as part of the thesis a written statement, endorsed by my supervisor, attesting to my contribution to the joint publication/s/scholarly work.

Signed

Nicole Jacqueline Camlin

Date: 6th October 2016

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Date: 6th October 2016

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(Secondary Supervisor)
Date: 6th October 2016

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Manuscripts Included as Part of Thesis


Conference Proceedings Relevant to Thesis

Camlin, N. J., Sobinoff, A.P., Sutherland, J.M., Hansbro, P.M., McLaughlin, E.A., and Holt, J.E. In utero exposure to mainstream cigarette smoke: long term effects on reproductive potential of female pups. 44th annual meeting for the Society of Reproductive Biology, August 2013, Sydney, Australia. (Oral)


Camlin, N. J., McLaughlin, E.A., and Holt, J.E. A kinesin motor protein is essential for normal mammalian oocyte meiosis. 47th annual meeting for the Society of Reproductive Biology, August 2016, Gold Coast, Australia. (Oral)
Awards

**Finalist for the Oozoa Award** for best oral student presentation, 45th Annual Conference for Society of Reproductive Biology, Melbourne, Australia (2014)

**Faculty of Science and I.T Conference Scholarship**, University of Newcastle, Australia (2015)


**Finalist for the Oozoa Award** for best oral student presentation, 47th Annual Conference for Society of Reproductive Biology, Gold Coast, Australia (2016)

**Winner of Science Meets Publican 60 Second Elevator Pitch**, 47th Annual Conference for Society of Reproductive Biology, Gold Coast, Australia (2016)
Additional Publications and Conference Proceedings


Mihalas, B.P., Camlin, N.J., McLaughlin, E.A., and Nixon B. Lipid peroxidation contributes to the ROS mediated deterioration of meiotic competency and quality of oocytes. 47th annual meeting for the Society of Reproductive Biology, August 2016, Gold Coast, Australia. (Poster)
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Abstract

Mammalian females are born with all the oocytes they will ever have. The quality and quantity of these gametes dictates the reproductive life span of a woman. In recent decades it has been well established that oocyte quality decreases with increasing maternal age primarily as a result of oocytes failing to separate chromosomes correctly. The outcome is a condition known as aneuploidy, which can result in increased rates of miscarriage and birth defects such as Downs Syndrome. Additionally, exposure of oocytes to chemotoxicants, including those found in cigarette smoke, has been found to prematurely reduce oocyte quality in women leading to early onset menopause. The aim of this thesis is to investigate potential causes of female fertility decline, including ageing and multigenerational exposure to cigarette smoke.

Research within this thesis highlights the role of in utero and multigenerational smoke exposure in reducing female fertility. Approximately 12% of Australian and American women smoke throughout their pregnancy (Australia’s mothers and babies, 2015; Tong et al., 2009). This has been linked to decreased birth weight, increased risk of sudden infant death syndrome, childhood cancers and asthma in in utero exposed offspring (maternal smoke exposed). Additionally, a growing body of evidence suggests that these maternal smoke exposed females have a reduction in fecundability in later life (Weinberg et al., 1989; Ye et al., 2010). However, the cause of this reduced fertility, and the fertility of the subsequent generations is largely unknown. Throughout this thesis, I detail how maternal and grandmaternal smoke exposure decreases female fertility in a mouse model, whilst great-grandmaternal smoke exposure appears to have little effect.

In addition to investigating the effects of multigenerational smoke exposure on female fertility, this thesis also explores the impact of maternal ageing on oocyte quality. It has been
well established that oocyte quality decreases with increasing age (Hassold et al., 2007; Jones, 2008). However, the molecular mechanisms underpinning this phenomenon are still being unravelled. In order to gain further insight into the fidelity of the oocyte cell cycle, I chose to examine a member of the kinesin motor protein family, Kif4. Kinesins are known to be important for the mitotic cell cycle, but little is known about how they function in mammalian oocytes. In mitosis Kif4 is involved in chromosome condensation and separation, metaphase and midzone spindle formation and cytokinesis (Hu et al., 2011; Mazumdar et al., 2004; Samejima et al., 2012). I show here for the first time that Kif4 has dynamic localisation throughout meiosis, and importantly, that it has essential roles in female meiosis, including spindle formation and polar body extrusion (cytokinesis). Furthermore, Kif4 appears to have roles in trafficking kinetochore proteins Ndc80 and CENP-C under the control of Aurora Kinase B and Cdk1. Finally, I show that Kif4 protein levels are elevated in metaphase I and II oocytes from reproductively aged mice, implicating a role for this protein in age related oocyte quality decline.

Collectively the data presented in this thesis helps build a clearer picture of the role of multigenerational smoke exposure and/or maternal ageing on reduced female fertility.
CHAPTER 1: Literature Review

Through the smoke: Use of in vivo and in vitro cigarette smoking models to elucidate its effect on female fertility

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Statement of Contribution

N.J.C., E.A.M. and J.E.H., contributed to the conception and design of the review. N.J.C was also responsible for drafting and revising the manuscript.
Chapter 1: Overview

The aim of following literature review was to evaluate the applicability of animal models of smoke exposure and to summarise findings to date of the effect of cigarette smoke or its constituents on female fertility.

This chapter explores the use of different exposure models, *in vivo* and *in vitro*, to cigarette smoke or its constituents on ovarian and oocyte quality. Throughout this review we highlight the strengths and weaknesses of each model. We conclude nose-only smoke inhalation best mimics human exposure and emphasise the need for future research using this model to elucidate the role of cigarette smoking on female fertility.
Through the smoke: Use of in vivo and in vitro cigarette smoking models to elucidate its effect on female fertility

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Abstract

A finite number of oocytes are established within the mammalian ovary prior to birth to form a precious ovarian reserve. Damage to this limited pool of gametes by environmental factors such as cigarette smoke and its constituents therefore represents a significant risk to a woman's reproductive capacity. Although evidence from human studies to date implicates a detrimental effect of cigarette smoking on female fertility, these retrospective studies are limited and present conflicting results. In an effort to more clearly understand the effect of cigarette smoke, and its chemical constituents, on female fertility, a variety of in vivo and in vitro animal models have been developed. This article represents a systematic review of the literature regarding four of experimental model types: 1) direct exposure of ovarian cells and follicles to smoking 2) in vitro, 2) direct exposure of whole ovarian tissue with smoking constituents in vitro, 3) whole body exposure of animals to smoking constituents and 4) whole body exposure of animals to cigarette smoke. We summarise key findings and highlight the strengths and weaknesses of each model system, and link these to the molecular mechanisms identified in smoke-induced fertility changes.

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Introduction: smoking and human fertility

Females are born with a finite number of oocytes, meiotically arrested at meiosis I in primordial follicles (Baker, 1963) (Fig. 1). Once puberty begins, a number of these follicles are recruited each cycle to mature into primary, secondary and finally antral follicles, ready for ovulation (Jones et al., 2013; Skinner, 2005). This recruitment and growth process continues until the follicular pool is exhausted and menopause occurs (Richardson et al., 1987). Accelerated depletion of this reserve can occur in response to a number of environmental and lifestyle insults, with cigarette smoking one of these factors (Hank et al., 2004; Cooper et al., 1999; Hayatbakhsh et al., 2012; Kinney et al., 2006; McKinlay et al., 1985; Midgette and Baron, 1990; Samç et al., 2010; Sobinoff et al., 2012; Sun et al., 2012; Willet et al., 1983).

Cigarettes contain over 4000 chemicals and are regarded as a reproductive hazard, associated with decreased conception rates, reduced assisted fertility success and premature ovarian failure (Neal et al., 2005; Sun et al., 2012). Despite the public promulgation of these facts, large numbers of women continue to smoke, with 25% of women in the United States, 23% in the United Kingdom and 19% in Australia documented smokers (WHO, 2009). Additionally, despite increasing knowledge of the adverse effect smoking has on lung and heart health, few women are aware of the negative effects it has on their fertility (Roth and Taylor, 2001). Compounding these issues are the handful of human studies that have found smoking has no effect on assisted reproduction success rates (Cinar et al., 2014; Fuentes et al., 2010; Petanovski et al., 2012; Wright et al., 2006). In order to assist in clarifying the effects of whole cigarette smoke and its constituents on female fertility numerous in vivo and in vitro animal models have been employed over the last decade, however the inherent differences between these models require careful interpretation.

The purpose of this review is to summarise the methodology and findings of the different animal models, both in vivo and in vitro, used to investigate cigarette smoke and/or its constituents and in so doing evaluate their strengths and weaknesses, summarised in Table 1. This review begins with an overview of follicle and oocyte maturation, followed by a discussion of studies that have used individual chemical constituents in vitro and in vivo and contrasts these with whole cigarette smoke exposure models. We also highlight molecular mechanisms underpinning the deleterious effects of cigarette smoke and its constituents, summarised in Table 2.

Oocyte and follicle maturation

Mammalian folliculogenesis and oocyte maturation are interlinked processes that are required for the production of oocytes capable of fertilisation. Quiescent primordial follicles consist of an immature oocyte meiotically arrested at prophase I surrounded by a single layer of granulosa cells (Skinner, 2000). As the follicle begins to grow through
primary, secondary and antral stages, granulosa cells multiply and theca cells develop at the basement membrane, while cumulus cells – a granulosa cell subtype, surround the oocyte (Skinner, 2005) (Fig. 1). Communication between these cell types, relies on gap junctions which are essential for oocyte and follicle maturation (Kidd and Mhawi, 2002). By late antral (pre-ovulatory) follicle stage, the oocyte has reached nuclear and cytoplasmic maturation having enlarged significantly, synthesised an acellular matrix, the zona pellucida, and is surrounded by specialised cumulus granulosa cells (McLaughlin and McIver, 2009). A surge of luteinising hormone (LH) triggers meiotic reprogramming and ovulation (Holt et al., 2013; Liu et al., 2013). The oocyte transitions from prophase I into metaphase I, with homologous chromosomes attaching to the spindle, and aligning on the metaphase plate (Holt et al., 2013; Jones et al., 2013). The chromosomes separate with half being extruded into the first polar body and the egg arrests once again, at metaphase II, until fertilisation (Holt et al., 2013; Jones et al., 2013). Following this event, the second polar body is extruded and the male and female pronuclei fuse to generate a zygote (Banaszynski et al., 2010).

Major constituents of cigarette smoke implicated in ovotoxicity

Cigarettes smoke contains over 4000 chemicals from a wide variety of chemical classes including hydrocarbons, alcohols, phenols, aldehydes, ketones, alkaloids, acids and heavy metals (Rodgman and Perfetti, 2013). Of these classes, the most widely studied with regard to female fertility are the hydrocarbons, specifically polycyclic aromatic hydrocarbons (PAHs) (Ganesan et al., 2013; Ganesan and Keating, 2014:

Table 1

<table>
<thead>
<tr>
<th>Experimental method</th>
<th>Strengths</th>
<th>Weaknesses</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular, oocyte and granulosa cell culture</td>
<td>- Fast turn over. - Exposure at specific time points in follicle/oocyte maturation - Mechanism of action easy to pin point - Reduced animal numbers</td>
<td>- Individual chemicals used - Extra follicular and/or cellular metabolism not accounted for - Cellular alterations from culture conditions</td>
</tr>
<tr>
<td>Ovarian explant culture</td>
<td>- Follicle-stromal interactions accounted for - Ovarian metabolism of toxicants - Mechanism of action easy to pin point - Reduced animal numbers</td>
<td>- Individual chemicals used - Difficult to achieve human doses - Difficult to achieve human doses</td>
</tr>
<tr>
<td>In vivo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injection (intraperitoneal, subcutaneous, intraovarian)</td>
<td>- Whole body metabolism - Hypothalamic-pituitary axis present</td>
<td>- Difficult to achieve human doses - Route does not mimic human exposure</td>
</tr>
<tr>
<td>Oral exposure</td>
<td>- Whole body metabolism - Hypothalamic-pituitary axis present</td>
<td>- Individual chemicals used - Route does not mimic human exposure</td>
</tr>
<tr>
<td>Whole-body smoking</td>
<td>- Exposure to all cigarette smoke components - Primary nasal exposure mimics human exposure - Whole body metabolism - Hypothalamic-pituitary axis present</td>
<td>- Secondary oral and dermal exposure</td>
</tr>
<tr>
<td>Nose-only smoking</td>
<td>- Exposure to all cigarette smoke components - Nasal exposure mimics human exposure - Whole body metabolism - Hypothalamic-pituitary axis present</td>
<td>- Animal restraint needed - Labour intensive</td>
</tr>
</tbody>
</table>
Table 2
Molecular mechanisms underpinning the deleterious effects of cigarette smoke and its constituents on ovarian folliculogenesis.

<table>
<thead>
<tr>
<th>Model</th>
<th>Ovarian parameters</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BeAP</td>
<td>Juvenile mouse, follicle culture</td>
<td>↑CyplA1, cyplB1, Hap90ab1 and Bax</td>
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<td></td>
<td>Juvenile rat, follicle culture</td>
<td>↑Antrol follicle development, E2 and AMH output</td>
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<td>Neonatal mouse, ovary culture</td>
<td>↑Follicle growth</td>
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<td></td>
<td>Neonatal mouse, ovary culture</td>
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<td></td>
<td>Rat, SC injection (PND 1-14)</td>
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<td></td>
<td>Neonatal mouse, IP injection</td>
<td>↑E2 responsiveness and E2 receptor beta</td>
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<td></td>
<td>Adult mouse, IP injection</td>
<td>↑Primary and secondary follicles</td>
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<td></td>
<td>Rat, SC injection (PND 1-14)</td>
<td>↑Corpora lutea</td>
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<td></td>
<td>Rat SC injection (PND 3-14)</td>
<td>↑E2 responsiveness and E2 receptor beta</td>
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<td></td>
<td>DMBA</td>
<td>↑Follicle numbers</td>
</tr>
<tr>
<td></td>
<td>Neonatal rat, ovary culture</td>
<td>↑β2 HAX and apoptosis (↑ATM, Xrcc6, Becn, Parp1, Rad51)</td>
</tr>
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<td></td>
<td>Neonatal mouse, ovary culture</td>
<td>↑Apoptosis (↑Bax)</td>
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<td></td>
<td>Neonatal mouse, ovary culture</td>
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<td>Juvenile mouse, follicle culture</td>
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<td>Adult rat IP injection</td>
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<td>Adult rat IP injection</td>
<td>↑Ovarian cholesterol</td>
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<td>Heat shock protein 25, SOHD2 and Bcl-2</td>
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<td></td>
<td>Orponceine (↑autophagosoma, Beclin 1 and LC3)</td>
<td>Autophagy (↑autophagosoma, Beclin 1 and LC3)</td>
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<td>Orponceine (↑autophagosoma, Beclin 1 and LC3)</td>
<td>Bcl-2 (protein)</td>
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<td>Ovary size, primordial and primary follicle numbers, corpus luteum, ovulated oocytes, sperm-egg interaction and litter size</td>
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<td>Ovary size, primordial and primary follicle numbers</td>
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<td>Ovary size, primordial and primary follicle numbers</td>
<td>Cumulus expansion and oocyte growth</td>
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Igawa et al., 2009; Neal et al., 2007, 2010; Sobinoff et al., 2011, 2012b). PAHs are a ubiquitous group of over 100 chemical pollutants formed by incomplete combustion, with cigarette smoking being a major source of human exposure (Lodziwicz et al., 2004). These chemicals are bioactivated into more reactive metabolites primarily within the liver, and to a lesser extent in the ovary, via aryl hydrocarbon receptor (AHR) mediated activation of the cytochrome P450 family of phase I detoxification enzymes (Otto et al., 1992; Shimada et al., 2003) (Fig. 2). P450 family members include the ubiquitously expressed cytochrome P450 1A1 and cytochrome P450 1B1. This bioactivation by enzymes of the cytochrome P450 family and others appears to be the primary cause of follicle loss and ovarian damage following PAH exposure (Igawa et al., 2009; Rajapaksika et al., 2007; Ramesh et al., 2010; Shimada et al., 2003).

The alkaloid nicotine, the major addictive compound in cigarettes has been found to negatively affect embryo implantation, oocyte quality and ovulation (Baldwin and Racowski, 1987; Maithes et al., 2000; Racowski et al., 1989). Metabolism of nicotine into its metabolites, such as cotinine, again mainly occurs in the liver, but via a different array of cytochrome P450 enzymes, including CYP2A6, CYP2A6 and CYP2B (Higashi et al., 2007). Intriguingly, ovarian expression of both CYP2A6 and CYP2B1 has also been detected (Cannady et al., 2003; Higashi et al., 2007; Malaiyandi et al., 2005). To a lesser extent the effects of cotinine on fertility have also been studied, as its in vivo half-life of 16 h is significantly greater than that of nicotine, at 2 h (Malaiyandi et al., 2005; Sanders et al., 2002; Vrnsanski et al., 2003).

Other chemicals found within cigarette smoke that appear to influence fertility include the heavy metal cadmium and the organic compounds anabasine, pyrazine, 2′-ethylpyridine (2-EP), and 3′-ethylpyridine (3-EP) (Vrnsanski et al., 2003; Wu and Liu, 2012).

Studies on the effect of smoking on human fertility

Most data regarding the effect of smoking on female fecundity are derived from studies of assisted reproductive technology (ART) patients, and reports present conflicting results. Several studies have found smoking decreases the number of oocytes retrieved for ART (El-Nemr et al., 1998; Fuentes et al., 2010; Zenzes et al., 1995). Those that are collected have a reduced ability to be fertilised, and subsequent embryo quality is decreased (B-Nemr et al., 1998; Gruber et al., 2008; Neal et al., 2005; Shiloh et al., 2004; Weigert et al., 1999). This has been proposed to ultimately lead to increased implantation failure, reduced clinical pregnancies, and decreased live birth rates (Benedict et al., 2011; Neal et al., 2005). However, other studies have found no alteration to oocyte number, fertilisation, embryo quality, clinical pregnancy and birth rates in smokers when compared to age matched controls (Cinar et al., 2014; El-Nemr et al., 1998; Gruber et al., 2008; Weigert et al., 1999; Wright et al., 2006).

A small number of human studies have also examined oocyte, cumulus cell and follicular fluid quality in smokers. Two consecutive studies by the same group found that smoking increases the number of meiotically immature oocytes retrieved, potentially decreasing fertilisation rates (Zenzes et al., 1995, 1997). Oocytes from smokers were found to have increased zona pellucida thickness, though the mechanism by which this occurs remains obscure (Shiloh et al., 2004). Cumulus cells collected from smoking women were also found to be abnormal, with increased levels of DNA damage (Snikó et al., 2005). Additionally, follicular fluid from smokers possessed higher levels of lipid peroxidation and reduced antioxidant potential (Paszkowski et al., 2002).

A growing body of evidence also suggests that smoking lowers the age of menopause in otherwise healthy women (Hanck et al., 2004; Cooper et al., 1999; Hayatbakhsh, et al., 2012; Jeck et al., 1977; Kinney et al., 2006; McKinlay et al., 1985; Midebar and Baron, 1990; Saruç et al., 2010; Sun et al., 2012; Willett et al., 1983). At least 3 studies have identified that active smoking lowers the age of natural menopause by 1–3 years (Hayatbakhsh et al., 2012; Kinney et al., 2006; McKinlay et al., 1985; Saruç et al., 2010). Interestingly, other studies have shown that menopause onset is the same in ex-smokers and women who had never smoked, suggesting that smoking cessation may be able to restore ovarian health and fertility in woman (Cooper et al., 1999; Hayatbakhsh et al., 2012; Kinney et al., 2006; Mikkelsen et al., 2007; Willett et al., 1983). However, it is likely, that despite a return to normal menopause onset in ex-smokers, ovarian damage could persist, particularly in oocytes due to their non-renewable nature, resulting in reduced fertility.

Conflicting results on the effect of smoking on human fertility is not unexpected. Many of these studies were performed as observational and/or retrospective studies only, and on a subgroup of subjects, ART patients. Therefore confounding factors are likely to have arisen due to human memory deficits or other infertility issues associated with ART patients. Additionally, single nucleotide polymorphisms could alter an individual’s ovarian response to cigarette smoke. This is highlighted by two studies that identified polymorphisms in genes associated with oestrogen modification, metabolism and free radical scavenging increased ovarian cancer risk, with cigarette smoking further elevating this risk (Goodman et al., 2001; Lurie et al., 2008). Furthermore the limited nature of human cell and tissue samples represents a difficulty for elucidation of molecular mechanisms underpinning ovarian functional changes. In an effort to address these limitations, several animal in vitro and in vivo models have been developed and are discussed below.

Oocyte, follicle and ovarian cell cultures: in vitro testing of ovarian cytotoxicity

In vitro culture of follicles, oocytes and isolated ovarian somatic cells, represent simple methods used to examine the direct effects of cigarette smoke constituents on fertility. These culture systems are well defined in rodent models and allow for toxicant exposure at very specific developmental time points. Additionally, ovarian cell and follicle cultures allow the mechanism of action to be more easily pinpointed in relation to the follicle/oocyte stage affected and the defined outcomes such as...
hormone production or apoptosis. Two distinct forms of follicle culture are most often used: 1) those involving mechanical removal of pre-antral follicles with theca cells intact that allow follicular staging and the study of ovulation in vitro and 2) enzymatically isolated primordial follicles consisting of granulosa cells and oocytes which allow oocyte-granulosa cell interactions to be observed (Cortvriendt and Smits, 2002). Gross morphological examination of rat and mouse pre-antral follicles cultured in either cigarette smoke condensate (CSC cigarette smoke trapped on filter paper and dissolved in DMEM) or the PAH benzo[a]pyrene (BaP) revealed altered size and growth characteristics from pre-antral to antral stages (Neal et al., 2007, 2010; Sadeu and Foster, 2011a,b). Follicle survival and cell viability were also reduced in both mouse and rat models exposed to PAHs (BaP and 7,12 dimethylbenz[a]anthracene (DMBA)) with an increase in proapoptotic markers Bax, activated caspase 3, Hsp90ab1 and DNA fragmentation (Neal et al., 2010; Sadeu and Foster, 2011b; Tsai-Turton and Luderer, 2006). Additionally, DMBA cultured rat follicles displayed an increase in oxidative stress-induced apoptosis while co-culture with glutathione, a naturally occurring ovarian antioxidant prevented cell death (Tsai-Turton and Luderer, 2006). In contrast, a second similar BaP study in a mouse culture system found no effect on antral follicle formation (Sadeu and Foster, 2013).

Follicular production of the hormones estradiol (E2) and anti-Müllerian hormone (AMH) is important for follicle growth, development and ovulation and therefore any alterations in hormone output can potentially affect not only the source follicle, but also surrounding follicles. In particular, E2 induces follicle stimulating hormone (FSH) and LH receptors in granulosa cells which are essential for follicle maturation (Brett et al., 2004; Richards et al., 1976; Salmon et al., 2004). CSC has been shown to decrease E2 production in pre-antral mouse follicles yet increase FSH in pre-ovulatory follicles (Sadeu and Foster, 2011a). E2 output was also decreased in rat and mouse antral follicles exposed to BaP alone (Neal et al., 2010; Sadeu and Foster, 2011b). This reduction in E2 production in pre-antral follicles following direct exposure to cigarette constituents, was further supported in a model examining cultured bovine granulosa cells which displayed decreased E2 output when exposed to nicotine but not its metabolite cotinine (Sanders et al., 2002). AMH production was also found to be altered in BaP treated murine follicles, which was predicted to be an indicator of reduced follicular functionality (Neal et al., 2010; Sadeu and Foster, 2011b).

Findings from another whole culture follicle study using BaP as the toxicant, revealed an increase in mRNA for bioactivation and phase I detoxification enzymes cytochrome p450 and cypp1b1 in mouse follicles (Sadeu and Foster, 2013). These data suggest that ovarian follicles are able to directly perform at least some bioactivation of PAHs into their more damaging metabolites. Direct bioactivation in somatic cells could indicate a mechanism for reduced oocyte quality in smokers.

The effect of cigarette constituents has also been examined directly on denuded oocytes from preovulatory follicles and enzymatically-isolated cumulus-oocyte complexes (OCs) (Racowsky et al., 1989; Vrnsaská et al., 2003; Wu and Liu, 2012; Zenes and Belecki, 2004). Maturation of porcine and mouse OCs in the presence of pyrazine, 2-EF and 3-EF combined, nicotine, cadmium or anabasine retarded both cumulus cell expansion and oocyte growth while cotinine had no effect (Vrnsaská et al., 2003; Wu and Liu, 2012). Maturation was also found to be altered in hamster and mouse oocytes cultured in nicotine (Racowsky et al., 1989; Zenes and Belecki, 2004). Racowsky et al. (1989) established that nicotine caused metaphase I blockage and disrupted proper chromosome segregation at anaphase I in hamster oocytes. Another study of nicotine treated mouse oocytes also identified altered metaphase I spindles and chromosome alignment leading to the majority of oocytes failing to progress past metaphase I. The few oocytes able to complete meiosis I were also altered with abnormal chromosome distribution, large polar bodies and multiple metaphase II spindles observed (Zenes and Belecki, 2004). These oocyte alterations have the potential to lead to aneuploid conceptions, with the majority of these errors occurring at meiosis I. Furthermore, increased incidence of oocyte aneuploidy is associated with maternal age (Jones and Lane, 2013), with cigarette smoking possibly replicating this phenomenon.

Ovarian explant culture: effects of smoking constituents

Ovarian explant culture has advantages over isolated follicle culture, as it mimics the effects of the metabolism of the constituents by all ovarian cells. Additionally it allows normal interaction between the follicle and the ovarian stromal cells. Mouse and rat neonatal ovaries have been cultured in the PAH BaP, 3-methylcholanthrene (3-MC), DMBA, and its metabolite DMBA-3,4-diol (Ganesan et al., 2013; Ganesan and Keating, 2014: Igawa et al., 2009; Sobinoff et al., 2011, 2012b,c; Tuttle et al., 2009). Loss of primordial and primary follicles occurred following in vitro treatment of rat ovaries with DMBA and DMBA-3,4-diol (Igawa et al., 2009). Conversely, 3-MC treatment resulted in an increase in primary follicle numbers in mouse ovaries, with a corresponding decrease in primordial follicles (Sobinoff et al., 2012b). Further investigation revealed that 3-MC treated ovaries demonstrated enhanced primordial to primary transition rates, which was suggested to be associated with the PI3K/Akt and mTOR pathways (Sobinoff et al., 2012b). Such a mode of ovotoxicant-induced follicle depletion was first identified in neonatal rat ovaries following treatment 4-vinylcyclohexane (Keating et al., 2009). Sobinoff et al. (2012b) went on to suggest that the increased primordial/primary transition was occurring to replace the later stage follicles, secondary and antral, that were undergoing elevated levels of atresia (Fig. 3). Two further mouse studies revealed that BaP and DMBA treatment also caused similar primordial follicle activation compensation with reduced primordial and growing follicles but increased primary follicles (Sobinoff et al., 2011, 2012c). These studies also found that the pro-apoptotic markers, activated caspases 2 and 3, and DNA fragmentation were elevated solely in growing follicles and were absent from primordial follicles, suggesting a specific targeting of later stage follicles by PAHs (Sobinoff et al., 2011, 2012b,c) (Fig. 4).

In agreement with these findings, studies by another group demonstrated that whole rat neonatal ovaries exposed to DMBA showed a marked increase in γH2AX in primary and secondary follicles (Ganesan et al., 2013). Additionally, DNA repair mechanisms were activated with an increase in ATM, Xrc6, Brcc1, Parp1 and Rad51 protein expression (Ganesan et al., 2013). DMBA treated rat ovaries also displayed a decrease in gap junction proteins connexin 37, and connexin 43. These proteins are essential for granulosa cell-oocyte and granulosaintracellular cell interactions, respectively, and their loss represents another potential cause of DMBA-associated follicular atresia (Ganesan and Keating, 2014).

Although the studies described above have yielded interesting insights into dynamics of the primordial and growing follicles in response to toxicants in cigarette smoke, it is important to note that these studies were performed on neonatal rodent ovaries. This is of particular importance as a growing body of evidence suggests that the primordial follicles that active shortly after birth are distinct from those that activate later, during adulthood (Zhang et al., 2014). The alterations observed therefore may not entirely reflect what occurs in the primordial follicles of adult ovaries. Note also that in many of these studies, both cell and ovary cultures, concentrations of tested compounds were at levels several times higher than those found in cigarette smokers and therefore interpretation of these findings must be considered with caution.

Direct systemic exposure to individual cigarette toxicants

Treatment of whole animal models with the individual constituents of cigarette smoke is another method commonly used to elucidate their
effects on ovarian health. The advantage of these models is their ability to incorporate metabolism of the chemicals by organs other than the ovary, such as the liver, against the backdrop of the hypothalamic-pituitary axis. Whole animal models also avoid potential artifactual changes that may occur as a result of the in vitro culturing process, such as increased oxidative stress (Halliwell, 2003). Intraperitoneal injection remains the most commonly employed method for substance administration; however, subcutaneous and intraovarian injections, and oral exposure have also been utilised.

In terms of changes to follicular pool dynamics, encouragingly: some in vitro observations have been replicated following in vivo exposure to different constituents of cigarette smoke. Intraperitoneal injections of neonatal mice to three different PAHs: \( \text{Ba}[\text{P}] \), \( 3\text{-MC} \) and \( \text{DMBA} \) demonstrated a markedly similar pattern of follicle pool dynamics to those observed in ovarian culture studies. A common finding was a decrease in the population of primordial follicles, with a corresponding increase in primordial–primary transition. Additionally, both primary and secondary follicle numbers were elevated, with a reduction in preantral and total follicle numbers (Sobinoff et al., 2011, 2012b,c). A reduction in total follicle numbers was also detected in both adult rats and mice exposed to \( \text{DMBA} \) and \( 3\text{-MC} \) (Shiromizu and Mattison, 1985; Ting and Pyttoff, 2010). Conversely, subcutaneous injection of \( \text{Ba}[\text{P}] \) and its sister compound \( \text{benz}[a]\text{anthracene} \) (\( \text{Ba}[\appa] \)) into neonatal rats caused an increase in antral follicles, suggesting increased follicle activation. However, the same study found that the PAH benzo[k]fluoranthene (\( \text{BkF} \)) had no effect on antral follicle numbers (Kummer et al., 2013). Further studies in adult mice and rats exposed to nicotine found no change in primordial follicles, but a decrease in primary, secondary and antral follicles, suggesting the demise of follicles during growth (Mohammadghasemi et al., 2012; Patil et al., 1998, 1999). As a result of these alterations to folliculogenesis, ovulation rates were decreased, with corpora lutea numbers reduced (Mailhes et al., 2000; Mohammadghasemi et al., 2012; Patil et al., 1998, 1999). A similar reduction in corpora lutea was found in \( \text{Ba}[\text{P}] \) exposed mice (Swartz and Mattison, 1983). Sobinoff et al. (2012c) also established that the quality of ovulated oocytes was decreased in adult mice exposed to \( \text{Ba}[\text{P}] \) neonatally. Oxidative stress was elevated and a decrease in sperm–egg binding and sperm–egg fusion observed (Sobinoff et al., 2012c). Furthermore exposure of mice to nicotine altered oocyte maturation and increased the incidence of premature sister chromatid separation at metaphase II, potentially increasing aneuploidy (Mailhes et al., 2000). These results indicate that different constituents of cigarette smoke can decrease both ovarian follicle numbers and quality, which has the potential to reduce the number of ovulated oocytes. As primordial follicle numbers are an indicator of ovarian reserve and reproductive lifespan, a decline in this follicle pool is concerning.
pool could lead to premature ovarian failure. Additionally, the remaining oocytes that are ovulated are likely to be of reduced quality, with decreased capacity for fertilisation (Mailhes et al., 2000; Sobinoff et al., 2012c).

Changes in ovarian hormone production and responsiveness, particularly that of $E_2$, have been implicated in altered ovarian function of animals exposed to cigarette smoking constituents. Nicotine exposure in adult mice reduced serum $E_2$, confirming similar findings in follicle and cell culture systems (Mohammadghasemi et al., 2012; Neal et al., 2010; Sadeu and Foster, 2011a,b; Sanders et al., 2002). This alteration is further supported with an increase in ovarian cholesterol, the $E_2$ precursor molecule, and decrease in glycogen, a polysaccharide stored in tissue under the influence of $E_2$, observed in similarly treated rat and mouse ovaries (Patil et al., 1998, 1999).

Ovarian responsiveness to this hormone may also be decreased, with exposure to PAHs $B[a]P$, $BaA$ and $BaF$ completely or partially preventing normal $E_2$ stimulation of follicle growth (Kummer et al., 2013). Further investigation of these rats found that this altered response, was in part due to reduced oestrogen receptor beta expression on both granulosa and theca cells (Kummer et al., 2013). Therefore it is possible that smoking results in a negative feedback cycle whereby follicle response to $E_2$ is attenuated such that further follicular production of $E_2$ is then limited thereby preventing further follicle growth and ovulation.

Despite having advantages over culture methods, direct exposure of animals to cigarette constituents has limitations. As with in vitro methods, achieving an appropriate human dose can be difficult to determine, given the relatively long life span of humans compared

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*Fig. 4. Neat ovarian exposure to smoking constituents display increased apoptosis. Immunofluorescence of neat ovaries exposed to $B[a]P$, DMBA and 3-MC revealed an increase in caspases 2 and 3 and DNA fragmentation, indicating an increase in apoptosis. These apoptotic markers were found to be absent from primordial follicles but present in later stages of follicle development; scale bar is equal to 100µm. Images published with permission from Sobinoff et al. (2011, 2012b,c).*
to rodents. Additionally, the administration routes do not mimic human exposure and could potentially alter pharmacokinetics of smoke constituents. Finally, examination of individual chemicals in isolation both in vitro and in vivo fails to take into account any potential interaction that could occur between the many constituents of cigarette smoke. This was highlighted by Wu and Liu (2012) who found that a combination of pyrazine, 2-EP, and 3-EP in culture was needed to alter oocyte maturation with individual chemical culture having no effect.

**Inhalation models of cigarette smoke exposure**

Recently mouse inhalation models of cigarette smoke exposure have been used to fully elucidate the negative impact smoking has on ovarian health (Gannon et al., 2012, 2013; Jennings et al., 2011; Sibinoff et al., 2013; Tuttle et al., 2009). These models have distinct advantages over those previously discussed as they allow exposure to all cigarette smoke components simultaneously via the nasal route, thus closely mimicking human exposure. Two models have been developed: whole body smoking (WB-S) and nose-only smoking (NO-S). Both models have been widely used to study the effect of smoking on non-reproductive organs, particularly the lungs, with over 155 studies on chronic obstructive pulmonary disease alone (Leberl et al., 2013). Of these two methods, nasal exposure most accurately mimics direct human exposure, with WB-S akin to secondary smoke exposure.

In WB-S, one or more animals are placed within a chamber that is filled with cigarette smoke allowing primarily nasal exposure, but also secondary oral and dermal exposure (Gannon et al., 2012, 2013; Wong, 2007) (Fig. 5A). In two consecutive studies by Gannon et al. (2012, 2013) mice were smoked twice daily, 5 days a week for 8 weeks. Tuttle et al. (2009) smoked the animals twice daily, 7 days a week for 8 weeks. Whole animal general health was unchanged with all three studies observing no difference in porphyria or lacrimation (Tuttle et al., 2009). Despite this, Gannon et al. (2012, 2013) but not Tuttle et al. (2009) observed a decrease in body weight. Ovaries from smoke-exposed animals were significantly smaller and had fewer primordial follicles (Gannon et al., 2012; Tuttle et al., 2009), however conflicting effects upon growing follicles were observed, with smoke exposure 5 days/week resulting in a decrease in total number while 7 days/week caused no change to the follicular pool (Gannon et al., 2012; Tuttle et al., 2009). Notwithstanding these findings, apoptosis in the ovary was consistently unchanged in WB-S animals, with no alteration in the pro-apoptotic markers Bax, activated caspase 3, caspase 8, Fas, FasLor DNA fragmentation (Gannon et al., 2012, 2013; Tuttle et al., 2009). As such, autophagy was proposed as the most likely method of follicle loss (Gannon et al., 2012; Tuttle et al., 2009). Supporting this hypothesis was the marked increase in autophagosomes, levels of the pro-autophagy markers Beclin 1 and light chain protein 3 (LC3), and decrease in the autophagy inhibitor Beclin 2, in granulosa cells (Gannon et al., 2012, 2013; Tuttle et al., 2009). Mitochondrial quality was also impacted, with increased mitochondrial fragmentation and degradation detected. Additionally mitochondrial specific antioxidant enzyme superoxide dismutase 2 (SOD2) levels were reduced and heat shock protein 25, a cellular defence against oxidative stress, elevated, indicating a potential increase in mitochondrial induced ROS leakage and oxidative stress (Gannon et al., 2013).

Nose-only treatment involves placing animals into individual tubes allowing smoke to be blown in puffs directly into the nose (Fig. 5B). NO-S is more labour intensive then WB-S and requires animals to be restrained (Wong, 2007). However, its advantage is in reducing dermal and oral exposure and therefore mimics human cigarette smoking more accurately (Wong, 2007). Two studies have examined fertility using the same smoking procedure with animals NO-S exposed twice daily, 5 days a week for 12–18 weeks (Jennings et al., 2011; Sibinoff et al., 2013). Lungs from these animals showed evidence of chronic obstructive pulmonary disease, indicating a smoke induced disease state (Jennings et al., 2011). In agreement with the above WB-S studies, ovaries were significantly smaller with a reduction in both primordial and primary follicle populations and subsequent decrease in the number of oocytes ovulated (Sibinoff et al., 2013). In contrast to WB-S, NO-S ovaries demonstrated an up-regulation of pro-apoptotic markers activated caspases 2 and 3 and DNA fragmentation, but no change in autophagy markers Beclin 1 and LC3 (Gannon et al., 2013: Sibinoff et al., 2013).

Similar to the other experimental models previously discussed (e.g. follicle and ovarian cultures), NO-S caused an increase in total ovarian and oocyte oxidative stress (Gannon et al., 2013; Sibinoff et al., 2012c; 2013; Tazi-Turtun and Iuderer, 2006). Oocytes displayed elevated lipid peroxidation and increased mitochondrial superoxide leakage, supporting the decrease in SOD2 observed in the WB-S model (Gannon et al., 2013: Sibinoff et al., 2013). Further investigation of oocyte quality revealed metaphase II spindle abnormalities, which could affect fertilisation (Jennings et al., 2011). Surprisingly, zona pellucida thickness was also increased, which has also been observed in the oocytes of human smokers, with a decrease in successful in vitro sperm-oocyte interaction also found (Jennings et al., 2011; Shiloh et al., 2004: Sibinoff et al., 2013).

**Conclusions**

It is widely known that cigarette smoking is linked to a number of diseases and pathologies, including decreased fertility. Human studies have shed some light on this phenomenon, however as the bulk of these studies are performed retrospectively or on ART patients, these findings can be compromised by numerous confounding factors. In an effort to truly understand how smoking affects female fertility, a variety of in vitro and in vivo animal models have been developed. Each of these models has its strengths and weaknesses. Cell and organ cultures are relatively easy and allow for fast turnover with high end numbers. Despite this, artifactual results may occur since culturing can alter cellular structure, function and smoking-constituent metabolism. Furthermore, the majority of these culture studies were performed using individual chemicals. The use of single compounds is useful for identifying their individual modes of ovotoxicity. However, as cigarette smoke contains thousands of different toxicants the use of models that allow exposure to all the chemical constituents are needed to determine the true physiological impact of smoking. Additionally, selecting doses of chemicals that correspond to relative human exposure can be a problem. The solution to many of these issues is the use of in vitro models – particularly nasal inhalation as it is the most accurate mimic of human smoking.

Collectively, the results of both animal and human research models examined in the last decade indicate that cigarette smoking and exposure to smoke constituents have a negative effect on female fertility, with an increase in follicle death and altered hormone output the most universal findings. Interestingly, human studies suggest that cessation of smoking can extend the age of natural menopause and potentially improve ovarian quality. In summary, studies to date strongly suggest that women of reproductive age should be encouraged not to smoke, or cease smoking, in order to preserve their fertility. Future studies using those models that best mimic human exposure, such as the nasal smoking model, are likely to provide detailed insights into the molecular mechanisms underlying loss of fertility following smoke exposure.

**Conflict of interest**

The authors declare that they have no conflict of interest.
Fig. 5. Whole body smoking (A) – One or more mice are placed within the exposure chamber on a vented base. Cigarette smoke is pumped into the chamber via the smoke inlet, causing whole body smoke exposure; nasal, dermal and oral. The smoke is then allowed to escape via the chamber exhaust, found under the vented base. Nose only smoking (B) – Individual mice are placed within tubes and held in place by a restraint at the rear of the mouse. Cigarette smoke is pumped into the smoke inlet in puffs, where the head chamber allows for both inhalation and exhalation of the smoke. This method allows for nose-only exposure to cigarette smoking, eliminating oral and dermal exposure.

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CHAPTER 2

Maternal Smoke Exposure Impairs the Long-Term Fertility of Female Offspring in a Murine Model

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Note: Supplementary figures are located in Appendix A, Pg 143.
Statement of Contribution

N.J.C. and A.P.S. contributed equally to the conception and design of the study, the acquisition of data and the analysis/interpretation of data. N.J.C was also responsible for drafting and revising the manuscript. J.M.S. contributed to data acquisition. The group of P.M.H including E.L.B, R.L.V. and A.G.J. performed the cigarette smoke exposures. P.M.H., E.A.M. and J.E.H., contributed to the conception and design of the study.
Chapter 2: Overview

Chapter 1 established that direct exposure to cigarette smoke leads to a reduction in female fertility, which can be partially restored upon smoking cessation, when exposed during adult life (Camlin et al., 2014). We next sought to determine what effect cigarette smoking could have on the developing fetal and neonatal mouse ovary and subsequent adult fertility. Therefore, the aim of the following manuscript was to establish a link between in utero/lactational exposure to cigarette smoke (maternal smoke exposure) and reduced female fertility in adult life.

A growing body of evidence suggests that in utero exposure to cigarette smoke or its constituents reduces female fertility. However, due to the nature of human epidemiological studies’ conflicting results have been reported on the adverse effects of maternal smoke exposure on the age of menarche and/or menopause and female offspring fecundability (Ernst et al., 2012; Jensen et al., 2006; Strohsnitter et al., 2008). To address this conflict and establish the potential mechanisms underpinning reduced fertility, this study employed a novel nose only smoke inhalation method, which mimics human cigarette smoking, during pregnancy and lactation.

Data reported within this manuscript strongly suggests that exposure of females to cigarette smoke during fetal/neonatal development reduces fertility in adult life. This subfertility is linked to a decline in ovarian folliculogenesis and oocyte quality, cumulating in an increased time to pregnancy and reduced litter size.
Maternal Smoke Exposure Impairs the Long-Term Fertility of Female Offspring in a Murine Model

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ABSTRACT

The theory of fetal origins of adult disease was first proposed in 1989, and in the decades since, a wide range of other diseases from obesity to asthma have been found to originate in early development. Because mammalian oocyte development begins in fetal life it has been suggested that environmental and lifestyle factors of the mother could directly impact the fertility of subsequent generations. Cigarette smoke is a known toxicant in active smokers, yet disturbingly 13% of Australian and 12% of US women continue to smoke throughout pregnancy. The focus of our investigation was to characterize the adverse effects of smoking on ova and oocyte quality in female offspring examined in early development. Pregnant mice were nasally exposed to cigarette smoke for 12 wk throughout pregnancy/lactation, and ova and oocyte quality of the F1 (maternal smoke exposed) generation was examined. Neonatal ovaries displayed abnormal somatic cell proliferation and increased apoptosis, leading to a reduction in follicle numbers. Further investigation found that altered somatic cell proliferation and reduced follicle number continued into adulthood; however, apoptosis did not. This reduction in follicles resulted in decreased oocyte numbers, with these oocytes found to have elevated levels of oxidative stress, altered metaphase II spindle, and reduced sperm–egg interaction. These ovarian and oocyte changes ultimately lead to subfertility, with maternal smoke-exposed animals having smaller litters and also taking longer to conceive. In conclusion, our results demonstrate that in utero and lactational exposure to cigarette smoke can have long-lasting effects on the fertility of the next generation of females.

cigarette smoke, in utero, oocyte, ovary, subfertility

INTRODUCTION

In utero exposure to cigarette smoke has been linked to adverse health effects in offspring, including an increased risk of premature birth, fetal growth retardation, and morbidity, and in later life obesity, asthma and congenital heart defects [1–4]. This is of particular concern, because despite increased public awareness of the adverse effects of smoking during pregnancy, 11%–14% of women at reproductive age (20–45 y) in Australia smoke daily [5]. Furthermore ~13% of Australian women and ~12% of women in the United States continue to smoke throughout their pregnancy [6, 7]. Furthermore, a growing body of evidence suggests that gestational exposure to cigarette smoke negatively affects the fertility of female offspring, leading to early-onset menarche, reduced fecundability, and premature menopause [8–13]. A number of studies examining human ovaries have explored the potential mechanism underlying such altered fertility during early ovarian development (5–21 wk gestation); however, longer-term studies in humans have been limited [14–16].

Mammalian females are born with a finite number of germ cells that begin their development during fetal life [17]. At birth, these germ cells, known as “oocytes,” are meiotically arrested at prophase I within primordial follicles, with their number dictating the reproductive lifespan of an individual [18, 19]. These quiescent primordial follicles consist of an immature oocyte surrounded by a single layer of granulosa cells. As folliculogenesis continues the number of granulosa cell layers increases, with these somatic cells providing important factors, such as inhibin, to the developing oocyte [20]. Once fully matured, a surge of luteinizing hormone causes ovulation and releases the oocyte from its prophase I arrest [21]. It is important to note, however, that most follicles are destined to die, with only a select few reaching ovulation, making them a precious resource.

In vivo and in vitro exposure of human and mouse fetal ovaries to cigarette smoke or its constituents nicotine, DMBA-DHD, DMBA, or BaP has been found to negatively affect oocyte and follicle development by altering proliferation and apoptosis of germ and granulosa cells [14, 15, 19, 22–24], and modifying hormone production of estrogen, progesterone, and inhibit [14, 25]. In this study we used an animal model to examine the effects of in utero/lactational exposure to whole
cigarette smoke on female fertility using our novel nose-only inhalation model [26–31]. Exposure of mice to cigarette smoke via this nose-only inhalation model for 8 wk leads to smoke-induced chronic obstructive pulmonary disease, which is evident in humans after years of cigarette smoking [26]. Our investigation focuses on both the short-term and long-term consequences of maternal cigarette exposure (MSE) on an offspring’s ovarian and oocyte quality, and ultimately their fertility.

MATERIALS AND METHODS

All reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Animals and Ethics Approval

All experiments were performed with the approval of the University of Newcastle Animal Care and Ethics Committee. C57BL/6 mice were obtained from Australian BioResources and housed with ad libitum food and water under a 12L:12D lighting regimen.

Smoke Exposure

Six-week-old female mice were exposed to smoke as previously described [26, 29, 31]. Briefly animals were nasally exposed to mainstream cigarette smoke (twelve 3R4F reference cigarettes; University of Kentucky) for 75 min twice daily, 5 days a week for 12–13 wk, in individual mouse chambers, preventing passive smoking of breeding males and pups. On Week 5 females were housed with non-cigarette smoke-exposed males (two females per male) until visibly pregnant (abdominal bulge observable from approximately Gestational Day 14). Smoke exposure continued throughout pregnancy and lactation, ceasing once pups (F1 generation) were weaned on Postnatal Days 21–23 (PNDs 21–23) [28].

Fertility Trial

Four F1 females (age 6 wk) per treatment group were housed with non-cigarette smoke-exposed males (age 8 wk) continuously. Date of birth and size of litters were recorded during a 3-mo period. Conception date was determined by subtracting 19 days from time of litter drop.

Follicle Counts and Staging

Three Bouin-fixed PND 6, 8- to 9-wk-old, and 9-mo-old ovaries per group were paraffin embedded and sectioned at 5 µm prior to hematoxylin and eosin staining. The PND 6 follicle counts were performed on every fifth section for half the ovary. The 8- to 9-wk-old and the 9-mo-old follicle counts were performed on every 15th section for the entire ovary. Only follicles with a visible oocyte nucleus were counted. The PND 6 follicles were staged through oocyte size. Oocyte diameters were measured using ImageJ software (freeware; National Institutes of Health); those with a diameter of < 15 µm were classed as primordial, 15–20 µm as activated follicles, and > 20 µm as primary follicles. Morphological features were used to stage follicles in adult ovaries. Briefly, primordial follicles have a single layer of squamous pregranulosa cells surrounding the oocyte. Primary follicles are characterized by a single layer of cuboidal granulosa cells. By secondary stage, a zona pellucida (ZP) has formed around the growing oocyte, which is surrounded by multiple layers of granulosa cells with a theca externa. Finally, a follicle fluid-filled antral cavity forms in antral follicles [19].

Immunofluorescence

Three Bouin-fixed PND 3, PND 6, 8- to 9-wk-old, and 9-mo-old ovaries per treatment were deparaffinized and rehydrated before antigen retrieval in Tris buffer (50 mM, pH 10.6). Sections were blocked in 3% bovine serum albumin (BSA) before incubation with primary antibodies active caspase 3 (1:200; ab13847, Abcam) for 1 h at room temperature or proliferating cell nuclear antigen (PCNA; 1:100; NA03; Merck Millipore) and AMH (1:20; MCA2246; AbD Serotec) overnight at 4°C. The appropriate secondary antibody conjugated to Alexa 594 (1:200; Life Technologies) was applied for 1 h at room temperature. Slides were again washed and counterstained with DAPI before mounting in citifluor (Citifluor Ltd.). An Axio Imager A1 epifluorescent microscope (Carl Zeiss MicroImaging Inc.) was used to visualize sections, with photomicrographs taken using an Olympus DP70 microscope camera (Olympus America). Fluorescence intensity analysis was performed using ImageJ on ovaries from at least three animals, across three replicates, as previously described by McClay et al. [32].

TUNEL Analysis

Sections were treated with 20 µg/ml proteinase K (Promega) prior to TUNEL analysis using ApopTag Fluorescein In Situ Apoptosis Detection Kit (S7110; Merck Millipore) according to the manufacturer’s protocol. DAPI was used to counterstain sections before mounting in citifluor (Citifluor) and imaging on an Axioscope A1 epifluorescent microscope with an Olympus DP70 microscope camera. Total cells and TUNEL-positive cells were counted using ImageJ and used to calculate the proportion of TUNEL-positive cells.

Metaphase II Egg Collection and Fixation

Female mice were intraperitoneally injected with 7.5 IU of pregnant mares’ gonadotropin (Intervet), followed by 5 IU of human chorionic gonadotropin (Intervet) 48 h later. Metaphase II arrested (MII) eggs were collected 12 h later from the ampulla into M2 media with BSA containing hyaluronidase (H4272) to remove cumulus cells. Oocytes were washed into M2 containing monastrol (200 µM; M8515) for 2 h to collapse the spindle for anxiously analysis or were fixed with 4% paraformaldehyde in PBS with 0.5% Triton X-100 for 30 min [33]. Following monastrol treatment, oocytes were fixed as described above.

Germinal Vesicle Oocyte Collection and In Vitro Maturation

Oocytes were dissected from 9-mo-old females, with one ovary per animal used for germinal vesicle (GV) oocyte collection. Briefly, preantral follicles were punctured with a 33.5-gauge needle, releasing cumulus-oocyte complexes into M2 with BSA and milrinone (M4659). Oocytes were denuded of cumulus cells, washed into MEM media (11900-024; Life Technologies), and allowed to undergo in vitro maturation in 5% CO2 at 37°C. After 16 h oocytes were scored for the presence of a polar body (MII oocytes), with MII oocytes treated with monastrol and fixed as described above.

Oocyte Oxidative Stress Determination

Oocyte lipid peroxidation and mitochondrial superoxide leakage were analyzed as previously described by Sobinoff et al. [27] on oocytes from three animals per treatment. Briefly, MII eggs were collected from 8-wk-old F1 females and incubated in either 10 mM 4,4-difluoro-5-(4-phenyl-1,3-buta dieny1)-4-bora-3a,4a-diazole-3-undecanitrile acid (581/591 C11 (BODIPY, D-22728; Life Technologies) or 5 mM MitoSOX red stain (M36008; Life Technologies) for 20 or 30 min, respectively. Positive control eggs were obtained by treatment with 5 µM menadione (MEN) prior to BODIPY or MitoSOX red exposure. Eggs were washed into M2, mounted, and imaged with an LSM510 laser-scanning microscope (Carl Zeiss). Lipid peroxidation was determined by the shift in BODIPY fluorescence from red to green (red-green ratio). Superoxide leakage was indicated by increasing MitoSOX red fluorescence.

Oocyte Immunocytochemistry

Immunocytochemistry was performed on oocytes from three animals to four animals per treatment using anti-α-tubulin (1:400; A11126; Life Technologies) or anti-CREST (1:400; 90C-CSI058; Bioclonel Australia) overnight at 48°C after blocking in 7% goat serum/PBS-0.1% Tween-20. Oocytes were washed through 1% BSA in PBS-0.1% Tween-20 prior to incubation with secondary antibodies conjugated with Alexa 555 or Alexa 633 (1:1000; Life Technologies). Oocytes were counterstained in Hoechst (20 µg/ml) and mounted in citifluor. Imaging was performed on an Olympus FV1000 using a 60X/1.2 N.A. UPLSAPO oil immersion objective lens (Olympus). ImageJ was used to measure spindle size and count kinetochores using a macro designed by Dr. Simon R.R. Lane [34]. For ZP analyses, four measurements were taken from the transmission image of each egg to calculate mean thickness (Supplemental Fig. S1; Supplemental Data are available online at www.bioreprod.org), as previously described by Jennings et al. [35].

Sperm-Egg Binding Assays

Freshly collected MII eggs were examined for sperm-zena and sperm-oolemma binding as previously described by Sobinoff et al. [27] on three
animals per treatment. Briefly, MI oocytes were collected in M2 media with hyaluronidase to remove cumulus cells. For sperm-oolemma binding assays, the zona was removed from eggs using Acid Tyrode solution (TT1788). Sperm was collected from the cauda epididymides of a mature untreated male mice and morphology analyzed. Morphologically normal samples were allowed to capacitate for 3 h at 37°C. Eggs with or without their ZP were incubated with capacitated sperm at 2 X 10^6 sperm per milliliter in M2 medium for 15 min before being washed into sperm-free media. Phase microscopy was used to count sperm heads bound to the zona or oolemma.

Statistics

Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software Inc.). For categorical data, Fisher exact test was used. All other data were tested from normal distribution using D'Agostino-Pearson omnibus normality test. For data found to follow a normal distribution, Student t-test or ANOVA with Tukey post hoc was used. For all other data, Mann-Whitney test or Kruskal-Wallis test with Dunn post hoc statistical test was performed. A P value <0.05 was considered statistically significant. Further information on statistics tests used for each data set can be found within the figure legends.

RESULTS

Maternal Smoke Exposure Alters Neonatal Follicle Population

Initially we investigated the neonatal ovarian consequences for female mice whose ovaries had been exposed to the products of inhaled cigarette smoke in utero (maternally smoke exposed, herein referred to as “MSE mice”). PND 6 ovaries that normally possess only primordial and newly activated primary follicles were examined. Strikingly, MSE ovaries only contained half the number of follicles compared with controls (305 ± 63 [MSE] vs. 595 ± 173 [control], P = 0.0079; Fig. 1A). Classification of follicles based on size found MSE ovaries contained a higher proportion of primordial follicles (< 15 µm: 46.6% ± 5.5% vs. 31.7% ± 4.5%, P = 0.0222; Fig. 1B), but activating (15–20 µm) and primary (>20 µm) follicle populations were unchanged (36.5% ± 3.4% vs. 40.4% ± 1.3% and 16.8% ± 5.6% vs. 27.9% ± 4.4%, respectively; Fig. 1B).

MSE Neonatal Ovaries Display Abnormal Levels of Cellular Apoptosis and Proliferation

To elucidate the mechanisms behind the altered follicle population observed at PND 6, examination of neonatal ovaries for markers of apoptosis revealed that DNA fragmentation as examined by TUNEL assay was significantly increased in MSE ovaries at PNDs 3 and 6 (positive cells, respectively: PND 3, 12.7% ± 8.6 vs. 1.0% ± 0.2%, P = 0.0090; and PND 6, 7.4% ± 3.5% vs. 1.3% ± 1.7%, P = 0.0013; Fig. 2A). To confirm this, a second marker of apoptosis, active caspase 3, was examined and was also found to be elevated in both the oocytes and granulosa cells of MSE females at PNDs 3 and 6 (~8-fold and ~52-fold, P = 0.0088 and P = 0.0007, respectively; Fig. 2B).

To determine whether proliferation of ovarian somatic cells, including granulosa cells, was affected by MSE, we analyzed PCNA expression, which has previously been correlated with the initiation of follicle growth [36]. Immunosignal, found primarily in ovarian somatic cells, was decreased in MSE ovaries at PND 3 (~0.5-fold, P = 0.0102; Fig. 2C); however, by PND 6, the signal was significantly increased relative to controls (~1.5-fold, P = 0.0194; Fig. 2C).

MSE Alters Adult Ovary Size and Follicle Population

To determine whether ovarian alterations persisted into adulthood, when animals were no longer lactationally exposed to cigarette smoke, ovaries from 8- to 9-wk-old (virgin) and 9-mo-old (nonvirgin) control and MSE females were examined. Ovarian weights of MSE females were found to be significantly lower than controls when normalized to body weight at 8–9 wk but not 9 mo (2.0 X 10^4 ± 2.8 X 10^3 vs. 4.5 X 10^4 ± 1.5 X 10^4, and 2.7 X 10^4 ± 6.8 X 10^3 vs. 2.4 X 10^4 ± 3.36 X 10^4 ovaries body weight ratio respectively, P = 0.0189; Fig. 3, A and C). Furthermore, the reduction in follicle numbers seen at PND 6 did not persist at 8–9 wk, with MSE ovaries containing equivalent follicle numbers compared with controls (39.3 ± 16.3 vs. 47.3 ± 12.0; Fig. 3B). Additionally, there was no change in the proportions of different follicle stages or corpus luteum (Fig. 3B). By 9 mo, however, there was once again a significant decrease in follicle numbers in MSE ovaries compared with controls (34.0 ± 19.4 vs. 80.3 ± 14.7, P = 0.0089; Fig. 3D).

MSE Adult Ovaries Display Abnormal Levels of Cellular Proliferation but No Apoptosis

To determine if changes in ovarian somatic cell proliferation continued into adulthood, 8- to 9-wk-old and 9-mo-old ovaries were examined for PCNA expression that localized primarily to granulosa cells in secondary and antral follicles. At 8–9 wk,
FIG. 2. Maternal smoke exposure increases neonatal ovarian apoptosis and alters proliferation. A) TUNEL staining (green) of neonatal control and MSE ovaries at X 200 magnification. Graphical representation of the number of TUNEL-positive cells in controls versus MSE at PND 3 (*p = 0.0090) and PND 6 (**p = 0.0013, Mann-Whitney test). B) Fluorescent immunolocalization of activated caspase 3 (red) at X 200 magnification. Graphical representation of average ovarian fluorescence per section in control versus MSE at PND 3 (*p = 0.0088) and PND 6 (**p = 0.0007, Mann-Whitney test). C) Fluorescent immunolocalization of cellular proliferation marker PCNA (red) at X 200 magnification. Graphical representation of average ovarian fluorescence per section in control versus MSE at PND 3 (**p = 0.0102) and PND 6 (**p = 0.0194, Student t-test). Nuclei of all sections counterstained with DAPI (blue). Sections analyzed from three control/smoked animals per age group. Values are shown as mean with SD marked. Bar = 50 µm.
FIG. 3. Maternal smoke exposure alters adult ovarian size and follicle population. A) Representative image of control and MSE ovaries at 8–9 wk. Graphical representation of average ovary size at 8 wk normalized to body weight (*p = 0.0189, Student t-test). B) Average number of follicles per ovary at 8–9 wk (p = 0.5307, Student t-test). Classification of follicles based on morphology revealed no change in follicle or corpora lutea population. C) Representative image of control and MSE ovaries at 9 mo, magnification X 100. Graphical representation of the average ovary size at 9 mo normalized to body weight (p = 0.4505, Student t-test). D) Average number of follicles per ovary at 9 mo (*p = 0.0089, Student t-test). Classification of follicles based on
MSE ovaries again had greatly decreased PCNA levels, presumably due to the lower numbers of proliferating follicles present, because PCNA at this age is most often associated with replicating granulosa cells of large preantral and antral follicles (~10-fold, P < 0.0001; Fig. 4A). Interestingly, however, by 9 mo MSE displayed an increase in PCNA fluorescence compared with controls (~2-fold, P = 0.0011; Fig. 4A). To investigate the possible cause of this elevated PCNA proliferation at 9 mo, ovaries were examined for AMH because it has been shown to reduce follicle proliferation [37]. Immunosignal of AMH that localized to granulosa cells in secondary and early antral follicles was significantly reduced in MSE ovaries (~2-fold, P = 0.0013; Fig. 4B). Further examination of ovaries for markers of apoptosis found no change in TUNEL-positive cells at 8–9 wk or 9 mo (Fig. 4C).

**Reduced Oocyte Number and Quality in MSE Animals**

Having established a reduction in preantral follicle number, as well as altered somatic cell proliferation and survival, in the ovaries of MSE females, we next assessed the number and quality of mature eggs isolated from these mice after hormonal stimulation. As expected, MSE females ovulated fewer MII eggs compared with controls at 8 wk (21 ± 2.8 vs. 28 ± 2.4, P = 0.0001; Fig. 5A). Furthermore, at 9 mo a reduced number of mature GV oocytes were collected from MSE ovaries (8.0 ± 0.8 vs. 13.2 ± 2.6, P = 0.0088; Fig. 5B). To determine the oxidative stress status of 8-wk-old eggs, we examined lipid peroxidation and mitochondrial superoxide leakage using the cell-permeable probes BODIPY and MitoSOX red, respectively. Superoxide leakage was increased approximately ~2-fold in MSE eggs (P = 0.0113; Fig. 5C), as was lipid peroxidation, which was 1.5-fold higher (P < 0.0001; Fig. 5D). Oxidative stress has been hypothesized to lead to poor oocyte quality, including increased incidence of aneuploidy [38]. Interestingly, however, there was no change in the aneuploidy rate between MSE and control animals at 4 wk or 9 mo of age (Supplemental Fig. S2), indicating normal chromosome segregation was likely to have taken place during meiosis I in these eggs.

Aneuploidy can also arise during completion of meiosis II, so we next examined the shape and size of the MII spindle, which can provide an indication of whether this second meiotic division is likely to occur normally. Both spindle pole-to-pole length (13.9 ± 1.2 vs. 15.1 ± 1.3 µm, P < 0.0001; Fig. 6) and spindle width (8.9 ± 1.0 vs. 10.7 ± 1.1 µm, P < 0.0001; Fig. 6) were smaller in MSE oocytes compared with controls, which suggests that completion of meiosis II after fertilization could be compromised.

**Decreased Sperm-Egg Interaction with MSE**

The interaction of eggs from MSE ovaries with wild-type sperm was also investigated to determine the likelihood of normal conception. The MSE animals had a reduction in both sperm-zona binding (14 ± 3.5 vs. 23 ± 5.2 bound sperm per egg, P = 0.0007; Fig. 7A) and sperm-oolemma binding (17 ± 4.6 vs. 24 ± 3.5 bound sperm per egg, P ≥ 0.0001; Fig. 7B) compared with controls. We also observed that ZP thickness was reduced in MSE animals at 4 wk (5.7 ± 0.6 vs. 7.0 ± 0.8 µm, P ≥ 0.0001; Fig. 7C). By 9 mo there was no difference between control and MSE ZP thickness (7.8 ± 0.6 vs. 7.9 ± 0.6 µm); however, ZPs of both groups were significantly thicker compared with 4-wk animals (P < 0.0001; Fig. 7C). Together these results suggest that maternal smoke exposure leads to reduced quality of the egg plasma membrane and glycoprotein extracellular matrix, which may impact the success of fertilization.

MSE Decreases F1 Female Fertility

Finally, we sought to establish the overall reproductive consequences for MSE females. Control and MSE female adult mice (age 6 wk) were mated with non-smoke-exposed males during a period of 3 mo, and the days to conception, litter sizes, and pup weights were recorded. Time to conception was significantly increased in MSE females (8.2 ± 7.7 vs. 3.3 ± 2.5 days, P = 0.0206; Fig. 8). Additionally, a small but significant reduction in litter size was also found in MSE mice compared with controls (6 ± 1.6 vs. 7 ± 1.8 pups per litter, P = 0.0190; Fig. 8) with no significant change in pup weights, suggesting phenotypically normal litters (Supplemental Fig. S3).

**DISCUSSION**

In utero exposure to cigarette smoke in humans has been linked to adverse health effects in offspring, including increased risk of obesity, asthma, and congenital heart defects, and reduced female reproductive capacity [1–3, 8–13]. Through the use of our novel animal model we have been able to avoid external confounding factors present in human epidemiological studies of smoking and have begun to unravel the effects of maternal smoke exposure directly on the fertility of the subsequent generation of females. In the current study, we have identified ovarian and oocyte changes associated with pregnancy and lactational smoke exposure in the mouse that may underlie the reduced fecundability and early-onset menopause observed following human maternal exposure. Interestingly, changes in oocyte and ovarian quality observed in MSE mice are similar to alterations detected in their mothers [27].

Smoke exposure during the gestational/weaning period resulted in the loss of nearly half the ovarian follicle population at PND 6. In the mouse, the entire cohort of primordial follicles, from which all mature oocytes are ultimately derived, is established during early postnatal life, with some follicles beginning to enter the growing pool. Therefore, the time after birth is crucial in determining the reproductive capacity of the adult female. At PNDs 3–6 we observed increased levels of TUNEL, which suggests that loss of primordial and/or activated follicles is occurring at this time through an apoptosis-mediated mechanism. Such a mechanism of germ cell depletion has been observed in in vitro studies where neonatal ovaries were exposed to individual chemical constituents of cigarette smoke. For example, culture of PND 3–4 ovaries in the presence of benzo[a]pyrene, 3-methylcholanthrene, or 7,12-dimethylbenz[a]anthracene resulted in increased follicle apoptosis with an elevation in activated caspase 2, caspase 3, and DNA strand breaks (TUNEL) [39–41]. It is possible that germ cell apoptosis in MSE females may be initiated prior to birth, in the developing fetal ovary when early meiotic events are taking place. This could contribute to the

morphology revealed that despite a decrease in total follicle number, there was no change to the proportion of follicle types or corpora lutea. Values are shown as mean with SD marked. Bar = 300 µm, magnification X 100.
reduction in follicle numbers observed in neonatal MSE ovaries. In support of this concept, the culture of mouse fetal ovaries with polycyclic aromatic hydrocarbons or in utero exposure of human fetuses to cigarette smoke has been shown to significantly elevate proapoptotic markers Bax and HRK [14, 24]. In addition, data from our male MSE model indicate
FIG. 5. Maternal smoke exposure reduces oocyte number and increases oocyte oxidative stress. A) Average number of oocytes ovulated per female at 8 wk (n = 8 control/MSE animals, \( *P = 0.0001 \), Student t-test). B) Average number of immature GV oocytes collected from one ovary per female at 9 mo (n = 4 control/MSE animals, \( *P = 0.0088 \), Student t-test). C) Representative image of MII oocyte at 8 wk probed with MitoSOX Red at X 600 magnification. n, number of oocytes examined from three control/MSE animals, \( P = 0.0113 \), Kruskal-Wallis test with Dunn post hoc test. D) Representative image of MII oocytes at 8 wk probed with BODIPY at X 600 magnification. n, number of oocytes examined from three control/MSE animals, \( P < 0.0001 \), Kruskal-Wallis test with Dunn post hoc test.
that meiotic spermatocytes are particularly susceptible to apoptosis following smoke exposure [28]. Future investigations examining the female MSE model at embryonic stages may assist in our understanding of how smoke exposure impacts early female meiosis.

Together with increased apoptosis in the neonatal ovary, we noted reduced proliferation of ovarian somatic cells at PND 3. Elevations in granulosa PCNA have previously been correlated with the initiation of follicle growth [36]. This reduced PCNA expression at PND 3, in conjunction with the increased proportion of primordial follicles at PND 6, indicated that in MSE ovaries, primordial follicles are failing to activate and transition to the growing pool in a timely manner. Interestingly, PCNA expression in both stromal and granulosa cells was elevated in MSE ovaries by PND 6. In addition to being a marker of S-phase, PCNA is involved in DNA repair [42], and so the elevation observed in MSE neonatal ovaries may reflect increased DNA repair activity. Our previous work in MSE males also revealed an increase in PCNA and DNA repair protein DCM1 in testis, indicating that gestational/neonatal exposure to cigarette smoke can lead to significant DNA damage and subsequent activation of repair and/or apoptotic pathways [28]. PCNA signal in control 8- to 9-wk ovaries was detected as expected, with numerous immunopositive granulosa cells present in preantral and antral follicles. We predict that the severe reduction in PCNA levels in adult MSE ovaries reflects abnormal granulosa cell proliferation, and therefore abnormal follicle growth. Despite this decreased PCNA expression, only a slight, nonsignificant reduction in the follicle population was observed at 8–9 wk. Because the ovarian follicle pool is established in neonatal life, it is therefore possible that this normalization in the follicle pool is a result of delayed follicle activation. During follicle activation and maturation the vast majority of follicles undergo atresia [43, 44]. The delay in follicle activation observed in PND 6 ovaries could result in the apparent follicular population catch-up in MSE ovaries at 8–9 wk; however, by late adult life (9 mo) follicle numbers were significantly reduced. Follicle-stimulating hormone (FSH) has been shown to increase PCNA expression in granulosa cells, and it is therefore possible that despite the apparently normal histological follicle population, the MSE ovaries are less sensitive to hormonal cues, resulting in the reduction of ovulated eggs observed [45, 46]. By 9 mo of age MSE ovaries contained significantly fewer follicles, resulting in a reduction of mature GV oocytes. Interestingly, however, PCNA expression was increased in MSE ovaries. We hypothesize that the elevation in follicular proliferation observed was the result of altered hormone dynamics within the ovary. One hormone in particular, AMH, which reduces follicular sensitivity to FSH and subsequently prevents follicle growth, is known to decrease in women near the end of their reproductive life [37, 47, 48]. In contrast to this, FSH levels are found to increase in these women [48]. Our investigation of AMH levels in reproducitively aged females (9 mo) revealed decreased AMH levels in MSE ovaries. It is therefore possible that MSE animals are displaying a more severe aging phenotype than controls. Reduced AMH levels may be leading to elevated granulosa proliferation as a result of increased sensitivity to raised FSH levels.

It is important to note, however, that a previous study indicated that smoke-exposed F0 females are significantly smaller than controls [26]. Several human studies have found altered serum cholesterol levels in women with premature ovarian failure [49, 50]. Cholesterol is an essential precursor for estrogen and is therefore important for normal fetal ovarian development [51]. As such, it is possible that the reduction in the ovarian reserve of MSE animals could be the result of exposure to ovotoxic cigarette smoke constituents in combination with reduced maternal body mass.

In addition to the loss of both immature and mature oocyte numbers, gestational/neonatal exposure to cigarette smoke also reduced mature oocyte quality, with respect to oxidative stress status. The key source of oxidative stress in MSE oocytes may well be endogenous from damaged mitochondria, which

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*FIG. 6. Maternal cigarette smoke exposure decreases the metaphase II spindle size in ovulated oocytes. Representative confocal images of control and MSE oocytes immunostained for β-tubulin (green) at 600x magnification. Chromosomes are counterstained with Hoechst (blue). Graphical representations of median MII spindle length/width (centerline); box outline 25th–75th percentiles and whiskers, 10th–90th percentiles. n, number of oocytes examined from three control/MSE animals. *p < 0.0001, Student t test, Bar = 5 μm.*
displayed increased rates of superoxide leakage. Oxidative stress has the potential to impact numerous aspects of oocyte maturation and development, such as meiotic spindle formation. Previous work has shown that exposure of oocytes to the pro-oxidant tertiary butyl hydroperoxide, or H$_2$O$_2$, significantly reduces spindle size in a manner similar to that observed in MSE mice [38, 52]. Correct spindle formation is critical for chromosome separation, and changes to normal MII spindle morphology in MSE oocytes could potentially lead to chromosome segregation errors during MII completion, causing aneuploidy, and ultimately embryo loss.

Furthermore, the increased lipid peroxidation in MSE oocytes, predicted to be another outcome of increased oxidative stress, may be the causative factor behind the decreased sperm-egg interactions we observed. We hypothesize that increased lipid peroxidation may have led to decreased fluidity of the oolemma membrane, potentially altering its protein composition and impacting proteins involved in sperm-egg interaction, such as CD9 or GM1 [53, 54]. Such scenarios have been previously reported in other cell systems, including in macrophages, whereby exposure to H$_2$O$_2$ decreased plasma membrane fluidity through increasing the frequency of lipid rafts [55].
Sperm-zona interaction also appeared to be reduced in MSE oocytes, and interestingly these oocytes possessed a thinner ZP matrix at 4 wk. In the mouse, the ZP consists of three proteins: ZP1, ZP2, and ZP3 [56]. The importance of this structure is indicated by the fact that deletion of the ZP2 or ZP3 genes in mice causes infertility due to abnormal follicle and oocyte development [57, 58]. Although ZP1-null females display relatively normal oocyte development, similar to our observations these oocytes had a significantly thinner ZP and females had smaller litters due to embryonic loss [59]. We predict that maternal smoke exposure might influence either 1) the production of ZP components through transcriptional or translational changes, or 2) ZP glycoprotein stability or turnover. It would be of particular interest to determine whether MSE influences the expression of the ZP or other key genes involved in oocyte development, which may impact the final quality of the mature oocyte. Interestingly, however, by 9 mo zona thickness was increased in both groups. In humans a correlation between zona thickening and increasing age has been found [60]. It is therefore possible that our aging MSE mice are showing a more severe aging phenotype with respect to zona thickness such that there is negligible difference from controls by 9 mo. To support this idea, we find that MSE oocytes have a 36% increase in zona thickness with age compared with the 12% increase observed in controls.

In summary, by using a model that avoids the external confounders present in human epidemiological studies, such as lifestyle and environmental factors, we have been able to provide strong evidence that maternal smoke exposure adversely affects the reproductive health of female offspring. Our results indicate that such smoke exposure results in reduced female fertility in adult life, which can be attributed to both a reduction in the number and quality of ovulated oocytes. These findings provide an important basis for understanding the decreased fecundability and early-onset menopause observed in humans following maternal smoking. Further studies using our MSE model could be instrumental in pinpointing the effects that smoking cessation prior to conception or at different embryonic stages of pregnancy has on the fertility of the subsequent generation, and our future work will focus on the potential of translational effects of MSE. Such knowledge will hopefully facilitate smoking cessation campaigns targeting young women, particularly those who are pregnant and/or breast-feeding.

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CHAPTER 3

Grandmaternal but not great-grandmaternal smoke exposure reduces female fertility in a murine model

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Note: Supplementary figures are located in Appendix B, Pg 146.
Statement of Contribution

N.J.C contributed to the conception and design of the study, performed data acquisition, data analysis/interpretation and was responsible for drafting and revising the manuscript. The group of P.M.H including R.L.V. and A.G.J. performed the cigarette smoke exposures. P.M.H., E.A.M. and J.E.H., contributed to the conception and design of the study.
Chapter 3: Overview

In Chapter 2 we established that \textit{in utero}/lactational exposure to cigarette smoke results in diminished female mouse fertility (Camlin et al., 2016). However, the effect \textit{in utero}/lactational exposure has on the subsequent generations in humans is unknown. Therefore, the aim of the following study was to determine if grandmaternal (multigenerational) or great-grandmaternal (transgenerational) exposure to cigarette smoke reduces female fertility in adult life using a mouse model.

The multi and transgenerational effects of environmental and lifestyle factors are an area of increasing research interest. Grandmaternal and great-grandmaternal exposure to bisphenol A and low protein diet in rodents, has been found to alter ovarian quality and female fertility (Aiken et al., 2015; Ziv-Gal et al., 2015). Furthermore, increasing evidence suggests that multi and transgenerational exposure to cigarette smoke or its constituent nicotine increases childhood cancer risk and negatively effects lung function and asthma outcomes in both humans and rats (Li et al., 2005; Magnus et al., 2015; Ortega-García et al., 2010; Rehan et al., 2012). Through the use of a novel nose only smoke inhalation method this study explores the effects of grandmaternal and great-grandmaternal cigarette exposure on female mouse fertility.

Data within this manuscript indicates that grandmaternal but not great-grandmaternal cigarette smoke exposure impacts female fertility in adult life, suggesting a multigenerational but not transgenerational effect.
Grandmaternal but not great-grandmaternal smoke exposure reduces female fertility in a mouse model

Key Words: Cigarette smoke/ subfertility/ grandmaternal/ great-grandmaternal/ multigenerational/ transgenerational/ ovary/ oocyte

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Abstract

A growing body of evidence suggests that multigenerational and transgenerational exposure to environmental and lifestyle factors can have significant health impacts. In particular, grandmaternal (F2) or great-grandmaternal (F3) exposure to cigarette smoke or its constituents appear to be linked to asthma and cancer incidence. Direct and in utero exposure to cigarette smoking is known to have detrimental impacts on ovarian quality and fertility. However, the impact of multi/transgenerational exposure on female fertility is largely unknown. The focus of our investigation was to characterise the adverse effects of multigenerational (F2) and transgenerational (F3) smoke exposure on ovary and oocyte quality. Female mice (F0) were nasally exposed to cigarette smoke for 12 weeks throughout pregnancy/lactation, with ovarian and oocyte quality of the F2 and F3 generations examined. F3 females displayed negligible oocyte and ovarian changes up to 9 months of age, and normal preimplantation embryo development. Conversely, although F2 females had no detectable change in ovarian quality at 8 weeks, by 9 months ovarian somatic cell proliferation was reduced. Further investigation revealed no change in F2 oocyte quality at 8 weeks, but a decrease in spindle pole to pole length at 9 months. Additionally, no change in preimplantation embryo development was observed following parthenogenetic activation. Ultimately F2 females displayed subfertility with significantly increased time to conception. In conclusion, our results demonstrate that grandmaternal but not great-grandmaternal cigarette smoke exposure reduces female fertility in mice, highlighting the clinical need to promote cessation of cigarette smoking in pregnant women.
Introduction

Cigarette smoke exposure is a known reproductive toxicant in female smokers (Gruber et al. 2008, Sobinoff et al. 2013, Camlin et al. 2014). Furthermore, in utero smoke exposure has been found to be detrimental to the fertility of both sons and daughters (F1 generation), exposed during this important developmental time period (Jensen et al. 2005, Lutterodt et al. 2009, Ernst et al. 2012, Fowler et al. 2014, Sobinoff et al. 2014, Camlin et al. 2016). Of further concern are the small number of studies which have shown that multigenerational (F2) and transgenerational (F3) exposure in humans and rats, to cigarette smoke or its constituents have negative effects on lung function, asthma outcomes and increase the risk of childhood cancers (Li et al. 2005, Ortega-García et al. 2010, Rehan et al. 2012, Rehan et al. 2013, Magnus et al. 2015). Interestingly, in rats, multi and transgenerational exposure to the cigarette smoke constituent benzopyrene, reduces females receptiveness to copulation (Csaba & Karabélyos 1997). This F2 multigenerational effect is only seen with environmental exposure of the F0 generation during pregnancy. Exposure of the F0 generation during non-pregnant periods results in an F2 transgenerational effect rather than the multigenerational effect examined here. However, little else is known about multigenerational effects on fertility in F2 and F3 female generations. As approximately 12% of Australian and US women smoke throughout pregnancy, this is a relevant and important topic that requires further investigation (Tong et al. 2013, 2015).

Mammalian oogenesis begins in fetal life, and in humans by birth, a finite oocyte pool is established containing all the functional oocytes a female will ever possess (Camlin et al. 2014). These immature oocytes are meiotically arrested at prophase I and surrounded by a single layer of granulosa cells forming the primordial follicle (McLaughlin & McIver 2009, Jones et al. 2013, Camlin et al. 2014). Following activation of the primordial follicle, preantral/antral follicular growth is promoted by follicle stimulating hormone (FSH).
Granulosa cells proliferate and the oocyte enlarges to eventually become a meiotically competent oocyte within an antral follicle. A surge of luteinizing hormone (LH) releases the oocyte from its prophase I arrest and leads to ovulation (Holt et al. 2013). The ovulated egg arrests again at metaphase II until fertilization, when calcium oscillations triggered by the sperm leads to meiosis II completion and subsequent embryo development (Nixon et al. 2002).

Due to the finite number of functional oocytes that form solely during fetal development, lifestyle and environmental factors of the grandmother, such as cigarette smoking, may directly affect the health of their grandchildren (multigenerational). It is not until the 3rd generation (‘great-grandchildren’) that a pregnant woman’s environmental and lifestyle factors do not directly impact a generation of offspring at the cellular level (transgenerational). In this study we used an established animal model to investigate the effect of cigarette smoking during pregnancy and lactation on the fertility of female grandchildren (F2 generation) and great-grandchildren (F3 generation) solely down the maternal line. Exposed F1 and F2 males were not used as breeding studs.

Materials and methods

All reagents were obtained from Sigma-Aldrich (St Louis, MO, USA) unless otherwise specified.

Animals and Ethical Approval

All experiments were performed with the approval of the University of Newcastle Animal Care and Ethics Committee. C57BL/6 mice were obtained from Australian BioResources (Mossvale, NSW) and housed with ad libitum food and water under a 12 hour light/12 hour dark lighting regimen.
Smoke Exposure and Breeding

Six week-old female mice (F0 generation) were exposed to cigarette smoke as previously described (Beckett et al. 2013, Sobinoff et al. 2013, Franklin et al. 2014, Hansbro et al. 2014, Chen-Yu Hsu et al. 2015, Haw et al. 2015, Camlin et al. 2016, Liu et al. 2016). Briefly, animals were exposed to mainstream cigarette smoke (twelve 3R4F reference cigarettes; University of Kentucky, USA) through the nose only for 75 minutes daily, 5 days/week for 12-13 weeks. This level of exposure equated to a pack-a-day (24 cigarettes/day) smoker (Fricker et al. 2014). Females were housed with non-cigarette smoke exposed males (2 females/male) from 5 weeks until visibly pregnant. Cigarette smoke exposure continued throughout pregnancy and lactation, ceasing once pups (F1 generation) were weaned on post-natal day (PND) 21-23 (Sobinoff et al. 2014, Camlin et al. 2016).

Six week-old F1 females of F0 exposed dams were housed with 8 week-old non-cigarette smoke exposed males (1 female/male) continuously for 3 months. F2 generation pups were weaned on PND 21-23. A subset of F2 females (6-week old) of F1 dams were housed with non-cigarette smoke exposed 8 week-old males (1 female/male) continuously for 3 months giving rise to an F3 generation of animals. F3 pups were weaned on PND 21-23. Control animals were F2 and F3 descendants of the non-smoke exposed female founders.

Fertility Trial

Four 6 week-old F2 females per treatment group were individually housed with non-cigarette smoke exposed males (8 weeks-old) continuously for 3 months. Litter size and date of birth was recorded throughout the trial. Days to conception was determined by subtracting 19 days from time of litter drop as previously described by Camlin et al. (2016).
**Tissue collection and histology**

Ovaries were fixed in Bouin’s solution, paraffin embedded and sectioned at 5 µm prior to hematoxylin and eosin staining or immunofluorescent processing.

**Follicle Counts and Staging**

Eight week and 9 month-old follicle counts were performed on every 15th section for the entire ovary. Only follicles with a visible oocyte nucleus were counted. Morphological features were used to stage follicles in adult ovaries as previously described by Camlin *et al.* (2016).

**Immunofluorescence**

Eight-week and 9 month-old ovaries were deparaffinised and rehydrated before antigen retrieval in Tris (50 mM, pH 10.6) or sodium citrate (10 mM, pH 6) buffer. Sections were blocked in 7% goat serum prior to overnight incubation with primary antibodies against PCNA (1:100; NA03, Merick Millipore) or AMH (1:20; MCA2246, AdB Serotec, UK) at 4°C. Secondary antibodies conjugated to Alexa-633 (1:200; Life Technologies, Australia) was applied for 1 hour at room temperature before being counter stained with DAPI and mounting in citifluor (Citifluor Ltd, UK). An Axio Imager A1 epifluorescent microscope (Carl Zeiss MicroImaging Inc., NY, USA) was used to visualize sections with photomicrograph taken using an AxioCam MRc microscope camera (Carl Zeiss MicroImaging Inc). Negative controls with only secondary antibodies were also examined (Supplementary data, Fig S1A).

**TUNEL Analysis**

Sections were treated with 20 µg/mL of proteinase K (Promega, WI, USA) prior to TUNEL analysis using ApopTag® Fluorescein In Situ Apoptosis Detection Kit (S7110, Merick
Millipore) according to the manufacturer’s protocol. DAPI was used to counterstain sections before mounting in citifluor and imaging on an Axio Imager A1 epifluorescent microscope with an AxioCam MRc microscope camera.

**Oocyte Collection**

Female mice (4 weeks-old) were intraperitoneally (i.p) injected with 7.5 IU of pregnant mares’ gonadotropin (Intervet, Australia) 44-52 hrs prior to germinal vesicle (GV) oocyte collection from the ovary or a 2nd i.p injection of 5 IU human chorionic gonadotropin (Intervet). 12 hours following the second injection MII stage eggs were collected from the ampulla. GV oocytes were collected into M2 media containing BSA and milrinone (M4659) while MII eggs were collected in media without milrinone. Cumulus cells were removed mechanically via repeated pipetting (GV oocyte) or enzymatically with hyaluronidase (300µg/ml; H4272; MII egg) (Mihalas et al. 2015). MII eggs were fixed in 2% paraformaldehyde in PBS with 0.5% triton X for 30mins or washed into M2 media with BSA containing monastrol (200µM; M8515) to collapse the metaphase spindle II as previously described by Camlin et al. and Holt et al. prior to fixation (Holt et al. 2012, Camlin et al. 2016). For collection from aged 9 month-old females, GV oocytes were retrieved directly from the ovary without prior hormonal stimulation and allowed to in vitro mature for 16 hours prior to fixation.

**Parthenogenetic activation**

MII eggs were collected as described above. Parthenogenetic activation was performed to induce calcium oscillations in MII eggs that mimic those that occur at fertilization, allowing for embryonic development without sperm (Zhang et al. 2005). For second polar body extrusion timing, eggs were washed into calcium free M2 media with 10 mM strontium chloride and BSA. MII eggs were then allowed to undergo parthenogenetic activation in a
Nikon Biostation IM with images taken every 10 mins for 12 hours. For timing of embryonic development to compaction stage MII eggs were activated in calcium free KSOM with 10mM strontium chloride and 1 ug/ml cytochalasin D (C2618) for 4 hours in 5% CO₂ at 37°C. Activated eggs were then washed into calcium containing KSOM media under mineral oil and incubated in 5% CO₂ at 37°C (Seah et al. 2012). Eggs were scored for development progress 9 hours post activation and then every 24 hours for 72 hours.

**Oocyte Immunocytochemistry**

Immunocytochemistry was performed on oocytes using anti-CREST antibody (1:400; 90C-CS1058, Bioclon Australia, Australia) for aneuploidy analysis or anti-α-tubulin (1:400; A11126, Life Technologies) for spindle analysis. Oocytes were blocked in 7% goat serum in PBS with 0.1% Tween-20 before overnight incubation in primary antibodies at 4°C. Secondary antibodies were conjugated with Alexa-488 or Alexa-555 (1:1000; Life Technology) and incubated with oocytes for 1 hour at room temperature. Finally, oocytes were counter stained in Hoechst (20 µg/ml) and mounted in Citifluor. Imaging was performed on an Olympus FV1000 using a 60x/1.2 NA UPLSAPO oil immersion objective lens (Olympus, Australia). ImageJ (freeware; National Institute of Health) was used to measure spindle size and count kinetochores using a macro designed by Dr Simon I. R. Lane (Lane & Jones 2014).

**Immunoblotting**

Whole ovary immunoblotting was performed as previously described by Holt et al. (2011). Briefly whole ovary protein was extracted using SDS lysis buffer. 5 µg of ovary protein was loaded onto a 4–12% NuPage gel and immunoblotting was performed using antibodies for PCNA (1:1000; ab29, Abcam) with anti- glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:5,000; G9545, Sigma-Aldrich) used as a loading control.
Serum Collection, Estrus Staging and Hormone Analysis

Three month old females were euthanized via cervical dislocation and blood collected via venipuncture from the heart. Whole blood samples were stored at room temperature over night to allow clotting. After clotting blood samples were centrifuged at RCF 11337 x g and the serum fraction collected for hormone analysis.

Following euthanasia vaginal lavage was immediately performed on females with 20 µL of sterile PBS. The lavage sample was smeared onto Poly-L-Lysine slides and air dried prior to methanol fixation. Slides were stained with Diff quik (LP-64851, Labs Aids Pty Ltd, Australia) according to manufacturer’s instructions prior to estrus staging using an Axio Imager A1 epifluorescent microscope as previously described by McLean et al. (2012). Serum from animals at diestrous was used for analysis of follicle stimulating hormone (FSH) and luteinizing hormone (LH); see Supplementary data, Fig S1B for examples of estrus staging. Serum FSH and LH levels were determined as previously described by van Casteren et al., and Jimenez et al. (van Casteren et al. 2000, Jimenez et al. 2005).

Statistics

Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc., CA, USA) or JMP (SAS, NC, USA) software. For categorical data Fisher’s Exact Test was used. All other data was tested from normal distribution using D’Agostino-Pearson omnibus normality test. For data found to follow a normal distribution Student’s t-test or ANOVA with Sidak’s Post-hoc was used. For all other data Mann-Whitney test or Kruskal-Wallis Test with Dunn’s post-hoc statistical test was performed. A p value <0.05 was considered statistically significant. For oocyte analysis linear mixed modelling was performed. Briefly, the fixed effect was set as treatment group (control or smoke exposed) with the effect of the
random variables replication and animal taken into account. Further information on statistics tests used for each data set can be found within the figure legends.

Results

No change in the ovarian follicular composition after grandmaternal or great-grandmaternal cigarette smoke exposure

We first sought to investigate whether cigarette smoke exposure grossly altered the folliculogenesis in the F2 and F3 generation by examining the number and type of ovarian follicles present in adult females. We analyzed F2 (grandmaternal smoke exposed: GM-SE) and F3 (great-grandmaternal smoke exposed: GG-SE) ovaries in young adult (8 week-old) and reproductively senescent (9 month-old) mice (Fig 1). GM-SE females had no change in total follicle number compared with control animals (8 weeks: 134.3±21.5 (GM-SE) vs 151.0±82.8 (control) follicles, p=0.7528, Fig 1A; 9 months: 33.0±8.2 vs 45.3±14.4 follicles, p=0.2674, Fig 1B) and no change in follicle composition at 8 weeks or 9 months. Similarly, no change was seen in the follicle number or composition of GG-SE females at 8 weeks and 9 months (8 weeks: 147.0±34.2 vs 159.7±11.2 follicles, p=0.5746, Fig 1C; 9 months: 36.0±4.9 vs 26.7±5.1 follicles, p=0.0785, Fig 1D). To determine whether numbers of mature/ovulated eggs might be altered in these mice, we also retrieved GV oocytes and MII eggs from hormonally stimulated GM-SE or GG-SE animals, at 8 weeks or 9 months. Similar numbers were retrieved from these mice compared with controls (Supplementary data, Fig. S2).

GM-SE and GG-SE ovaries display no or subtle alteration in follicular dynamics

We investigated ovarian quality of GM-SE and GG-SE mice using markers of apoptosis (TUNEL) and somatic cell proliferation (PCNA and AMH). At 8 weeks GM-SE had no
change in PCNA immunosignal following ovarian immunofluorescence or immunoblotting (1.2±0.5 vs 1±0.0, p=0.6213, Fig 2A and D). However, by 9 months there was a significant reduction in PCNA immunosignal (0.7±0.1 vs 1.0±0.0, p=0.0197, Fig 2A and E). No change in AMH immunofluorescence or TUNEL positive cells was observed in GM-SE ovaries at 8 weeks or 9 months (Fig 2B and C).

In contrast to GM-SE ovaries no change in PCNA immunosignal was observed at either 8 weeks or 9 months in GG-SE ovaries (8 weeks: 0.9±0.2 vs 1.0±0.0, p=0.3825, Fig 2F and I; 9 months: 0.9±0.1 vs 1.0±0.0, p=0.2401, Fig 2F and J). Additionally, there was no detectable change, in AMH immunofluorescence or TUNEL positive cells, in 8 week or 9 month GG-SE ovaries (Fig 2G and H).

GM-SE and GG-SE has little effect on oocyte quality

Having found no gross ovarian abnormalities, we began investigating the quality of oocytes from GM and GG smoke-exposed mice. Metaphase II spindle structure and chromosome number of MII eggs of 4-week old animals was investigated. Both GM-SE and GG-SE eggs from hormonally stimulated animals had normal ploidy (Fig. 3A and C). Further examination of the metaphase II spindle found no change in spindle width (11.7±2.2 vs 13.2±1.2 µm, p=0.3474, Fig. 3B) or spindle pole to pole length (16.2±2.9 vs 18.1±1.8 µm, p=0.3982, Fig. 3B) in GM-SE oocytes. However, GG-SE metaphase spindles were found to have significantly smaller width (11.7±2.2 vs 13.2±1.2 µm, p=0.0243, Fig. 3B) but no change in spindle pole to pole length (16.2±2.9 vs 18.1±1.8 µm, p=0.2849, Fig. 3B). In addition to metaphase II spindle structure, the distance between kinetochores of sister chromatids can be a marker of how faithfully chromosomes will segregate during anaphase (Chiang et al. 2010, Merriman et al. 2012, Merriman et al. 2013). We therefore compared interkinetochore distances of GM-SE/GG-SE eggs with control eggs with no change observed (GM-SE:
0.88±0.17 vs 0.77±0.12 µm, p=0.2624, Fig. 3E; GG-SE: 0.63±0.12 vs 0.61±0.07 µm, p=0.5306, Fig. 4F).

Eggs from reproductively aged animals (9 months old) were next examined to determine if age related oocyte quantity and quality decline was more pronounced after multigenerational (F2) or transgenerational (F3) cigarette smoke exposure. As expected aged animals produced a lower number of euploid eggs than young animals, however no increase in aneuploidy above that of aged controls was observed in either GM-SE or GG-SE oocytes (Fig. 4A and B). Additionally, aged GM-SE eggs displayed smaller metaphase II spindles for pole to pole length (19.8±3.2 vs 23.9±3.8 µm, p=0.0109; Fig. 4C) but not width (8.9±1.5 vs 11.3±2.1 µm, p0.0527; Fig. 4C). Interestingly, GG-SE at 9 months old displayed no metaphase II spindle changes unlike their 4 week old counterparts (length: 21.5±1.9 vs 20.9±2.2 µm, p=0.4017; width: 8.1±1.0 vs 8.5±1.7 µm, p=0.9748; Fig. 4D).

**GM-SE delays meiosis II completion but both GM-SE and GG-SE have normal preimplantation embryo development**

Having established that GM-SE MII eggs appeared relatively normal, we next sought to examine whether completion of the 2nd meiotic division was affected following parthenogenetic activation. Polar body (PB2) extrusion was used as a marker of MII completion. We noted a ~20-minute delay in PB2 extrusion in GM-SE eggs compared with controls (197.4±113.6 vs 176.3±123.3 minutes, p=0.0337, Fig. 5A). We then continued to follow preimplantation development and observed that neither multigenerational (F2) or transgenerational (F3) smoke exposure effected development at any stage up to 72 hours post activation, with the majority of embryos reaching normal developmental milestones in a timely fashion (Fig. 5B-C).

**GM-SE increases time to conception but not litter size**
To investigate the reproductive consequences of GM-SE upon female mice, we established a fertility trial using GM-SE females paired with non-smoke exposed male mice over a period of 3 months. We observed that litter sizes remained similar (7.1±1.7 GM-SE vs 6.7±1.5 control pups/litter, p=0.4075, Fig. 6), however, interestingly time to conception was significantly increased in GM-SE females compared with controls (9.3±8.9 vs 3.8±2.0 days, p=0.0042, Fig. 6).

**GM-SE and GG-SE have no effect on serum FSH or LH levels at diestrous**

Having established the oocyte and ovarian consequences of GM-SE and GG-SE, we next sought to determine whether gonadotrophin levels were altered since this may explain the increased time to conception. For these analyses, due to animal availability, we were restricted to using females in diestrous which provided an indicator of basal gonadotrophin levels. GM-SE females had both serum FSH and LH concentrations that were similar to controls (FSH: 5.9±3.0 v 6.3±2.4, p=0.8652, LH: 0.7±0.9 v 0.7±0.7, p=0.9618, Fig 7A). Similarly, no change was observed for serum FSH or LH in GG-SE females (FSH: 3.1±1.1 v 3.8±2.2, p=0.9517; LH: 0.7±0.7±0.9, p=0.6715, Fig 7B).

**Discussion**

Recent studies have implicated transgenerational and multigenerational cigarette smoke exposure in elevated rates of diseases such as asthma and childhood cancers (Ortega-García *et al.* 2010, Rehan *et al.* 2012, Rehan *et al.* 2013, Magnus *et al.* 2015). In the current study, we have used a novel nose-only inhalational method to assist in unravelling the effect of grand and great-grandmaternal smoke exposure on female fertility. To the best of our knowledge we have shown for the first time that grandmaternal, but not great-grandmaternal smoke exposure reduces female fertility.
The most noticeable effect of cigarette smoke exposure we observed related to the subfertility of GM-SE females which manifested as increased time between litters. Litter sizes were normal which was consistent with the unaltered follicle composition and numbers of oocytes/eggs retrieved from treated animals. These findings suggest that GM-SE mice could have alterations in their estrus cycles. We found that basal gonadotrophins were normal in these mice; however, it remains possible that the length of individual estrus stages could have been altered. Ovulation occurs during the estrus stage of the cycle, therefore an extended period in non-estrus stages could result in reduced pregnancies over time.

Interestingly, previous studies noted that direct exposure of female rats to the cigarette smoke constituents benzo(a)pyrene or nicotine increased the time animals spent in non-estrus stages of the estrus cycle (Patil et al. 1999, Xu et al. 2010). Furthermore, multigenerational but not transgenerational exposure to synthetic estrogens genistein and ethinyl estradiol increased estrus cycle length (2008, 2010). Although genistein and ethinyl estradiol are not found within cigarette smoke, cigarette smoke condensate has been found to have estrogenicity, suggesting that a similar mechanism of action could be at play in GM-SE females (Takamura-Enya et al. 2003). Another conclusion, could be modifications to courtship and sexual behaviors in GM-SE females. Multi and transgenerational exposure of female rats to the cigarette smoke constituent benzopyrene significantly altered females receptivity to copulation as measured by lordosis frequency (Csaba & Karabélyos 1997). It is therefore possible that altered mating and cyclicity of animals could all compound to result in the observed GM-SE delayed conception.

Abnormalities in GM-SE and GG-SE spindle size were also observed. Of note is that neither GM-SE nor GG-SE resulted in an elevation in aneuploidy in young (4 weeks) or reproductively aged mice (9 months) above that of control mice. This was particularly surprising for the 9 month GM-SE cohort since an spindle abnormalities have been shown to
increase with age and are thought to contribute to aneuploidy (Battaglia et al. 1996). It is important to note, however, that the strain of mouse used during this study has been found to be particularly resilient to age related aneuploidy. Oocytes from 17-19 month old females displayed 9% aneuploidy only which was not significantly increased from young females (Yun et al. 2014). Human oocytes, on the other hand are particularly error prone with aneuploidy rates as high as 60% reported (Fragouli et al. 2011, Kuliev et al. 2011). Therefore, although age related aneuploidy and its associated reproductive consequences were not observed in GM-SE/GG-SE mice, it is possible that the altered spindles observed could lead to chromosome segregation errors in similarly exposed humans.

No aberrations in ovarian development were observed in GM-SE or GG-SE animals at 8 weeks. By 9 months, however, GM-SE but not GG-SE showed a reduction in PCNA expression. PCNA is a marker of both cellular proliferation or DNA damage repair (Essers et al. 2005). Furthermore, PCNA has been found to be an important marker of folliculogenesis and initiation of follicle growth (Oktay et al. 1995, Xu et al. 2011). Previous studies have noted changes in ovarian PCNA expression after ovarian in vitro culture or in utero exposure to cigarette smoke or its constituents (Petrik et al. 2009, Sobinoff et al. 2012, Camlin et al. 2016). Neonatal ovarian culture with benzo(a)pyrene significantly increases PCNA expression (Sobinoff et al. 2012). Conversely in utero exposure to nicotine decreased follicular PCNA in adult ovaries (Petrik et al. 2009). Finally, our research has found that the F1 smoke exposed generation had a decrease in follicular PCNA at 8 weeks and an increase at 9 months (Camlin et al. 2016). These studies indicate the susceptibility of ovarian follicle proliferation to alterations via cigarette smoke and its constituents. It is therefore likely that the decrease in PCNA observed in GM-SE ovaries is indicative of reduced follicular growth at 9 months, and potentially an indication of accelerated ovarian ageing. In support of this, in vitro culture of granulosa cells from IVF patients has found a significant correlation between
diminished granulosa proliferation and patient age (Seifer et al. 1993, Wu et al. 2015). Alternatively, this PCNA reduction may be a result of altered cumulus-oocyte communication. Oocytes produce granulosa cell growth factors including GDF9 and BMP-15, which stimulate granulosa cell proliferation in part by PCNA upregulation (Kedem et al. 2011). It is therefore possible that the decreased PCNA expression in GM-SE females with age could be the result of deficient signaling from the oocyte.

In summary, through the use of a novel smoke-only inhalation method we have shown that GM-SE but not GG-SE reduces female fertility. This data adds to the growing body of evidence which suggests that cigarette smoking during pregnancy can have multigenerational effects on female offspring (Csaba & Karabélyos 1997, Li et al. 2005, Ortega-García et al. 2010, Rehan et al. 2012, Rehan et al. 2013, Magnus et al. 2015). In contrast transgenerational exposure to cigarette smoke appears to have no effect of female fertility. Our studies provide further evidence of the negative impact of smoking upon subsequent generations with such knowledge needed to facilitate smoking cessation programs among pregnant women.

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Conflict of interest

The authors declare no conflict of interest.

Note: Supplementary figures are located in Appendix B, Pg 146.
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Figure 1: Unchanged follicle numbers with grandmaternal (GM-SE) and great grandmaternal cigarette smoke exposure (GG-SE). (A) Follicle numbers and type per 8 week ovary after
GM-SE. Graphical representation of the average number of follicles per ovary (p=0.7528, n=3, Students t-test) and average number of follicle types based on morphological classification (ANOVA with Tukey’s Post-hoc). (B) Follicle numbers and type per 9 month ovary after GM-SE. Graphical representation of the average number of follicles per ovary (p=0.2674, n=3, Students t-test) and average number of follicle types based on morphological classification (ANOVA with Tukey’s Post-hoc). (C) Follicle numbers and type per 8 week ovary after GG-SE. Graphical representation of the average number of follicles per ovary (p=0.5746, n=3, Students t-test) and average number of follicle types based on morphological classification (ANOVA with Tukey’s Post-hoc). (D) Follicle numbers and type per 9 month ovary after GG-SE. Graphical representation of the average number of follicles per ovary (p=0.0785, Students t-test) and average number of follicle types based on morphological classification (ANOVA with Tukey’s Post-hoc). Values are shown as mean with SD marked.
Figure 2: Unchanged ovarian quality with GG-SE but not GM-SE. (A) Fluorescent immunolocalisation of PCNA (red) in control and GM-SE ovaries at 8 week and 9 month. (B)
Fluorescent immunolocalisation of AMH (red) in control and GM-SE ovaries at 8 week and 9 month. (C) TUNEL staining (green) of control and GM-SE 8 week and 9 month ovaries. Positive control was DNase treated (D) Immunoblot of PCNA (red arrow) control and GM-SE whole ovary lysate at 8 week. Graphical representation of PCNA densitometry; p=0.6213, Students $t$-test, n=3. (E) Immunoblot of PCNA (red arrow) control and GM-SE whole ovary lysate at 9 month. Graphical representation of PCNA densitometry; p=0.0197, Students $t$-test, n=3. (F) Fluorescent immunolocalisation of PCNA (red) in control and GG-SE ovaries at 8 week and 9 month. (G) Fluorescent immunolocalisation of AMH (red) in control and GG-SE ovaries at 8 week and 9 month. (H) TUNEL staining (green) of control and GG-SE 8 week and 9 month ovaries. Positive control was DNase treated. (I) Immunoblot of PCNA (red arrow) control and GG-SE whole ovary lysate at 8 week. Graphical representation of PCNA densitometry; p=0.3825, Students $t$-test, n=3. (J) Immunoblot of PCNA (red arrow) control and GG-SE whole ovary lysate at 9 month. Graphical representation of PCNA densitometry; p=0.2401, Students $t$-test, n=3. Nuclei of all sections counterstained with DAPI (blue). Sections and immunoblots analysed from three control/treated animals per age group. Values are shown as mean with SD marked. Scale bar=100µm.
Figure 3: Unchanged ploidy status but altered MII oocyte spindle size with GG-SE in young females. (A) Graphical representation of GM-SE MII oocytes ploidy status at 4 week (p=0.9776, Fisher’s Exact Test). (B) Representative confocal images of 4 week control and GM-SE oocytes immunostained for α-tubulin (green). Graphical representations of median MII spindle length (p=0.3982, linear mixed model) and width (p=0.3474, linear mixed model). (C) Graphical representation of GG-SE MII oocytes ploidy status at 4 week (p=0.5168, Fisher’s Exact Test). (D) Representative confocal images of 4 week control and
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immunostained for α-tubulin (green). Graphical representations of median MII spindle length (p=0.4017, linear mixed model) and width (p=0.9748, linear mixed model). Box plots show mean (centerline) with box outline 25-75th percentiles and whiskers 10-90th percentiles, bar graphs show mean; n=number of oocytes examined from 3 animals. Chromosomes are counterstained with Hoechst (blue), scale bar=10µm.
Figure 5: GM-SE but not GG-SE alters MII oocyte parthenogenetic activation. (A) Phase contrast images of control and GM-SE oocytes undergoing MII completion after strontium activation as determined by second polar body (PB2; indicated by red arrow) extrusion, scale bar=10µm. Graphical representation of the time taken for oocytes to extrude PB2; p=0.0337, Mann-Whitney Test. (B-C) Graphical representation of parthenote development in ctrl, GM-SE and GG-SE parthenotes at key developmental milestones; 9 hours (1-cell), 24 hours (2-cell), 48 hours (3/4 cell) and 72 hours (compaction (comp)) post activation. ANOVA with Sidak’s Post-hoc. Box plots show mean (centerline) with box outline 25-75th percentiles and
whiskers 10-90\textsuperscript{th} percentiles; bar graphs show mean with SD marked. \(n\) = number of oocytes examined from 3 animals.
Figure 6: GM-SE causes reduced fertility in adult life. (A) Average number of days to conception in control and GM-SE females continually housed with wild-type males (p=0.0042, Mann-Whitney test). (B) Average litter size for control versus GM-SE females (p=0.4075, Students t-test). Results represent n=4 control/GM-SE animals per group over 3 months; Box plot shows mean (centerline) with box outline 25-75th percentiles and whiskers 10-90th percentiles.
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Note: Supplementary figures are located in Appendix B, Pg 146.
CHAPTER 4

Kif4 is essential for mouse oocyte meiosis

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Note: Supplementary figures and tables are located in Appendix C, Pg 148.
Statement of Contribution

N.J.C contributed to the conception and design of the study, performed data acquisition, data analysis/interpretation and was responsible for drafting and revising the manuscript. E.A.M. and J.E.H., contributed to the conception and design of the study inputted into manuscript.
Chapter 4: Overview

Chapters 1-3 explored the role of direct, in utero, multigenerational and transgenerational exposure to cigarette smoke on female fertility. It is well appreciated that chemotoxicant exposure has adverse effects on female fertility, however, maternal ageing is the leading and the least understood cause of reduced oocyte quality and spontaneous abortion.

A large body of research shows that maternal ageing reduces oocyte quality, ultimately leading to increased oocyte aneuploidy, and therefore elevated spontaneous abortions or trisomy conceptions in women over 35 years of age (Hassold and Hunt, 2001). Furthermore, research suggests that this reduced oocyte quality is multifactorial with evidence pointing to reduced chromosomal cohesion, decreased cell cycle checkpoint sensitivity and alterations in number and function, to name a few (Chiang et al., 2010; Eichenlaub-Ritter et al., 2011; Yun et al., 2014).

To gain further insight into how oocyte quality is maintained, the role of the kinesin motor protein Kif4 was examined in young healthy oocytes, in addition to its expression in maternally aged oocytes. Kif4 is a chromokinesin that has essential roles in mitosis, including chromosome condensation and separation, spindle and midzone formation and cytokinesis (Hu et al., 2011; Mazumdar et al., 2004). However, the role of this protein in mammalian meiosis has not been examined to date. To address this, the following study used anti-sense technology knock down of Kif4, and small molecular inhibitor approaches, to determine the function and interacting partners of Kif4 throughout meiosis.

Data within this manuscript suggests Kif4 has essential roles throughout oocyte meiosis, and maybe be involved in kinetochore dynamics, under the control of Aurora Kinase B and Cdk1.
Kif4 is essential for mouse oocyte meiosis

**Key Words:** oocyte/meiosis/Kif4/CENP-C/Ndc80/Aurora kinase B/Cdk1

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Abstract

Progression through the meiotic cell cycle must be strictly regulated in oocytes to generate viable embryos and offspring. During mitosis, the kinesin motor protein Kif4, is indispensable for chromosome condensation and separation, midzone formation and cytokinesis. Additionally, the bioactivity of Kif4 is dependent on phosphorylation via Aurora Kinase B and Cdk1, which regulate Kif4 function throughout mitosis. Here, we examine the role of Kif4 in mammalian oocyte meiosis. Kif4 localized in the cytoplasm throughout meiosis I and II, but was also observed to have a dynamic subcellular distribution, associating with both microtubules and kinetochores at different stages of development. Co-localization and proximity ligation assays revealed that the kinetochore proteins, CENP-C and Ndc80, are potential Kif4 interacting proteins. Functional analysis of Kif4 in oocytes via antisense knock-down demonstrated that this protein was not essential for meiosis I completion. However, Kif4 depleted oocytes displayed enlarged polar bodies and abnormal metaphase II spindles, indicating an essential role for this protein for correct asymmetric cell division in meiosis I. Finally, investigation of the phosphoregulation of meiotic Kif4, revealed that Aurora Kinase and Cdk activity is critical for Kif4 kinetochore localization and interaction with Ndc80 and CENP-C.

Introduction

The fidelity of chromosome segregation during meiosis is key to producing high quality oocytes, capable of creating healthy offspring. In mammalian oocytes, errors in chromosome separation result in embryonic aneuploidy, which may lead to spontaneous abortion or trisomy births such as Downs Syndrome. A known risk factor associated with oocyte aneuploidy is maternal ageing, with the aneuploidy incidence approximately 2% for women in their 20s increasing to 35% for women in their 40s.\textsuperscript{1-3} In recent years, a growing body of
Evidence suggests that alterations in chromosome architecture with age result in abnormal kinetochore structure, aberrant kinetochore microtubule (KT-MT) interaction and ultimately aneuploidy. To further elucidate how such events arise, we investigated a kinesin motor protein, Kif4 which has known roles in microtubule flux.

The kinesin motor protein Kif4 (human homologue KIF4A) is a member of the Kinesin-4 subfamily, consisting of KIF4A and B in humans and Kif4 in mice. Studies in mitotic systems have revealed key roles for Kif4 in chromosome condensation and separation, metaphase and midzone spindle formation as well as cytokinesis. Such functions are achieved through the ability of Kif4 to interact with condensin I, influence kinetochore protein loading, regulate microtubule length throughout metaphase and telophase and control microtubule kinetochore flux. A growing body of evidence suggests that Kif4 function is mediated by cell cycle kinases Cyclin-dependent kinase 1 (Cdk1) and Aurora Kinase B (AurB). Loss of Kif4 has also been associated with multiple tumours and is evident in cancer cell lines, indicating an important role for this protein throughout the cell cycle.

To date the role of Kif4 in mammalian fertility has not been investigated. However, the Drosophila homologue Klpl3A is essential for male and female fertility. In males, mutations in Klpl3A results in midzone instability and cytokinesis failure in meiotically dividing spermatocytes. In contrast, females are able to successfully complete meiosis, however, oocytes are unable to support embryogenesis. In meiotic Xenopus egg extracts, the Xenopus homologue XKlp1, limits microtubule growth and is important for correct spindle shape. Thus, in the present study we sought to characterize the localization and role of mammalian Kif4 in female meiosis. Additionally, we present evidence indicating Kif4 is involved in kinetochore dynamics under the control of both AurB and Cdk1.
Materials and Methods

All reagents were obtained from Sigma-Aldrich unless otherwise specified.

Animals and Ethics Approval

Animal use for this study was approved by the University of Newcastle Animal Care and Ethics Committee. C57BL/6 x CBA F1 hybrid cross were obtained from the University of Newcastle Animal Resources and housed with ad libitum water and food under a 12hrs light: 12hrs dark cycle.

Oocyte Collection and Maturation

4-6 week old female mice were intraperitoneally (i.p) injected with 7.5 IU of pregnant mares’ gonadotropin (Intervet) 44-52 hrs prior to a second i.p injection of 5 IU human chorionic gonadotropin (Intervet) or germinal vesicle (GV) oocyte collection from the ovary. 12 hrs after the second injection MII stage eggs were collected from the ampulla. Oocytes were collected into M2 media containing BSA. For GV collections media was supplemented with 1mM milrinone (M4659) to prevent meiotic resumption. Cumulus cells were then removed enzymatically with hyaluronidase (300 µg/ml; H4272) for MII or mechanically via repeated pipetting for GV oocytes. For collection from aged (12-19 months) females and corresponding young controls, GV oocytes were retrieved directly from the ovary without prior hormonal stimulation.

To allow meiotic maturation in vitro, GV oocytes were washed into milrinone free M2 media at 37°C (GVB/MI) or MEM (MII; 11900024, Gibco) with 20% FCS at 37 ℃ in 5% CO2. GV oocytes underwent IVM for 1.5 hrs for GVB stage, 7.5hrs for MI stage or 16 hrs for MII stage oocytes. For inhibitor experiments 10 µM ZM447439 (2458, Tocris), 100 µM
Roscovitine (R7772), 100 nM BI 2539 (S1109, Selleck) or DMSO was added to media at 3.5 hrs post washout.

**Immunocytochemistry and Proximity Ligation Assay**

For all immunocytochemistry oocytes were fixed in 2% paraformaldehyde in PBS with 0.5% triton X for 30 mins. Oocytes were blocked in 7% goat serum PBS-0.1% Tween for 1hr prior to overnight incubation with primary antibody (see Supplementary Table 1). Secondary antibodies were conjugated with Alexa-488, Alexa-555 and Alexa-633 (1:1000; Life Technology) and incubated with oocytes for 1hr at room temperature prior to Hoechst (20 µg/ml) counter staining and mounting in Citifluor (Citifluor Ltd). Secondary only negative controls were also performed to confirm the specificity of immunocytochemistry.

Proximity ligation assay (DUO92101; PLA) was performed as per manufactures instructions to detect proteins within 40 nm of each other, and therefore potential protein-protein interactions. Oocytes were then counterstained in Hoechst and mounted in Citifluor as above. Specificity of PLA was confirmed through the use of negative controls including Kif4 antibody only, secondary antibody only or Kif4 antibody with testis specific protein Piwil1 antibody.

Imaging was performed using an Olympus FV1000 confocal using a 60x/1.2 NA UPLSAPO oil immersion objective lens (Olympus, Australia). ImageJ (freeware; National Institute of Health) was used to measure fluorescent intensity and count PLA foci as previously described.

**Oocyte qRT-PCR**

Five denuded oocytes were used per reaction. The zona pellucida was removed with Acid Tyrodes solution (T1788) prior to being washed in PBS/ polyvinylpyrrolidone. Following
lysis, cDNA was prepared using the TaqMan Gene Expression Cells-toC\textsubscript{T} Kit (AM1728, Ambion) as per manufactures instructions. A whole cDNA sample was used per one reaction. qRT-PCR was performed with a Light Cycle 96 SW 1.1 (Roche) using TaqMan mRNA assay (4369016, ThermoFisher Scientific) as per manufactures instructions. P\textsubscript{pi}a was employed as an endogenous control to normalize the expression levels of Kif4. The relative expression levels of Kif4 were calculated using the $\Delta C_t$ method.

**Oocyte Microinjection**

GV oocytes were microinjected as previously described by Holt et al., with either Kif4 knock-down or mis-match morpholino oligo (Gene-tools; see Supplementary Table 2 for targeting sequence).\textsuperscript{30,31} Oocytes were then incubated in MEM (11900024, Gibco) with 20% FCS at 37°C in 5% CO\textsubscript{2} for 24 hrs to allow protein knock-down.

**Phosphorylation Site Prediction**

Potential AurB and Cdk1 phosphorylation sites for Kif4 (accession BAA02167.1) were predicted using GPS 3.0 (freeware, The Cuckoo Workgroup).\textsuperscript{32-34}

**Statistics**

GraphPad Prism 6.0 software (GraphPad Software, Inc) was used for statistical analysis. For numerical data D’Agnostino-Pearson omnibus normality test was performed. For normally distributed data Student’s $t$-test was performed. For data that was not normally disturbed Mann-Whitney test or Kruskal-Wallis Test with Dunn’s post-hoc statistical test was performed. For categorical data Fisher’s Exact Test was used. A p value $<0.05$ was considered statistically significant.

**Results**
Kif4 has dynamic localization throughout meiosis

The localization of Kif4 through four distinct stages of female meiosis was investigated, using an antibody directed against the N-terminal motor domain. At each stage examined, Kif4 was found dispersed throughout the cytoplasm and in cytoplasm aggregates (Fig 1A). Additionally, it displayed distinct changes in subcellular localization dependent upon meiotic stage. In GV arrested oocytes, Kif4 was found to be enriched around the nuclear envelope and chromatin (Fig 1A). During prometaphase (post-GVB), Kif4 was distributed around the chromosomes, presumably associated with the microtubule ball, which forms as the meiotic spindle develops (Fig 1A). By MI, Kif4 had relocated and was now associated with the chromosomes were it co-localized with centromere/kinetochore marker ACA (Fig 1B.) To study this change in localization more closely, we analyzed fixed oocytes every 30 minutes throughout late prometaphase-MI and confirmed that, as Kif4 was lost from the microtubules, it become associated with the kinetochore region of the chromosomes (Fig 1C).

Once the polar body was extruded and oocytes had reached MII arrest, Kif4 was once again associated with the microtubules on the metaphase II spindle (Fig 1A). To confirm Kif4 localization throughout meiosis, immunocytochemistry was performed using a second antibody directed against the C-terminal cargo domain with a similar pattern observed (Supplementary Fig 1A).

Kif4 interacts with both inner and outer kinetochore proteins

Having established that Kif4 was co-located with the centromere/kinetochore at MI, we next sought to investigate whether this kinesin might interact directly with key kinetochore proteins. Immunolocalization revealed Kif4 signal overlap with the inner kinetochore protein, CENP-C at all meiotic stages examined. CENP-C associated with Kif4 aggregates in GV and MII oocytes (Fig 2A) and as expected, CENP-C and Kif4 foci overlapped at the kinetochore,
at both MI and MII (Fig 2A). To determine whether these proteins interacted, we performed a proximity ligation assay (PLA) on fixed oocytes using the CENP-C and Kif4 antibodies, and observed positive PLA signal throughout the cytoplasm, at all three stages of meiosis examined (Fig 2B). Furthermore, PLA foci were also associated with the chromosomes, and therefore presumably the kinetochore at MI and MII (Fig 2B and Supplementary Fig 1B).

To gain further insight into the role of Kif4 at the kinetochores, we next investigated its interaction with the outer kinetochore protein Ndc80. As with CENP-C, Ndc80 co-localized with Kif4 aggregates throughout GV oocytes (Fig 3A). At MI Kif4 foci overlapped with ACA fluorescence, however, Ndc80 foci flanked either side, indicative of its role as an outer kinetochore protein (Fig 3A and Supplementary Figure 1C). By MII, both Ndc80 and Kif4 were found on the metaphase spindle. Confirmation of a potential protein-protein interaction was achieved via PLA, with proteins interacting cytoplasmically throughout meiosis (Fig 3B). Additionally, as observed previously, these PLA foci were associated with chromosomes at MI and MII (Fig 3B and Supplementary Fig 1D).

**Kif4 is essential for normal oocyte meiosis**

The spindle/kinetochore localization and potential binding partners of Kif4 suggested it may be important for meiotic progression. To examine its role more closely Kif4 knock-down (KD) was performed via oligo morpholino translational repression. Using immunocytochemistry we confirmed Kif4 protein expression was reduced 2.5 fold 24hrs post morpholino injection when compared to mis-match morpholino injected controls (0.39±0.25 AU KD v 1±0.07 AU control, p<0.0001; Fig 4A). IVM of KD oocytes found no abnormalities in meiosis timing or maturation rates (data not shown). However, investigation of MII oocyte quality found KD oocytes had a significantly higher proportion of abnormal MII spindles compared to controls (48% knock-down v 12% controls, p=0.0017; Fig 4B).
Spindle abnormalities included mild and severe chromosome misalignment (27% of oocytes), and microtubule aggregates attached to spindles (7% of oocytes). Additionally, KD oocytes were observed to have abnormal cytokinesis as determined by a significant increase in polar body size (5489±1692 µm² KD v 2661±522 µm² control; Fig 4C).

**Kif4 and kinetochore protein interaction is mediated via kinase activity**

Since meiosis is driven by the activity of several important kinases, we next sought to determine if the function of Kif4 was regulated in this manner. Previous research has found that Cdk1 and AurB mediate the interaction of Kif4 with condensin I. Furthermore, AurB has been found to directly interact with Kif4 at mitotic midzones. Group based prediction of AurB and Cdk1 phosphorylation sites on Kif4 found 9 potential Cdk1 sites and 13 potential AurB sites located throughout the three major domains; motor, coiled-coil and cargo (Fig 5A). Of note is that 4 of these sites, serine 816, 1224, 1230 and threonine 800, are known Kif4 phosphorylation residues. Additionally threonine 800 is a well categorized AurB phosphorylation site.

To examine whether Cdk1 and AurB kinase activity did in fact regulate Kif4, oocytes were allowed to mature in the presence of either the pan Aurora Kinase inhibitor ZM 447439 (ZM) or the pan Cdk inhibitor Roscovitine (Ros) for 4hrs from prometaphase to MI. Interestingly immunolocalization of Kif4 to the kinetochore was lost after treatment with both ZM and Ros (Fig 5B). Furthermore, PLA analysis found that both treatments significantly reduced Kif4/CENP-C and Kif4/Ndc80 interaction compared to the vehicle control (CENP-C: 0.58±0.27 PLA foci ZM v 0.54±0.37 PLA foci Ros v 1±0.50 PLA foci Veh, p=0.0013; Fig 5C and Ndc80: 0.44±0.12 PLA foci ZM v 0.60±0.19 PLA foci Ros v 1±0.25 PLA foci Veh, p<0.0001; Fig 5D).
To confirm that these results were the consequence of specific Aurora Kinase or Cdk regulation oocytes were allowed to mature in the presence of the Plk1 inhibitor BI 2536. Unlike Ros or ZM treatment, BI 2536 had no effect on Kif4 kinetochore localization consistent with the lack of Plk1 and Kif4 co-localization off the kinetochores (Supplementary Fig 2).

**Kif4 expression increases with maternal ageing**

Finally, we sought to determine if Kif4 protein was altered with maternal age in oocytes which might be associated with their reduced quality. Immunocytochemistry revealed that Kif4 expression was unchanged in young vs aged GV stage oocytes (0.95±0.39 AU Aged v 1±0.28 AU Young, p=0.6891; Fig 6). However, by MI there was a significant increase in protein expression (1.73±0.93 AU Aged v 1±0.26 AU Young, p=0.0025; Fig 6) which continued into MII (1.81±0.28 AU Aged v 1±0.28 AU Young, p<0.0001; Fig 6). Quantitative gene expression (qRT-PCR) showed no change in transcript level at GV (p=0.0741; Supplementary Fig 3A) or MII (p=0.6964; Supplementary Fig 3B) indicating the increase of Kif4 may have been a result of elevated translation or reduced protein turnover with age.

**Discussion**

In the current study we have demonstrated an essential role for Kif4 in mammalian oocyte meiosis and highlighted its potential interaction with key kinetochore proteins and regulation by meiotically important kinases.

The localization of Kif4 in oocytes is dynamic: we observed that as meiosis I progressed Kif4 left the metaphase I spindle and became enriched on the chromosomes and kinetochores. This enrichment at the kinetochore towards the end of metaphase I occurs at time when kinetochore-microtubule (KT-MT) attachments are stabilized and so could indicate a role for
Kif4 in this stabilization. In line with this, is the known role of Kif4 in microtubule stabilization in mitotic cells and *Xenopus* egg extracts. Ablation of Kif4 from cells lines or XKlp1 from *Xenopus* egg extracts significantly altered microtubule dynamics including microtubule overgrowth, decreased KT-MT flux and altered kinetochore oscillations.\(^7, 27, 36, 37\)

These altered microtubule dynamics ultimately lead to abnormal spindle formation and misaligned chromosomes, cumulating in aneuploidy with misaligned chromosomes as observed in our KD oocytes.\(^9, 36, 37\) Evidence in mitotic extracts suggests that the abnormal microtubule dynamics is a likely cause of XKlp1 limiting microtubule growth via allosteric inhibition of microtubule dynamic instability.\(^38\) Alterations in microtubule stability, growth and kinetochore oscillations could account for the metaphase spindle abnormalities observed in KD oocytes. Increased microtubule length, and reduced kinetochore oscillations would be expected to cause the chromosome misalignment we observed. Further to this, microtubule overgrowth was detected in Kif4 ablated oocytes, with the addition of microtubule spheres attached to spindle poles.

Cytokinesis abnormalities were also a common feature in Kif4 KD oocytes. Ablation of Kif4, XKlp1 or Klp3A mutation results in cytokinesis failure in HeLa cells, mitotic *Xenopus* egg extracts or *Drosophila* spermatocytes respectively.\(^15, 24, 37\) Conversely, we have shown that depletion of Kif4 in oocytes results in abnormal cytokinesis as demonstrated via enlarged polar bodies. This indicates that Kif4 is not essential for cytokinesis but is necessary for asymmetric cytokinesis in oocytes.

Interestingly, enlarged polar bodies have previously also been observed in oocytes depleted of Ndc80.\(^39\) Of note is that Ndc80 is crucial for correct spindle formation and chromosome alignment at metaphase I and II in mouse and porcine oocytes.\(^39-41\) The similar phenotype observed between Ndc80 and Kif4 ablated oocytes in conjunction with their continued co-localization and potential interaction throughout meiosis highlights a probable role for Kif4 in
correct Ndc80 function throughout the cell cycle. Interestingly, Kif4 was also found to interact with CENP-C throughout meiosis. KD of CENP-C in DT40 chicken cells results in abnormal chromosome alignment similar to that seen in our Kif4 KD oocytes. It is therefore likely that Kif4 depletion from oocytes results in alterations to both CENP-C and Ndc80 localization, which may be responsible for the observed abnormal metaphase II oocyte phenotype.

It is also tempting to speculate that CENP-C and Ndc80 are potential cargo proteins of Kif4, with Kif4 involved in the correct shuttling of these proteins throughout the oocyte. In support of this theory, KD of Kif4 in DT40 cells significantly altered the expression of a large number of kinetochore proteins, including CENP-C, Ndc80 and AurB specifically on chromosomes. We found that interaction of kinetochore proteins CENP-C or Ndc80 with Kif4 was not restricted to chromosomes, but was also found throughout the cytoplasm of meiotically cycling oocytes. As kinesins have traditionally been associated with roles in cargo shuttling, it is possible that Kif4 is at least partially responsible for shuttling CENP-C/Ndc80 to and from the chromosomes. Ablation of Kif4, therefore, could result in incorrect loading of CENP-C/Ndc80 to the kinetochore which might account for the chromosome misalignment observed.

Intriguingly, the movement of Kif4 to the kinetochores or its interaction with CENP-C and Ndc80 appears to be cell cycle dependent and under the control of Aurora Kinase and Cdk activity. CENP-C and Ndc80 expansion has been found to be AurB dependent and essential for correct KT-MT attachments. Further to this, AurB is responsible for phosphorylation of human Kif4 T799 (mouse T800), with an additional predicted AurB phosphorylation sites identified. AurB/C is essential in meiosis I for correct KT-MT attachment and spindle formation, with AurB/C destabilizing KT-MT attachments to allow error correction. The phosphatase PP2A is a known antagonist of AurB and has also been found to interact with
Kif4, reversing T799 phosphorylation. Furthermore, Kif4 has been found to regulate PP2A localization throughout mitosis. In contrast, Cdk1 appears to regulate KT-MT attachment stabilization. Cdk1 activity increases throughout meiosis I with Cdk1 inhibition during prometaphase/metaphase I leading to a reduction in stable KT-MT attachments. Conversely, over activation of Cdk1 leads to accelerated stabilization of KT-MT during meiosis I. It is therefore possible that, AurB and Cdk1 recruits Kif4 to the kinetochore during the later stages of prometaphase when microtubule stabilization is occurring. This accumulation, could in turn recruit PP2A. Stabilization of correct KT-MT attachments via Kif4 directly, and PP2A dephosphorylation of AurB/C substrates could then occur. In support of this, KD of Kif4 in oocytes led to a significant increase in spindle abnormalities including misaligned chromosomes, likely a result of incorrect KT-MT attachments. In addition, the localization of Kif4 to the kinetochore and its interaction with kinetochore proteins appears to be under kinase control.

It is important to note, however, that inhibitors used throughout this study are pan Cdk and Aurora Kinase inhibitors. Research into the role of other cyclin dependent kinases in oocyte meiosis is limited, however it appears probable that Cdk1 is the key Cdk in oocyte meiosis. As Cdk1 has been found in numerous studies to interact with Kif4, it is also the most likely candidate for Kif4 regulation in oocytes. Additionally, to date there is evidence for AurB but not AurC, directed regulation of Kif4. ZM inhibits both AurB and AurC and unlike somatic cells AurC regulation has been found to have essential roles in oocyte meiosis, with multiple proteins being controlled via both AurB and C kinases. Therefore it cannot be ruled out that AurC may be involved in Kif4 functioning throughout meiosis, although this requires further investigation.

Finally, maternal ageing is a well-established cause of reduced oocyte quality, which ultimately leads to subfertility. Considering the overexpression of Kif4 at the protein level in
reproductively aged mice at the MI/MII stage, this indicates a potential role for Kif4 in age related oocyte quality decline. Interestingly, aged GV oocytes had normal Kif4 levels, suggesting that Kif4 upregulation occurs as a result of its increased translation or reduced turnover post meiotic resumption. This increased expression of Kif4, a known microtubule stabilizing protein, could result in increased microtubule stabilization throughout meiosis in aged oocytes. In support of this, Kif4 overexpression in migrating fibroblasts has been shown to increase the number of microtubules resistant to the microtubule destabilizing agent nocodazole - consistent with a role for Kif4 in microtubule stabilization. Furthermore, maternally aged oocytes are less sensitive to nocodazole treatment, with aged oocytes having increased ability to complete meiosis I in the presence of nocodazole compared to young oocytes. Additionally, maternally aged oocytes have a higher frequency of KT-MT attachment errors, ultimately leading to aneuploidy. It is therefore possible that overexpression of Kif4 with age partially desensitizes oocytes to spindle abnormalities and incorrect KT-MT attachment. However, further investigation is needed to determine the potential cause and consequence of Kif4 upregulation with maternal age.

In conclusion we have found that Kif4 is expressed throughout oocyte meiosis and has essential roles in cytokinesis and spindle formation. Furthermore, Kif4 localization and interaction with kinetochore proteins appears to be regulated via AurB and Cdk1. In addition, its upregulation with age makes Kif4 a promising lead protein in our understanding of age-related oocyte quality decline.

Acknowledgements

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Note: Supplementary figures and tables are located in Appendix C, Pg 148.
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Figure 1: Kif4 has dynamic localization throughout oocyte meiosis. (A) Fluorescent immunolocalization of Kif4 (grey) at GV, GVB, MI and MII. Scale bar=10µm. (B) Fluorescent immunolocalization of Kif4 (grey/green) to the kinetochore at MI (red arrow). Kinetochores are counter labelled with the inner kinetochore marker ACA (red). Scale bar=2µm. (C) Fluorescent immunolocalization of Kif4 throughout MI at 6hrs to 8.5hrs post milrinone wash-out. Scale bar=10µm. DNA is counterstained with Hoechst (blue).
Figure 2: Kif4 co-localizes and interacts with inner kinetochore protein CENP-C throughout meiosis. (A) Fluorescent immunolocalization of Kif4 and CENP-C at GV, MI and MII. MI and MII oocytes are counter labelled with the inner kinetochore marker ACA (red). Red arrows highlight Kif4 and CENP-C aggregates. Secondary antibody only controls reveal nonspecific antibody binding to the zona pellucida only. (B) Positive proximately ligation
assay (red) between Kif4 and CENP-C at GV, MI and MII is indicative of protein-protein interaction. Insets highlight interaction on chromosomes (See Supplementary Figure 1B for additional examples). PLA negative control of Kif4 with testis specific Piwil1 antibodies reveal no nonspecific signal amplification. DNA is counterstained with Hoechst (blue). Scale bar=10µm.
Figure 3: Kif4 co-localizes and interacts with outer kinetochore Ndc80 throughout meiosis. (A) Fluorescent immunolocalization of Kif4 and Ndc80 at GV, MI and MII. MI oocytes are counter labelled with the inner kinetochore marker ACA (red). Secondary only controls reveal nonspecific antibody binding to the zona pellucida only. (B) Positive proximately ligation assay (red) between Kif4 and Ndc80 at GV, MI and MII is indicative of protein-protein interaction. Insets highlight interaction on chromosomes (See Supplementary Figure 1D for additional examples). PLA negative control of Kif4 with testis specific Piwil1 antibodies reveal no nonspecific signal amplification. DNA is counterstained with Hoechst (blue). Scale bar=10µm.
**Figure 4**: Kif4 is essential for correct spindle formation and cytokinesis. (A) Fluorescent immunolocalization of Kif4 (grey) in control and knock-down (KD) oocytes. Graphical representation of normalized Kif4 fluorescence; p<0.0001, Mann-Whitney test. (B) Fluorescent immunolocalization of α-tubulin at MII in control and Kif4 KD. Kif4 KD oocytes were found to have a higher percentage of abnormal spindles (orange arrow) and chromosome misalignment (red arrow). Graphical representation of abnormal spindle
percentages per group; \( p=0.0017 \), Fishers Exact test. (C) Phase contrast images of control and Kif4 KD MII oocytes. Graphical representation of PB1 size between control and Kif4 KD oocytes; \( p<0.0001 \), Mann-Whitney test. Box plots show mean (centerline) with box outline 25-75\(^{th}\) percentiles and whiskers 10-90\(^{th}\) percentiles, bar graphs show mean. \( n=\) number of oocytes examined from 3 replicates. DNA is counterstained with Hoechst (blue), scale bar=10\(\mu\)m.
Figure 5: Kinase inhibition reduces Kif4 kinetochore protein interaction. (A) Schematic representation of mouse Kif4 including motor (pink), coiled-coil (purple) and cargo (green) domains. Serine (S) and threonine (T) predicated phosphorylation sites for Cdk1 (red) and
AurB (blue) are labelled. * indicates known Kif4 phosphorylation sites. (B) Fluorescent immunolocalization of Kif4 (grey) at MI following 3.5hrs of treatment with vehicle (Veh) DMSO, aurora kinase inhibitor ZM 447439 (ZM) or CDK inhibitor Roscovitine (Ros). (C) PLA (red) of Kif4 and CENP-C at MI following 3.5hrs of treatment with the Veh, ZM or Ros. Graphical representation of normalized PLA foci per oocyte; p=0.0013, Kruskal-Wallis with Dunn’s Post-hoc. (D) PLA (red) of Kif4 and Ndc80 at MI following 3.5hrs of treatment with the Veh, ZM or Ros. Graphical representation of normalized PLA foci per oocyte; p<0.00031, Kruskal-Wallis with Dunn’s Post-hoc. Box plots show mean (centerline) with box outline 25-75th percentiles and whiskers 10-90th percentiles; n=number of oocytes examined from 3 replicates. DNA is counterstained with Hoechst (blue). Scale bar=10µm.
**Figure 6:** Kif4 protein expression increases with maternal ageing. Fluorescent immunolocalization of Kif4 (grey) in GV, MI and MII oocytes from young and maternally aged animals. Graphical representation of normalized Kif4 fluorescence at GV (p=0.6891, Student’s *t*-test), MI (p=0.0025, Student’s *t*-test) and MII (p<0.0001, Student’s *t*-test). Box plots show mean (centerline) with box outline 25-75th percentiles and whiskers 10-90th percentiles; n=number of oocytes examined from 4 animals. DNA is counterstained with Hoechst (blue). Scale bar=10µm.

Note: Supplementary figures and tables are located in Appendix C, Pg 148.
CHAPTER 5: Literature Review

Motoring through: The role kinesin superfamily of proteins in female meiosis

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Statement of Contribution

N.J.C., E.A.M. and J.E.H., contributed to the conception and design of the review. N.J.C was also responsible for drafting and revising the manuscript.
Chapter 5: Overview

Having established in Chapter 4 the essential role of Kif4 in oocytes, I reviewed the role of all known kinesin motor proteins in female meiosis.

This chapter explores the function(s) of all 14 classes of mammalian kinesin motor proteins, throughout female meiosis. Where research has not been undertaken in oocytes, mitotic functions are examined and extrapolated to potential meiotic roles. Throughout this review, we discuss all major model organisms; mouse, *Xenopus* egg extracts, *Drosophila*, and *C. elegans*, and highlight the potential clinical importance of kinesin proteins. From this we conclude that many kinesin motor proteins are essential for oocyte meiosis and that more research is needed into their roles throughout meiosis which may yield information of clinical importance.
Motoring through: The role of kinesin superfamily proteins in female meiosis

Key Words: Kinesin motor protein/ oocyte / meiosis / cell cycle

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Abstract

The kinesin motor protein family consists of 14 distinct subclasses and 45 kinesin proteins in humans. A large number of these proteins or their orthologues have been identified to have essential functions throughout the cell cycle in both mitotic and meiotic cells. Kinesins have important roles in chromosome separation, microtubule dynamics, spindle formation, cytokinesis and cell cycle progression. This article represents a systematic review of the literature regarding the role of kinesin motor proteins in female meiosis in model species. Throughout we discuss the function of each class of kinesin proteins during oocyte meiosis, and where such data is not available their role in mitosis is considered. Finally, this review highlights the potential clinical importance of this family of proteins for human oocyte quality.

Introduction

The production of haploid gametes relies on two rounds of nuclear cell division following one round of DNA synthesis in a process called meiosis. Correct chromosome division during meiosis is essential to establish the next generation of offspring, with aneuploidy the leading cause of pregnancy loss in humans.\(^1\) This aneuploidy is largely the result of chromosome separation errors in the oocyte rather than the sperm, with oocytes being surprisingly error prone. Furthermore, the frequency of aneuploid oocytes increases dramatically with maternal age.\(^1,2\)

The kinesin (Kif) superfamily of proteins have proven essential in macromolecule transport, mitosis and even meiosis.\(^3\) Kif proteins are a group of motor proteins that can be divided into 14 classes.\(^4\) The majority comprise two domains, an ATP hydrolysis domain that allows it to traverse microtubules usually in a plus-end directed manner and a tail domain that is able to bind to structures and/or cargos.\(^3,4\) In this review, we focus on the role of Kif proteins in
female meiosis of model animal species and highlight their potential clinical role in human oocytes.

**Female Meiosis**

Meiosis is a unique cell division that gives rise to haploid germ cells. In males, spermatogenesis is generally continuous, however, in females meiosis contains one to two arrest phases dependent on species (Figure 1). In all species meiosis I begins and oocytes enter prophase I, where they undergo their first meiotic arrest. This arrest is overcome by various stimuli and the nuclear envelope breaks down (NEB). In *C. elegans*, oocytes are fertilized at this point and continue through meiosis without further stopping. During metaphase I spindle formation, chromosomes attach to microtubules and line up along the metaphase plate. It is here at metaphase I that *Drosophila* oocytes undergo their second arrest, until ovulation. Next chromosomes separate at anaphase I and in all but *Drosophila*, half the chromosomes are extruded into the polar body at telophase I. Metaphase II continues on from this with no intervening S-phase. Mammalian and *Xenopus* oocytes undergo their second meiotic arrest at metaphase II until fertilization. As before, chromosomes are separated and extruded into a second polar body with *Drosophila* an exception. Similar to mitosis, meiosis is strictly controlled to ensure correct chromosome segregation via the anaphase promoting complex (APC/C) and the spindle assembly checkpoint (SAC) (Figure 2). For a detailed review of the APC/C and SAC in mammalian female meiosis see Homer, 2013, Holt and Jones, 2009 (APC/C) and Jones and Lane, 2013, Touati and Wassmann, 2016 (SAC).

**The kinesin superfamily and their roles in the cell cycle**

Kinesin motor proteins are a large group of enzymes, with 45 recorded in human cells; See Table 1 for mammalian kinesin proteins and their orthologues. These kinesin proteins have
varied functions and have been sub-classified into 14 classes based on the sequence of their motor domain.\textsuperscript{16}

Kinesins have been found to be indispensable throughout the cell cycle in both mitotic and meiotic cells including regulation of chromosome condensation and alignment, spindle formation, cytokinesis and cell cycle progression. The activity of each family of kinesin motor proteins in female meiosis is explored, with mitotic functions considered when no meiotic information exists.

\textbf{Roles of kinesin motor proteins in oocyte meiosis}

\textbf{Kinesin 1 Family}

Kinesin 1 motor proteins are heterotetramers consisting of two kinesin heavy chains and two kinesin light chains which have been found to be important throughout meiosis.\textsuperscript{17} Mammalian cells have 3 kinesin 1 proteins, Kif5A, Kif5B and Kif5C, with \textit{C.elegans} having 1 kinesin 1 consisting of the heavy chain UNC-116 and the light chains KLC-1 and 2.\textsuperscript{3,18}

The function of the mammalian orthologue Kif5B and \textit{C.elegans} heavy and light chains during meiosis has been investigated. Throughout meiosis I, porcine Kif5B has a distinct localization pattern.\textsuperscript{19} Prophase I arrested oocytes contained little to no Kif5B, however, after NEB (nuclear envelope breakdown) Kif5B was present at the oocyte periphery and then throughout the cytoplasm at metaphase I, only to be lost from the oocyte again by metaphase II. Interestingly in porcine oocytes with low meiotic competence Kif5B was retained at the periphery.\textsuperscript{19} An essential role of Kif5B in normal meiosis has been confirmed in both mouse and \textit{C.elegans} oocytes.\textsuperscript{18,20} In mouse oocytes, knock-down of Kif5B delays NEB and prevents the extrusion of the first polar body.\textsuperscript{20} Similarly, knock-down of kinesin 1 heavy chain UNC-116 or both light chains in \textit{C.elegans}, also causes failure of one or both polar
bodies to be extruded. Further investigation found that UNC-116 was essential for timely spindle migration and rotation at both metaphase I and II. The other kinesin 1 proteins Kif5A and Kif5C are unlikely to be important in meiosis as their expression is limited solely to neuronal cells.

Kinesin 3 Family

The largest subfamily of kinesin motors, the kinesin 3 family, comprises 8 proteins; Kif1A, Kif1B, Kif1C, Kif13A, Kif13B, Kif14, Kif16A and Kif16B. Of these only the *Xenopus* Kif14 paralogue NabKin and the *Drosophila* orthologue Klp38B have been studied in oocyte meiosis. NabKin was found to bind to both microtubules and F-actin in *Xenopus* oocytes throughout meiosis, including the actomyosin contractile ring at cytokinesis. Knock-down of NabKin during meiosis results in failed cytokinesis, with all chromosomes retained within the oocyte. Additionally metaphase II oocytes contained spindles that were abnormally large or had two spindles. Of interest is that in somatic cells, the mammalian paralogue Kif14 has a similar localization pattern around the actomyosin contractile ring in HeLa cells with depletion preventing cytokinesis, potentially indicting a role for this protein in mammalian meiosis. Furthermore, mutation of *Drosophila* orthologue Klp38B, leads to increased chromosome nondisjunction rates, which is further increased in Klp38B/Nod double mutants. Although NabKin and Klp38B have distinct roles it is clear that both are essential for the development of a viable haploid gamete.

Investigation in somatic cells of the other 7 kinesin 3 motor proteins has established that Kif1B, Kif13A, Kif13B and Kif16A have roles in mitosis. Depletion of Kif1B in human cell lines was found to lengthen metaphase as a result of prolonged Aurora Kinase B (AurB) phosphorylation, and increased Mad2 at the kinetochores which was responsible for maintaining the spindle assembly checkpoint (SAC). Both Kif13A and Kif13B are essential...
for cytokinesis with *in vitro* knock-down resulting in multinucleated cells.\(^{25, 26}\) Finally, in human cell lines, depletion of Kif16A led to SAC-mediated prometaphase arrest.\(^{27}\) The role of these kinesins in meiosis has not yet been examined.

**Kinesin 5 Family**

The kinesin 5 family consists of 1 member in mammals and *Xenopus*, Kif11 (Eg5) and 1 in *C. elegans*, BMK-1.\(^{28-30}\) These kinesin 5 orthologues are highly conserved and involved in crosslinking parallel spindle fibers found near spindle poles and antiparallel fibers at the spindle equator throughout the cell cycle.\(^{31}\)

The vertebrate orthologue Kif11 is expressed during oogenesis and meiosis, with protein levels increasing throughout prophase I arrest and prometaphase I in *Xenopus* oocytes, indicating a role throughout meiosis.\(^{30}\) In confirmation of this, transient exposure of mouse oocytes to the Kif11 inhibitor monastrol during the first 6 hours of meiosis I leads to delayed NEB and increased numbers of metaphase I arrested oocytes.\(^{28}\) Furthermore, the metaphase I spindles exhibited abnormal morphology and misaligned chromosomes.\(^{28}\) Oocytes which did reach metaphase II, had an increased incidence of aneuploidy as a result of premature sister chromatid separation, rather than bivalent nondisjunction (Figure 3).\(^{28}\) Of note, is the changing distribution and role of this protein in *in vitro* (IVM) and *in vivo* (IVV) matured metaphase II mouse oocytes. IVM metaphase II spindles have abundant Kif11 and are particularly sensitive to monastrol compared to IVV metaphase II spindles, suggesting a role for Kif11 in oocyte quaility.\(^{32}\)

Intriguingly, this sensitivity to Kif11 loss appears to be limited to mammalian oocytes, with Kif11 having functional redundancy with Kif15 in mammalian somatic cells.\(^{33}\) Additionally, mutation or knock-down of BMK-1 in *C. elegans* oocytes has no effect on spindle formation or shape, with these oocytes producing viable embryos, suggesting that BMK-1 may also
have functional redundancy, as in mammalian somatic cells.\textsuperscript{29} Despite this, the \textit{C.elegans} AurB orthologue AIR-2, but not the Aurora Kinase A orthologue AIR-1, was found be essential for normal BMK-1 localization to the spindle throughout meiosis.\textsuperscript{29} Furthermore, AIR-2 was found to directly interact with BMK-1 whilst causing phosphorylation of its C-terminal.\textsuperscript{29} Direct interaction between Aurora Kinases and Kif11 has previously been found in \textit{Xenopus} mitotic cells.\textsuperscript{34} Moreover, use of the pan Aurora Kinase inhibitor ZM447436 in mouse oocytes leads to a similar phenotype as observed in monastrol treated oocytes, implicating Aurora Kinases in the correct functioning of Kif11 in mammalian female meiosis.\textsuperscript{35, 36}

\textbf{Kinesin 6 Family}

The kinesin 6 class of motor protein consists of 3 proteins Kif20A, Kif20B and Kif23, with this group of kinesins essential in mitotic cytokinesis.\textsuperscript{3, 37, 38} Investigation into the role of Kif20A in mammalian meiosis, has also found it is also vital for oocyte cytokinesis. Mouse oocytes treated with the Kif20A inhibitor paprotrain, triggered metaphase I to anaphase/telophase I transition arrest and failure to extrude the first polar body.\textsuperscript{39, 40} Interestingly, this occurred in the presence of a normal metaphase I spindle.\textsuperscript{39, 40} Loss-of-function mutation of subito, the \textit{Drosophila} Kif20 orthologue, led to a similar phenotype. Unlike other species, \textit{Drosophila} oocytes do not extrude polar bodies, but rather “polar body” chromosomes degrade post-fertilisation.\textsuperscript{6} As a consequence, rather than cytokinesis failure, \textit{Drosophila} oocytes are unable to separate chromosomes at meiosis I due to a lack of bipolar spindle formation in loss-of-function subito mutants.\textsuperscript{41} Metaphase I spindles in subito mutants have abnormal midzones, resulting in the mislocalization of midzone proteins AurB and Incenp, both of which are essential for cytokinesis in mouse oocytes.\textsuperscript{41-43}
Kif23 is also an obligatory protein for cytokinesis. In oocyte meiosis, loss of *C. elegans* orthologue ZEN-4 prevents polar body extrusion. Further investigation revealed that ZEN-4 knockdown results in failure of the Aurora Kinase AIR-2 to localize to the spindle midzone during meiosis I similar to that seen in subito mutants. In mitotic *C. elegans* embryos and HeLa cells, AIR-2/AurB phosphorylate ZEN-4/Kif23 on the spindle midzone, with inhibition of this phosphorylation preventing cytokinesis without disrupting midzone spindle formation or morphology. Additionally, recruitment of Kif20A to the equatorial cortex in HeLa cells is essential for AurB localization to this region, with inhibition resulting in failed cytokinesis. As inhibition of AurB in mouse or surf clam oocytes prevents polar body extrusion, it is possible that a similar mechanism of action is in play in oocyte cytokinesis.

Similar to both Kif20A and Kif23, Kif20B depletion leads to cytokinesis failure in somatic cells. Kif20B protein has been found to be expressed within the human ovary and therefore could potentially play a role in female meiosis. Despite species difference in oocyte meiosis, results collectively point to a key interaction between kinesin 6 proteins and Aurora Kinases culminating in an essential role in chromosome separation and cytokinesis.

**Kinesin 7 Family**

The kinesin 7 class of motor proteins are centromere-associated proteins that form part of the kinetochore. Most species including mammals and *Xenopus* have 1 kinesin 7 family member Cenp-E (also known as Kif10). However, *Drosophila* have 2 kinesin 7 proteins CMET and CANA. Cenp-E is essential for the completion of mouse meiosis I. Depletion via antibody or morpholino oligo injection leads to delayed meiosis I, a reduction in polar body extrusion rates and an increase in metaphase I arrest. Furthermore, these oocytes contained severely misaligned chromosomes and incorrect kinetochore orientation. This
phenotype was also observed in CMET mutant or CANA/CMET double mutant in *Drosophila* oocytes.\textsuperscript{52} Interestingly, in *Drosophila* with loss-of-function mutations in both CMET and the kinetochore scaffold protein SPC105R (KNL-1 orthologue), which is responsible for lateral kinetochore microtubule attachment, chromosome alignment is restored. Conversely, the chromosome misalignment defect remains in CMET/NDC80 double mutants.\textsuperscript{52} NDC80 is a kinetochore protein which is essential for end-on kinetochore microtubule attachment.\textsuperscript{52} Together this suggests that in *Drosophila* oocytes CMET is involved in SPC105R rather than NDC80 mediated microtubule-kinetochore attachments.

In Cenp-E depleted mouse oocytes, these misaligned chromosomes recruit Mad2 to the kinetochore and oocytes display a reduction in BubR1 and stabilization of APC/C substrates cyclin B1 and securin. This leads to a SAC mediated arrest at metaphase I (see Figure 2 for SAC / APC/C overview).\textsuperscript{53} Such a phenotype is reminiscent of that observed in BubR1 depleted oocytes, suggesting that the loss of BubR1 after Cenp-E knockdown could be a major cause of oocyte dysfunction.\textsuperscript{54} However, in a small subset of oocytes, Mad2 was still lost allowing for SAC fulfilment and the completion of meiosis I. Finally, in both mouse and *Xenopus* oocytes at metaphase II arrest, Cenp-E is masked in a Mos dependent manner, implicating its importance in the resumption of meiosis II in vertebrate species.\textsuperscript{51}

**Kinesin 10 Family**

Kif22 (also known as Kid) is a chromokinesin due to its ability to directly interact with chromosome arms and is the only known kinesin 10 motor protein.\textsuperscript{55} Nod, the *Drosophila* orthologue, localizes to the oocyte chromosomes and has two DNA binding domains, HGMN and HhH(2)/NDD.\textsuperscript{56,57} Interestingly, while the HGMN domain is essential for DNA binding in both oocytes and somatic cells the HhH(2)/NDD domain is only required for DNA binding in oocytes.\textsuperscript{57} This difference in binding sites between meiotic and mitotic cells suggests that
Nod may have slightly different functions between the two cell cycles. Functionally Nod has been found to be important for normal chromosome separation.\textsuperscript{57-60} Mutations in this motor protein cause an increase in aneuploidy via chromosome nondisjunction, but has no effect on chromosome recombination.\textsuperscript{57-60} Furthermore, natural populations of \textit{Drosophila} have been found to have two distinct polymorphisms in the Nod gene that account for increased chromosome nondisjunction.\textsuperscript{60} It is therefore intriguing to speculate that polymorphisms in the human orthologue Kif22, could account for increased susceptibility of certain women to oocyte aneuploidy.

**Kinesin 12 Family**

Kif12 and Kif15 make up the kinesin 12 family of proteins and have been found to have distinct roles throughout mitosis.\textsuperscript{3} In somatic cells, Kif15 is functionally redundant with the kinesin 5 Kif11. However, in Kif15 somatic cell knockouts with partial reduction of Kif11, there are effects on spindle elongation and bipolarity.\textsuperscript{33} Nonetheless, as with Kif11, Kif15 is not functionally redundant in oocytes. In \textit{C.elegans} the Kif15 orthologue KLP-18 is essential for spindle formation since knock-down in meiotic embryos results in failure to form a bipolar spindle.\textsuperscript{61, 62} Additionally, chromosomes are captured and congress around a monopolar spindle but fail to form the classical metaphase plate alignment.\textsuperscript{61, 62} Despite abnormalities in chromosome alignment and spindle shape there is completion of meiosis I. However, correct chromosome separation fails to occur with all the chromosomes extruded in the first polar body or kept within the embryo.\textsuperscript{61, 62}

Kif12, on the other hand appears to be important for cytokinesis in mitotically dividing Dictyostelium (slime mould).\textsuperscript{63, 64} In Kif12 null strains, accumulation of myosin II to the cleavage furrow is suppressed whilst INCENP fail to localize there ultimately preventing...
cytokinesis.\textsuperscript{63, 64} Investigation into the role of this kinesin in vertebrate somatic cells and oocytes is required to determine if Kif12 function is conserved across all eukaryotes.

**Kinesin 13 Family**

The kinesin 13 family consists of 4 proteins Kif2A, Kif2B, Kif2C (also known as MCAK) and Kif24, however, MCAK is the only kinesin 13 that has been studied to date in oocyte meiosis.\textsuperscript{3} In mitosis MCAK is not essential for bipolar spindle assembly but is necessary for correct microtubule-kinetochore attachments.\textsuperscript{65} In MCAK mitotic knockdown models incorrect microtubule-kinetochore attachments are not repaired yet are able to satisfy the SAC leading to lagging chromosomes at anaphase and presumably aneuploidy.\textsuperscript{66, 67} In mouse oocytes, loss of MCAK lead to an increase in chromosome misalignment at metaphase I.\textsuperscript{68} However, these MCAK depleted oocytes have apparently normal metaphase I spindles and no change in ploidy status, suggesting functional redundancy of this kinesin in mammalian oocyte meiosis I.\textsuperscript{68} Interestingly, however, knock-down of shugoshin-like 2 (SGOL2), a protein involved in cohesion protection, prevents MCAK accumulation at the kinetochores during meiosis I in mice oocytes. SGOL2 recruitment of MCAK is mediated by AurB/C and prevents bivalent overstrecthing and reduces interkinetochore distance.\textsuperscript{69} Increased interkinetochore distance in mouse oocytes is associated with aneuploidy and is elevated with maternal age.\textsuperscript{70, 71} The involvement of MCAK in maintaining correct bivalent stretching/interkinetochore distance could, therefore, highlight a role in correct chromosome separation at meiosis II or in age related aneuploidy in mammalian oocytes.

Conversely, the *C.elegans* orthologue KLP-7 and the *Drosophila* orthologue Klp10A were found to be necessary for normal spindle assembly.\textsuperscript{72, 73} Depletion of KLP-7 resulted in multipolar spindles. These extra functional poles led to chromosomes frequently separating into three masses at anaphase I and therefore aneuploidy.\textsuperscript{72} Loss of Klp10A on the other hand
caused metaphase I spindle elongation and mis-positioning. Furthermore, end binding 1(EB1) attachment to, and release from microtubule plus ends was delayed. EB1 promotes the growth of microtubules and is essential for normal metaphase spindle formation in mitotic cells. It is therefore possible that Klp10A facilitates the addition and removal of EB1 from microtubules plus ends, resulting in increased spindle elongation with loss of this protein.

MCAK is also essential for correct metaphase I spindle formation in *Xenopus* oocytes. Phosphorylation of MCAK by AurB allows the formation of a bipolar spindle. However, inhibition of this phosphorylation event leads to monopolar spindle formation. Differences in phenotypes described here could indicate differences in the function of this protein between species, with MCAK appearing to be functionally redundant in meiosis I mammalian oocytes.

In mitotic human cell lines, Kif2A and Kif2B have been identified as essential for spindle assembly, with depletion leading to monopolar or disorganized spindles. Despite MCAK appearing to be functionally redundant in mammalian female meiosis, it is possible the other kinesin 13 family members, such as Kif2A and Kif2B, which are important for mitosis, may play an essential role in oocytes. In contrast, Kif24 is involved cilia formation via microtubule stabilization, and appears to have no role in cell cycle progression.

**Kinesin 14 Family**

Unlike other kinesins, the kinesin 14 subfamily has a C-terminal motor domain rather than an N-terminal motor domain, triggering them to slide along microtubules in a minus-end directed manner. In mammalian cells, this family consists of 4 proteins (Kif25, KifC1, KifC2 and KifC3), however, to date only the role of ncd, the *Drosophila* orthologue for KifC1 has been investigated in female meiosis. Oocytes from ncd null mutants or ncd partial loss-of-function mutants have the same phenotype, abnormal spindle formation at
metaphase I and II. These spindles take significantly longer to form, are unstable and have elevated numbers of spindle poles as a result of pole splitting. Furthermore, due to their unstable nature these spindles continually change shape. As a consequence, aneuploidy is induced with chromosome segregation occurring more than twice. This highlights the key role of ncd protein in female meiosis. Of interest, is that in mammalian mitotic cells, this kinesin has been found to be an APC/C substrate, with both overexpression of KifC1 or stabilization of the APC/C specific destruction motif (D-Box) resulting in monopolar spindles.

Of the other C-terminal motor proteins in this family, KifC2 is unlikely to have an essential role in female meiosis since null mice develop and reproduce normally. In contrast, depletion of KifC3 in HeLa cells leads to an increase in the time to completion of the final cytokinetic event, abscission. It is therefore plausible that KifC3 may act in this final stage in female meiosis. Finally, the Kif25 gene, which is present in human cells is completely missing from the mouse genome and its function has not been investigated.

Roles of kinesin families 2, 4, 8, 9 and 11 in mitosis

To date research into role of the kinesin subfamilies 2, 4, 8, 9 and 11 has not been performed in female meiosis. However, many of these kinesins have important roles in mitosis, and are therefore may also play a role in meiosis.

The kinesin 2 family consists of Kif3A, Kif3B, Kif3C and Kif17. Of the Kinesin 2 family Kif3A and Kif3B are normally found in a heterodimer together with the protein KAP3 linking the Kif3A/3B dimer (Kif3) to a cargo. In HeLa cells mutations in Kif3 that prevents its interaction with KAP3 lead to abnormal spindle formation and aneuploidy. Kif3C appears to have a nonessential role with null mice developing normally with no change in their fertility. Finally, Kif17 appears to only be expressed in grey matter, lung, heart, spleen,
liver, testis and skeletal muscle, with no evidence to date that it is expressed in oocytes or even ovaries. Therefore Kif3C and Kif17 are unlikely to play a role in female meiosis.

Of the kinesin 4 subfamily; Kif4A, Kif4B, Kif7, Kif21A, Kif21B and Kif27, only 3 of the 5 proteins have known roles in mitosis. Kif4A and Kif4B are 2 closely related chromokinesins, which have similar roles in mitosis. Depletion of Kif4B from human cell lines, results in abnormal anaphase spindle morphology and failed cytokinesis, ultimately cumulating in binucleated cells. Kif4A knockdown cells demonstrate this phenotype in addition to abnormal metaphase spindles, chromosome misalignment and mis-segregation. Interestingly, Kif4A is also a predicted substrate of the APC/C and therefore could have a role in anaphase onset regulation. Conversely, Kif7 appears to be important for cell cycle regulation. Ablation of Kif7 prematurely upregulates key cell cycle regulators cyclin dependent kinases Cdk1 and Cdk2 leading to early APC/C activation and ultimately increasing cellular proliferation. The bioactivity of Kif4A, Kif4B and Kif7 in mitosis suggests that these kinesin 4 proteins are important for normal cell cycle completion and therefore could play important roles in female meiosis.

The kinesin 8 family of proteins (Kif18A, Kif18B, Kif19A and Kif19B) are essential in microtubule depolymerisation. Kif19B only localizes to cilia tips and is therefore unlikely to play a role in female meiosis. Kif18A and Kif18B in contrast are essential for normal mitosis. Kif18A is a known APC/C substrate that is degraded at anaphase onset. Depletion of Kif18A in human cell lines reduces microtubule-kinetochore tension leading to mitotic arrest via Mad2 activation of the SAC. In meiotically dividing Drosophila primary spermatocytes, knockdown of the Kif18A orthologue Klp67A causes defects in metaphase I spindle formation, chromosome congression and separation and cytokinesis. The importance of Kif18A for mitosis and spermatocyte meiosis, strongly implies that this protein may also have a role in female meiosis. Kif18B also has known roles in mitosis interacting.
with EB1 and the kinesin 13 MCAK.\textsuperscript{97, 98} Via EB1 interaction, Kif18B is able to bind to the plus-ends of microtubules, with MCAK interaction needed to promote microtubule depolymerisation.\textsuperscript{97, 98} It is of interest to note that this interaction of MCAK is controlled by Aurora Kinases.\textsuperscript{97} Furthermore depletion of Kif18B leads to spindle defects as a result of elevated numbers and size of microtubules.\textsuperscript{98}

Very little research has been undertaken on the function of kinesin 9 proteins Kif6 and Kif9. No research has been performed on the role of Kif6 during the cell cycle. However, Kif9 has been found to play a role in metaphase spindle length and chromosome alignment.\textsuperscript{100} Furthermore, depletion of Kif9 extended mitosis due to a prometaphase delay.\textsuperscript{100} Finally, to date, no data regarding kinesin 11 proteins, Kif26A and Kif26B during the cell cycle is available.

**Clinical Role of Kinesin Motor Proteins**

Many kinesin motor proteins have a clear and important role in female meiosis, and so are of great clinical interest. Mammalian oocytes are unique in that they are prophase I arrested from perinatal life until puberty months (rodents) or years (humans) later.\textsuperscript{11, 12} Because of this mammalian oocytes, including human oocytes are highly vulnerable to environmental factors and ageing.\textsuperscript{1, 2, 7} In a 2008 study by Pan et al., mRNA expression of five kinesin genes were found to be altered in reproductively aged prophase I or metaphase II mouse oocytes. A kinesin 1 (Kif5B), kinesin 3 (Kif13A) and kinesin 4 (Kif21A) were upregulated and a kinesin 6 (Kif23) and kinesin 13 (Kif2C/MCAK) were downregulated.\textsuperscript{101}

Similarly, comparison of polar bodies from aged and young mouse metaphase II oocytes also found MCAK mRNA decreased.\textsuperscript{102} Importantly, in young oocytes mRNA levels of MCAK were comparable between oocyte and polar body.\textsuperscript{102} Furthermore, a protein study between young and aged GV mouse oocytes found a kinesin 3 (Kif16B) to be upregulated.\textsuperscript{103} These
studies potentially highlight that the kinesin family of proteins may be involved in age related decline in oocyte quality.

Additionally, changes in expression of this family of proteins have been found in both human and mouse oocytes after cryopreservation via controlled slow freezing or ultra-rapid cooling (vitrification). After slow freezing metaphase II human oocytes mRNA expression levels of a kinesin 2 (Kif3A), kinesin 3 (Kif14), kinesin 5 (Kif11/Eg5) and kinesin 13 (Kif2C/MCAK) were downregulated.\textsuperscript{104} Similarly, in mouse oocytes vitrification led to an upregulation in kinesin 5 (Kif11/Eg5) and downregulation of kinesin 7 (Kif10/Cenp-E) mRNA.\textsuperscript{105-107} Interestingly, supplementation of vitrification media with anti-freeze proteins found within arctic fish restored the levels of mRNA, including Kif11/Eg5 and Kif10/Cenp-E to fresh unfrozen mouse oocyte levels.\textsuperscript{105-107} This supplementation also resulted in increased meiotic competency and oocyte quality.\textsuperscript{105-107}

Remarkably, excessive alcohol consumption in Rhesus monkeys (akin to human ‘binge’ drinking) twice weekly for 6 months decreased the mRNA expression of a kinesin 3 (Kif14) in oocytes.\textsuperscript{108} This highlights the possibility for kinesin motor proteins to be altered in oocytes via other environmental and lifestyle impacts such as cigarette smoking, alcohol consumption and obesity, potentially contributing to reduced oocyte quality.

**Conclusion**

Kinesin motor proteins are essential for female meiosis, having roles in cell cycle progression, spindle formation, chromosome separation and cytokinesis. Importantly, recent studies have shown the expression of certain kinesins are altered in oocytes in response to ageing, cryopreservation and environmental exposures. With increasing numbers of women in the western world delaying child bearing to the latter half of their reproductive life and cryopreserving oocytes for personal reasons (“social freezing”) becoming more popular,
understanding the function of this family of proteins is increasingly important. Despite this, the role of only 10 of the 45 human kinesin motor proteins or their orthologues has been investigated in female meiosis. This represents a large opportunity for research into the roles this diverse and interesting family of proteins play in oocyte meiosis. Furthermore, it could lead to an increased understanding of the mechanisms underpinning the decline in oocyte quality with maternal ageing, exposure to environmental toxicants and cryopreservation.
References

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Andrieu G, Quaranta M, Leprince C and Hatzoglou A. The GTPase Gem and its partner Kif9 are required for chromosome alignment, spindle length control, and mitotic progression. The FASEB Journal 2012; 26:5025-34.


Figure 1: Comparative representation of meiosis in *C. elegans*, *Drosophila*, *Xenopus* and Mammalian oocytes. In all species meiosis I begins and arrests at prophase I. Various stimuli overcome this arrest, allowing for meiotic resumption and nuclear envelope breakdown (NEB). The metaphase I spindle forms, and chromosomes attach to spindle fibers before begin separated at anaphase I with half extruded in the first polar body. Meiosis II continues on without an intervening S-phase, where chromosomes attach to a second spindle at metaphase II. Chromosomes are again halved at anaphase II and extruded in the second polar. Of note is that *Drosophila* do not extrude a polar body, but rather degrade excess chromosomes. The oocyte then transitions into embryonic development. Throughout this process each species has its own unique timing for the second meiotic arrest, ovulation and fertilization.
**Figure 2:** Correct timing of chromosome separation is controlled via SAC inhibition of the APC/C. During prometaphase Mad2 localizes to unattached and/or incorrectly attached kinetochores (grey) where it is activated (purple; * denotes activation). Activated Mad2 binds to Cdc20 (green), with this complex and BubR1 (pink) binding to the APC (orange) and preventing its activation, maintaining the pool of APC substrates (blue). This inhibits anaphase onset, allowing time for the formation of correct kinetochore-microtubule attachments. Once all kinetochores are correctly attached to spindle fibers at metaphase SAC
signal is lost. This activates the APC and allows it to degrade its substrates, such as cyclin B1 and securin, resulting in anaphase onset and chromosome separation.
Figure 3: Correct chromosome separation at meiosis I is needed to produce a normal meiosis II oocyte. (A) Correct chromosome composition of meiosis II oocyte, chromosomes are found in pairs (bivalents) with half extruded in the polar body. Aneuploidy can be caused by either (B) bivalent nondisjunction or (C) premature sister chromatid separation. Bivalent nondisjunction occurs when an extra bivalent is kept within the oocyte (shown) or extruded into the polar body. Alternatively, premature sister chromatid separation occurs when bivalents prematurely individualize into chromosomes allowing them to independently separate.
Table 1: Kinesin motor protein families, protein orthologues in model animal systems and human ovarian follicle expression.

<table>
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<tr>
<th>Kinesin Family</th>
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<th>Xenopus</th>
<th>Drosophila</th>
<th>Ovarian follicle localisation of human protein*</th>
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<td>1</td>
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<td>Neb (Klp38B)</td>
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*Protein expression retrieved from The Human Protein Atlas.*
Concluding Remarks and Future Directions

6.1 Impact of maternal and grandmaternal smoke exposure on female fertility

The research presented in this thesis highlights the impact of multigenerational exposure to environmental toxicants and ageing on oocyte quality and female fertility. Direct exposure to cigarette smoke is a known reproductive toxicant; however there has been a paucity of knowledge regarding the potential multigenerational effect on female fertility. Evidence presented here suggests that both in utero and grandmaternal exposure to cigarette smoke can negatively affect female fertility outcomes. To fully elucidate the cause of this subfertility further investigation into the molecular mechanisms underlying this process is needed.

Epidemiological studies on maternal smoke exposure in humans have found early onset menarche/menopause and decreased fecundability (de Barros et al., 2016; Ernst et al., 2012; Jensen et al., 2006). Conversely, grandmaternal exposure to the cigarette smoke condensate benzopyrene in rats reduces a females receptiveness to copulation (Csaba and Karabélyos, 1997). Chapters 2 and 3, outlines the effect of maternal and grandmaternal smoke exposure on mouse female fertility, however, only begins to shed light into the potential cause of this altered fertility. Alterations to the estrous cycle in rodents have been documented with in utero exposure to the environmental estrogens BPA and fipronil and grandmaternal exposure to genistein and ethinyl estradiol (de Barros et al., 2016; National Toxicology Program, 2008; National Toxicology Program, 2010; Suvorov and Waxman, 2015). Although these estrogenic compounds are absent in cigarette smoke, cigarette smoke condensate has been found to have estrogenic properties (Takamura-Enya et al., 2003). Furthermore, direct exposure of rats to the cigarette smoke constituent benzo[a]anthracene, benzo[a]pyrene or benzo[k]fluoranthene significantly reduce ovarian responsiveness to estrogen and estrogen receptors (Kummer et al., 2013). It is therefore attractive to speculate that maternal and
grandmaternal smoke exposure could affect hormone levels and therefore courtship behaviours and/or the estrous cycle. In Chapter 3, I began to explore this but observed no change in basal levels of FSH or LH levels at diestrus. However, estrogen and progesterone levels also fluctuate throughout the estrous cycle with these hormones having roles in ovulation and lordosis (Rodriguez et al., 2010; White and Uphouse, 2004). Additionally, the length of the estrous cycle was not examined with maternal or grandmaternal smoke exposure. Further investigation into the cyclicity and mating behaviors of maternal and grandmaternal smoke exposed animals is needed to begin to fully elucidate the mechanism behind the reduced fertility seen in these females.

Abnormalities in oocyte quality were also observed after maternal, grandmaternal and great-grandmaternal exposure to cigarette smoke. Consistently, altered metaphase II spindles were detected in all generations, however, elevated aneuploidy at this stage in both reproductively young and aged mice was never observed. Despite the lack of aneuploidy in our mouse model it is possible that elevated levels of chromosome missegregation may be observed in humans or other mouse strains. Within this thesis all cigarette smoke exposure experiments were performed on C57Bl/6 mice. This strain of mouse is particularly resilient to age related aneuploidy with only 9% of oocytes aneuploid at 17-19months which was not significantly different from 1 month animals (Yun et al., 2014). Conversely, human oocyte aneuploidy rates have been reported to be as high as 60% (Fragouli et al., 2011; Kuliev et al., 2011). Despite the low levels of aneuploidy observed in C57Bl/6 mice with age other strains have been shown to have elevated rates of aneuploidy similar to that observed in humans including CD1 mice (42.5% at 19-25months) and MF1 mice (33.3% at 15-17months) (Sebestova et al., 2012; Shomper et al., 2014). Investigation into the effect of multigenerational smoke exposure on oocyte quality in these strains of mice could reveal a role for this in oocyte aneuploidy.
Finally, humans are exposed to multiple environmental and lifestyle factors throughout their pregnancy while in animal models exposure is limited to one factor. Research into multifactorial exposure of known reproductive and developmental hazards such as alcohol, cigarette smoke, and high fat/sugar diet needs to be performed to determine if these factors combine to increase the detrimental reproductive consequences of either insult alone.

6.2 Role of kinesins in oocyte quality with increasing maternal age

Reproductive ageing on the other hand, is a known hazard to oocyte quality that is unavoidable. In order to gain insight into how oocyte quality declines with increasing maternal age a greater understanding of female meiosis is needed, in particular the role of kinesin motor proteins throughout oocyte meiosis. Within human cells 45 kinesin motor proteins have been discovered with the role of only 10 of these proteins or their orthologues investigated in female meiosis. Furthermore, of these 10 only half have been examined in mammalian oocyte meiosis despite alterations in mRNA transcript or protein levels for many of these kinesins with maternal ageing or cryopreservation (Jiao et al., 2014; Jo et al., 2011; Jo et al., 2012; Monzo et al., 2012; Pan et al., 2008; Schwarzer et al., 2014; Wen et al., 2014). Furthermore, several of these proteins including Kif1B, Kif16A, Kif4A, Kif7, Cenp-E, Kif18A, MCAK and KifC1 are known or predicted APC/C substrates or have been found to be involved in SAC mediated cell cycle arrest (Coles et al., 2015; Gui and Homer, 2012; Singh et al., 2014; Torres et al., 2011; Vitiello et al., 2014). APC/C and SAC control of cell cycle progression is key for correct oocyte meiosis, with a growing body of evidence to suggest alterations in these pathways with maternal ageing (Pan et al., 2008; Yun et al., 2014).

To begin to elucidate the role of kinesin motor proteins in meiosis the mouse homologue (Kif4) of the predicted APC/C substrate, Kif4A, was investigated. Evidence presented within
this thesis shows for the first time that the kinesin motor protein Kif4 is essential for normal oocyte meiosis, and is controlled by key meiotic kinases, Aurora Kinase B/C and Cdk1. Interestingly, despite interacting with Aurora Kinase B/C and Cdk1, Kif4 does not appear to be a direct substrate of the APC/C in oocytes with knock-down having no effect on meiotic timing. Bioactivation via Aurora Kinase B/C and Cdk1, however, does appear to be important for the interaction of Kif4 with the kinetochore and kinetochore proteins. Investigation into the role of Kif4 in kinetochore dynamics and how it is controlled throughout the cell cycle is needed with particular focus on kinetochore construction and microtubule-kinetochore attachments.

Additionally, Kif4 upregulation after meiotic resumption in maternally aged oocytes highlights its potential clinical importance. Kif4 has known roles in microtubule stabilisation and its elevation reduces microtubule sensitivity to nocodazole (Morris et al., 2014). Maternally aged oocytes are less sensitive to nocodazole induced meiotic arrest (Yun et al., 2014) and so this presents an interesting question: does increased Kif4 with age result in increased microtubule stabilisation, manifesting as reduced sensitivity to nocodazole? Investigation into this area is key as it may provide further insight into the molecular cause of chromosomal separation errors with age.

Finally, further investigation into the role of other kinesin motor proteins during meiosis and in age related fertility decline is needed to gain a clearer picture of how this family of motor proteins regulates oocyte quality.
References


Appendices
Supplementary Figure S1: The zona pellucida was measured at four points evenly dispersed around the oocyte as displayed. The solid line represents the angle measurements were taken at with the dashed line indicating the edges of the zona pellucida. The mean of these measurements was used as the oocyte's zona thickness.
Supplementary Figure S2: MSE caused no change in ploidy status of MII oocytes at 4 weeks or 9 months. Percentage of euploid oocytes; n, number of oocytes examined from 3 control/MSE animals, Fisher’s Exact Test.
**Supplementary Figure S2:** MSE caused no change in pup body weight. MSE and control litters were weighed every 2 days from birth until PND 36 with no change in size observed (Kruskal-Wallis test with Dunn's Post-hoc). Graph shows mean and SD.
Supplementary Figure S1: (A) Secondary only immunofluorescent controls for PCNA and AMH. Scale bar=100µm (B) Estrus staging of vaginal lavage. Representative brightfield images of mouse vaginal lavage at diestrus, proestrus, estrus and metestrus. Scale bar=50µm.
**Supplementary Figure S2:**GM-SE and GG-SE has no effect on oocyte numbers. (A) Graphical representation of the number of GV oocytes or ovulated oocytes collected from 1 week old control or GM-SE hormonally primed females; p=0.3491 and 0.3746 respectively, Student’s $t$-test. (B) Graphical representation of the number of GV from 9 month old control or GM-SE females; p=0.3182, Student’s $t$-test. (C) Graphical representation of the number of GV oocytes or ovulated oocytes collected from 1 week old control or GG-SE hormonally primed females; p=0.4227 and 0.8907 respectively, Student’s $t$-test. (D) Graphical representation of the number of GV from 9 month old control or GG-SE females; p=0.0561, Student’s $t$-test. Box plot shows mean (centerline) with box outline 25-75th percentiles and whiskers 10-90th percentiles, bar graph shows mean with SD marked. n=number of animals examined.
Appendix C: Chapter 4 Supplementary Data

Supplementary Figure 1: (A) Immunolocalization of Kif4 at GV, GVB, MI and MII with C-terminal direct antibody. (B) Kif4 and CENP-C PLA foci in MI oocytes were found throughout the cytoplasm and associated with chromosomes (yellow arrow). (C) Fluorescent
immunolocalization of Kif4 and Ndc80 at MI. Oocytes are counter labelled with the inner kinetochore marker ACA (red). (D) Kif4 and Ndc80 PLA foci in MI oocytes were found throughout the cytoplasm and associated with chromosomes (yellow arrow). DNA is counterstained with Hoechst (blue). Scale bar=10µm (A, B & D) or 5µm (C).
**Supplementary Figure 2:** (A) Immunolocalization of Kif4 and Plk1 at MI at the kinetochores and spindle poles (B) Fluorescent immunolocalization of Kif4 (grey) at MI following 3.5hrs of treatment with the vehicle (Veh) DMSO or Plk1 inhibitor BI 2536. Scale bar=10µm.
Supplementary Figure 3: (A) Relative expression (ΔCt) of Kif4 mRNA between young and aged females in GV oocytes; p=0.0741, Student’s t-test. (B) Relative expression (ΔCt) of Kif4 mRNA between young and aged females in MII oocytes; p=0.6964, Student’s t-test. Bar graphs show mean with SD marked, n=number of animals.
**Supplementary Table 1:** Antibodies used for immunocytochemistry and proximity ligation

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**Supplementary Table 2:** Morpholino target sequence

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Appendix D: Abstract and Chapter Overview

References

Abstract


Hassold, T., H. Hall, and P. Hunt. 2007. The origin of human aneuploidy: where we have been, where we are going. *Human Molecular Genetics*. 16:R203-R208.


Chapter 2 Overview

Chapter 3 Overview


**Chapter 4 Overview**


Appendix E: Additional Publication

**Germ cell specific overactivation of WNT/βcatenin signalling has no effect on folliculogenesis but causes fertility defects due to abnormal foetal development**

**Authors:** Manish Kumar\(^1\), Nicole J Camlin\(^2\), Janet E. Holt\(^2\), Jose M. Teixeira\(^3,4\), Eileen A. McLaughlin\(^2\) and Pradeep S. Tanwar\(^1*\)

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*Corresponding Authors

Nicole J. Camlin performed the oocyte and preimplantation embryo experiments found in Figure 1B-E and Figure 5A-B
Germ cell specific overactivation of WNT/β-catenin signalling has no effect on folliculogenesis but causes fertility defects due to abnormal foetal development

Manish Kumar1, Nicole J. Camlin2, Janet E. Holt2, Jose M. Teixeira3,4, Eileen A. McLaughlin2 & Pradeep S. Tanwar5

All the major components of the WNT signalling pathway are expressed in female germ cells and embryos. However, their functional relevance in oocyte biology is currently unclear. We examined ovaries collected from TCFGFP mice, a well-known Wnt reporter mouse model, and found dynamic changes in the Wnt/β-catenin signalling activity during different stages of oocyte development and maturation. To understand the functional importance of Wnt signalling in oocytes, we developed a mouse model with the germ cell-specific constitutive activation of β-catenin using cre recombinase driven by the DEAD (Asp-Glu-Ala-Asp) box protein 4 (Ddb4) gene promoter. Histopathological and functional analysis of ovaries from these mutant mice (Ctnnb1+/- cko) showed no defects in ovarian functions, oocytes, ovulation and early embryonic development. However, breeding of the Ctnnb1+/- cko female mice with males of known fertility never resulted in birth of mutant pups. Examination of uteri from time pregnant mutant females revealed defects in ectoderm differentiation leading to abnormal foetal development and premature death. Collectively, our work has established the role of active WNT/β-catenin signalling in oocyte biology and foetal development, and provides novel insights into the possible mechanisms of complications in human pregnancy such as repeated spontaneous abortion, sudden intrauterine unexpected foetal death syndrome and stillbirth.

WNT signalling is involved in various developmental processes such as cell proliferation and differentiation. β-catenin is an important mediator of the WNT pathway. In the absence of WNT ligands, the Adenomatous Polyposis Coli (APC) complex binds to β-catenin and causes its phosphorylation at highly conserved Ser/Thr residues. This phosphorylated form of β-catenin is then recognised by the E3 ubiquitin ligase complex and degraded by the proteasome. In the presence of WNT ligands, the APC complex is no longer able to target β-catenin, leading to the stabilization and subsequent nuclear localization of β-catenin. In the nucleus, β-catenin interacts with various factors, including the members of the TCF/LEF family to activate the transcription of targeted genes.

We and others have established the significance of WNT signalling in gonadal functions and reproductive tract cancers. Overexpression of the Wnt4 gene, a well-known WNT ligand, causes suppression of steroidalogenesis and abnormal testis development in both humans and mice. In contrast, loss of the Wnt4 gene leads to female-to-male sex reversal in mice. In male mice, overactivation of WNT signalling by the conditional deletion of exon 3 of the β-catenin gene (Ctnnb1) in Sertoli cells of testis causes disorganization of seminiferous tubules, premature germ cell loss, infertility and stromal tumours. Loss of Apc, a negative regulator of β-catenin, in stromal cells induces an epithelial-mesenchymal transition-like state in Sertoli cells leading to defects in testicular development.

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WNT/β-catenin signalling has been shown to be involved in ovarian development and diseases. Many members of the WNT pathway including ligands, receptors, co-receptors and negative regulators are expressed in the mouse ovary, oocyte and embryos. Somatic cell-specific loss of the Wnt4 gene in the mouse ovary using Anti Mullerian hormone receptor 2 cre (Amhr2cre) causes abnormalities in follicular development resulting in premature ovarian failure and subfertility. Similar to WNT4, Fzr2, a known receptor for WNT ligands, is expressed in the mouse ovary and its loss causes impaired corpus luteum development and infertility without affecting follicular formation and functions. Stabilization of β-catenin in postnatal granulosa cells increases follicle stimulating hormone (FSH)-induced growth of ovarian follicles but suppresses luteinizing hormone (LH)-induced oocyte maturation, ovulation and luteinisation. In prenatally granulosa cells, constitutive activation of β-catenin defects in the differentiation of granulosa cells, leading to the development of stromal tumours. Activation of PI3K signalling by deleting Pten in these mice results in the development of a highly aggressive and metastatic form of ovarian stromal tumours. Collectively, these findings highlight the importance of the WNT pathway in stromal cells of the ovary.

Although much is known about the functions of WNT signalling in the stromal cells of the ovary, limited information is available regarding its involvement in ovarian germ cell biology. To determine the significance of WNT signalling during different stages of oogenesis, we monitored real-time changes in WNT signalling activity in the mouse ovary using a well-known WNT reporter mouse model. We found dynamic changes in the levels of WNT activity during different stages of oocyte development and maturation suggesting a requirement for this pathway in follicle/oocyte functions. To further understand the requirement of WNT signalling in oocyte biology, we developed a mouse model with overactive β-catenin in female germ cells and showed that abnormal WNT/β-catenin signalling leads to defects in female fertility.

**Results**

**Activation of WNT signalling during different stages of follicular development.** To determine the physiological activity of WNT signalling during follicular development, we examined oocytes from a well-characterized Wnt reporter mouse model, TCFGFP. In this model, nuclear GFP is only present in cells with active WNT signalling. Examination of oocytes from pre-pubertal TCFGFP mice revealed GFP expression in the nucleus of oocytes (Fig. 1A; N = 3). Both GFP positive and negative oocytes were present in TCFGFP oocytes. Consistent with previous reports showing active WNT signalling in the ovarian surface epithelium (OSE) and stromal cells, GFP expression was also observed in OSE and some stromal cells of oocytes collected from TCFGFP mice (Fig. 1A; N = 3). Next, we collected oocytes from unprimed TCFGFP mice (N = 3) and found that 81% (N = 21/26) of oocytes were positive for GFP expression (Fig. 1B,C). To study dynamic changes in WNT signalling during oocyte maturation, oocytes collected from primed TCFGFP ovaries were subjected to undergo in vitro maturation. We observed no difference in the meiotic competency of GFP positive and negative oocytes during different stages of oocyte maturation (Fig. 1D). This suggests that active WNT signalling is not a predictor of oocyte meiotic competency. Time lapse imaging of in vitro maturation of oocytes revealed changes in GFP expression during different stages (Fig. 1E). High GFP expression was also found in the extruding polar body (Fig. 1Ee–f) indicating a role for WNT signalling in asymmetric division. Collectively, these results showed that dynamic changes in WNT signalling activity occur during several stages of oocyte development and maturation.

**Conditional activation of WNT/β-catenin signalling in ovarian germ cells.** To further investigate the role of active WNT signalling in oocyte development, we developed a mouse model with germ cell specific overactivation of WNT/β-catenin signalling using DEAD (Asp-Glu-Ala-Asp) box protein 4 cre (Ddx4cre). In our previous study, we have shown that Ddx4cre causes fetal recombination in ovarian germ cells beginning from embryonic day 15. We crossed Ddx4cre mice with Cnmb1fl/fl mice to generate mice with deletion of exon 3 of the β-catenin gene (Fig. 2A; Cnmb1−/−d), Exon 3 of the β-catenin gene harbours phosphorylation sites targeted by the APC destruction complex leading to abnormal accumulation of β-catenin in the cytoplasm that subsequently translocates to the nucleus leading to the transcription of targeted genes. Using PCR, we confirmed that Ddx4cre specifically causes recombination of Cnmb1fl/fl mice (Fig. 2B). Examination of β-catenin protein expression showed a significant increase in cytoplasmic and nuclear accumulation of β-catenin in the oocytes of mutant ovaries compared to controls (Fig. 2C,D; N = 3). No change in localization of β-catenin was observed in both control and mutant ovarian somatic cells (Fig. 2C). To further confirm the specificity of Ddx4cre, we developed another mouse model (Cnmb1fl/fl) and found presence of LacZ/β-gal expression only in germ cells of mutant ovaries (Sfig. 1A–P; N = 3/3). Colocalization of β-gal and β-catenin revealed cytoplasmic/nuclear accumulation of β-catenin only in the β-gal positive oocytes of the mutant ovaries (Sfig. 2A–D; N = 3/3). Co-immunostaining of β-gal with a germ cell marker (GCNAs, Germ Cell Nuclear Antigen) showed that β-gal expression in the mutant ovaries is limited to the GCNA-positive cells (Sfig. 2E–H; N = 3/3). In summary, these results demonstrated that Ddx4cre mediated deletion of exon3 of β-catenin gene causes a greater accumulation of β-catenin specifically in germ cells of the ovary.

**Oocyte specific constitutive activation of WNT/β-catenin signalling causes subfertility but has no effect on folliculogenesis and early embryonic development.** To study the effects of overactivation of WNT/β-catenin signalling, control (N = 14) and mutant females (Cnmb1−/−d) were mated with the wild type and Cnmb1fl/fl males of known fertility. Cotriculated plugs were observed in both control and mutant females, suggestive of normal mating behavior in these mice. Control females bred normally and produced 5.09±1.97 pups per litter during four months of breeding period, whereas, mating of mutant females...
Figure 1. Active WNT signalling during follicular development in mouse ovary. Nuclear GFP expression (white arrowhead), indicative of active Wnt signalling, is present in oocytes of TCFGFP ovaries (Aa,b). GFP expression was also observed in ovarian surface epithelium (Ab; yellow arrowhead). Brightfield and GFP images of oocytes collected from ovaries of unprimed TCFGFP mice (Ba,b). Both GFP positive and negative oocytes were present. Graphical presentation of percentage of GFP positive oocytes collected from non-cycling TCFGFP ovaries (C; N = 26). No significant difference between GFP positive and negative oocytes were observed post in vitro maturation for the different oocyte stages that were: immature oocytes (with intact nuclear membrane), Germinal Vesicle (GV; competent to undergo nuclear membrane breakdown), MI-MII (full meiotic competence-extrudes polar body in vitro) (D). Sequential imaging of a TCFGFP oocyte undergoing in vitro maturation showed higher GFP expression in the extruding polar body compared to the rest of the oocyte. Bars: 100 μm (A); 10 μm (E).

with Ctnnb1-/- males produced no litters (Table 1). Fewer pups were born from the mating of mutant females with wild type males (Table 1). Genotyping of pups resulting from this mating showed none of the pups were of the mutant genotype (Ctnnb1-/-cKO).

Histological examination of pre- and post-pubertal ovaries showed no difference between control and mutant mice (Fig. 3A–D; N = 5/each). Corpora lutea were present in adult control and mutant ovaries suggesting that ovulation was normal in these females (Fig. 3C,D; N = 5/each). To determine if overactive β-catenin affects the germ cells, we performed immunostaining for GCNA and found no obvious difference in GCNA expression in oocytes from ovaries in both groups (Fig. 3E,F; N = 5/each). Examination of inhibinα (Fig. 4A–D; N = 5/each),
Figure 2. Overactivation of Wnt signalling in oocytes does not affect oogenesis. (A) Schematic representation of mouse model with germ cell-specific overactivation of Wnt signalling. (B) PCR based detection of the mutant form (arrow) of the β-catenin gene from DNA isolated from ovaries of control and Ctnmb1<sup>−/−</sup> k.o mice. Presence of 700bp band with DNA from ovaries of mutant animals shows successful recombination. (C) Increased expression of β-catenin in oocytes of mutant mice (arrowheads) as compared to control (arrowheads) indicating of overactive Wnt signalling in germ cells of mutant ovaries. No change in β-catenin expression was observed in ovarian somatic cells in both control and mutant animals. (D) Quantification of β-catenin expression in oocytes of control and mutant ovaries showing a significant increase in intensity of β-catenin staining in oocytes of mutant ovaries. Nuclei are marked blue by DAPI. Bars: 100 μm.

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Table 1. Ctnmb1<sup>−/−</sup> k.o female mice are subfertile.

a marker for antral follicles, and Müllerian Inhibiting Substance/Anti Müllerian hormone (AMH) (Fig. 4E-H; N = 5/each), a marker for developing follicles, expression in ovaries also revealed no difference between control and mutant mice. These findings suggest that germ cell and follicular development is not affected in mutant mice. Our analysis of TCFGFP mice showed dynamic changes in WNT/β-catenin signalling occurs during different stages of oocyte maturation (Fig. 1E). To determine the effect of overactive WNT signalling on oocyte maturation, we collected oocytes from hormonally primed control and mutant mice (Ctnmb1<sup>−/−</sup> k.o; N = 3/each). The number of oocytes collected from mutant ovaries was comparable to controls (Fig. 5A). To evaluate the potential of the mutant oocyte to undergo embryogenesis, we subjected both control and mutant oocytes to parthenogenetic activation (N = 3/each). Both control and mutant oocytes showed comparable potential to undergo parthenogenesis and subsequent embryonic development to blastocyst stage (Fig. 5B). Collectively, these data suggest that the process of oocyte maturation and early embryonic development is not affected by hyperactive WNT signalling.
Sustained activation of WNT signalling in oocytes leads to abnormal development of ectoderm and foetal loss. To determine whether abnormalities during the later stages of embryonic development cause defects in fertility of the mutant mice, we time mated control and mutant females with Cmnb1-/- males of proven fertility. Control and mutant uteri from time pregnant females were collected during different stages of foetal development. Examination of 8.5 day post coitum (dpc) pregnant uteri showed no obvious differences. GCNA, a marker of germ cells, expression in ovaries of 3-day-old control (B) and mutant (F) mice. Nuclei are marked blue by DAPI. Bars: 100μm.

Figure 3. Normal morphology of pre-pubertal and adult ovaries in control and mutant mice. Histology of early postnatal (A, B; 3-day-old) and adult (C, D; 7-weeks-old) ovaries from control and mutant mice showed no obvious differences. GCNA, a marker of germ cells, expression in ovaries of 3-day-old control (E) and mutant (F) mice. Nuclei are marked blue by DAPI. Bars: 100μm.
Figure 4. Normal follicular development in ovaries of control and mutant mice. Inhibin, a marker for antral follicles, expression in control (A,C) and mutant (B,D) ovaries. Panel C and D are high magnification images of boxed areas in panel A and B, respectively. Expression of Mullerian Inhibiting Substance (MIS), a marker for preantral follicles, was also similar among control (E,G) and mutant ovaries (F,H). Nuclei are marked blue by DAPI. Bars: 100 µm.

(3.8±1.13; Table 1). None of the pups (N = 38) from 10 litters were of the mutant genotype. To confirm if foetal mortality is the reason for reduced litter size, we collected uteri from 15.5 dpc pregnant mutant females and found a significant number of dead foetuses (4.5±1/litter) in mutant mice (Fig. 5C–I).

To investigate the reasons for foetal lethality in mutant females, we performed histological examination of 8.5 dpc embryos and found a disorganised development of all three germ layers in mutants compared to controls (Fig. 6A–C). We selected the 8.5 dpc time point because at this stage there was no gross difference in the number or size of implantation sites (Fig. 5Ca–c). Analysis of β-catenin expression in embryos collected from 8.5 dpc time mated control females depicted only membranous expression of β-catenin in controls (Fig. 7A–C). However, both cytoplasmic and nuclear accumulation of β-catenin was seen in embryos collected from mutant mice (Fig. 7D–I).
Figure 5. Overactivation of Wnt signalling in oocytes does not affect oocyte maturation but leads to fertility defects due to embryonic death. (A) No obvious differences were observed in oocytes (viable, meiosis arrested, degenerating and good quality) that were collected by hormonal priming of control and mutant ovaries. (B) No differences were observed among various stages of parthenogenetic development (1-cell, degenerating cells, 2-cells, 3–4-cells, morula and blastocyst) of oocytes collected from control and mutant animals. (Ga,b) Normal gross morphology of pregnant uterus from control and mutant females at 8.5 dpc. (Gc) Graph represents no significant difference between the number of implantation sites in control (N = 7) and mutant (N = 6) animals at 8.5 dpc. (Cd,g) Normal foetal development was observed in 15.5 dpc control females (N = 10). Blackened and shrunken uterus without any viable foetuses collected from a 15.5 dpc mutant female mated with a Ctnnb1tm1 male (Ceh; N = 5). (Gf) Graph depicts significant difference in the number of foetuses at 15.5 dpc between control and mutant females. (Gh,i) Both live and resorbed foetuses were present in the pregnant uteri of mutant females that were mated with wild type males at 15.5 dpc. (Ck,l) Total number of foetuses and the number of resorbed foetuses in 15.5 dpc control and mutant females mated with wild type males. Panel (Gj) is high magnification image of boxed area in panel (G). Bars: 1 cm (C, a,b,d,e and i) and 100 μm (Cg–h and j).
La2 expression only in the mutant embryos from the Cntnb1<sup>−/−</sup> clo dams confirmed recombination in embryonic tissues (Fig. 7a,b). Next, we examined the expression of Ecadherin, a marker for the endodermal layer of the gut and the surface ectoderm of 8.5 dpc embryos<sup>6</sup>, in control and mutant mice (Fig. 8A–I). Compared to controls (Fig. 8A–C), abnormally increased Ecadherin expression was detected in mutant embryos (Fig. 8D–I). A similar expression pattern for Batenin and Ecadherin was also observed in 10.5dpc mutant embryos (SFigs 5 and 6). This suggests that the embryonic germ layers are not formed normally in mutant embryos. To rule out the possible loss of progesterone to the fetal death phenotype, we analysed control and mutant ovaries from 8.5 and 15.5 dpc pregnant females and confirmed the presence of corpora lutea in oocytes from both groups (Fig. 9A–D). In summary, these findings have demonstrated that constitutive activation of WNT/Batenin in oocytes causes defects in the development of embryonic germ layers, leading to infertility/subfertility due to fetal death.

**Discussion**

WNT signalling plays a major role in organogenesis and oncogenesis of the reproductive tract<sup>1,14,15,22–24</sup>. We have previously shown that balanced WNT signalling is essential for the proper development of reproductive tract organs and fertility<sup>6</sup>. Deregulated WNT signalling causes defects in gonadal cell proliferation and differentiation resulting in the genesis of ovarian and testicular cancers<sup>1,14,15,22</sup>. Importantly, 71% of Sertoli cell tumour patients present with activating mutations in the Cntnb1 gene and show cytoplasmic/nuclear accumulation of this protein<sup>6</sup>. Similarly, 16–38% of human ovarian endometrioid adenocarcinoma patients, a subtype of ovarian epithelial cancer, harbour activating mutations in the Batenin gene<sup>5</sup>. In mouse models, activation of WNT/Batenin signalling by the deletion of exon 3 of Batenin or truncation of Apc results in the development of similar ovarian and testicular tumours<sup>12,23</sup>, providing strong evidence for the involvement of this pathway in the pathogenesis of these cancers. Whether dysregulated WNT signalling also contributes to the formation of ovarian germ cell tumours is currently unknown. In this study, we have shown that sustained activation of Batenin in ovarian germ cells is unable to initiate tumorigenic growth of these cells, suggesting limited involvement of this pathway in ovarian germ cell tumours.

In mice, primordial germ cells (PGCs) differentiate from the epiblast at ~7 dpc and migrate through the hindgut and dorsal mesentery to reach the genital ridges by 10.5 dpc<sup>28</sup>. During early development, these cells undergo massive expansion and their number increases from ~45 at 7.5 dpc to ~25000 at 13 dpc<sup>29</sup>. In the gonads, PGCs acquire male or female fate under the influence of sex-specific signalling pathways. Any disruption to these signalling molecules leads to aberrations in proliferation and/or differentiation of these cells<sup>30</sup>. In mice, loss of Resolin 1, an antagonist of WNT signalling, leads to impairment of germ cell proliferation and meiosis<sup>30</sup>. Constitutive activation of WNT signalling by stabilization of Batenin also causes germ cell deficiency through decreasing primordial germ cell proliferation at the G1/S phase of the cell cycle<sup>5</sup>. These findings suggest that balanced WNT signalling in germ cells is essential for their proliferation and their commitment to meiosis. Surprisingly, in this study, we found no adverse effects from the overactivation of WNT/Batenin signalling in female germ cells on oocyte development and maturation, ovulation, and normal ovarian function (Figs 3 and 4). The use of a different gene promoter driving recombinase expression might explain differences in the phenotype between our study and others<sup>30</sup>. Both of the previous studies have used Tissue Non-specific Alkaline phosphatase cre (TNAcre), which is expressed in pre-meiotic PGCs from 9.5 dpc and also in other organs including the placenta and the intestine<sup>28</sup>. In comparison, Dlx3/4 is known to induce recombination in meiotic germ cells
Figure 7. Embryonic lethality in mutant females is associated with overactivation of Wnt signalling in embryos. (A–C) 8.5 dpc embryos from control females showed membranous expression of $\beta$-catenin. (D–I) Cytoplasmic and nuclear accumulation of $\beta$-catenin, indicative of overactive Wnt signalling, in 8.5 dpc embryos from mutant females. (Ja,b) lacZ expression was present only in embryos and not in other cells of uterus collected from 8.5 dpc Ctnnb1 cKO females. No lacZ expression was observed in control Ctnnb1 cKO; ROSA26 mice. White arrow marks lacZ positive embryo. Nuclei are marked blue by DAPI. Bars: 100 $\mu$m.

of the ovary from 15 dpc onwards\(^7\). Collectively, these findings suggest differential requirements of WNT/$\beta$-catenin signalling during different stages of germ cell development.

The follicles are the functional units of the ovary that provide a nourishing environment for normal oocyte development\(^8\). In each oestrous cycle, some follicles are recruited from the primordial follicle reserve to grow under the influence of gonadotropins and only a few selected ones are ovulated in mice\(^8\). There are no specific markers to predict which follicles will be recruited to grow and ovulate or undergo atresia. A recent study showed that high WNT signalling activity marks non-ovulatory follicles in mouse ovary\(^9\). Using TOPGAL mice, a $\beta$-galactosidase (lacZ) based WNT signalling reporter mouse model, these authors showed that lacZ staining is mainly present in the oocytes of the post-pubertal ovaries. Both lacZ-positive and -negative follicles are present in TOPGAL ovaries\(^9\). However, only lacZ-negative oocytes are ovulated and lacZ staining was limited to the atretic oocytes\(^9\). In this study, we have used TCFGFP mice that are similar to TOPGAL mice except lacZ is replaced with the histone 1 H2bb enhanced green fluorescent fusion protein gene\(^6\). In TCFGFP mice, highly specific nuclear GFP is directly detectable in cells where WNT/$\beta$-catenin signalling is activated\(^6\). Using this model, we showed that active WNT signalling is present in both ovulatory and non-ovulatory oocytes (Fig. 1). We observed no differences in meiotic competency of GFP-positive and -negative oocytes (Fig. 1). We believe the differences between our results and the TOPGAL study\(^9\) can be attributed to the lack of sensitivity of the standard lacZ staining procedure. For example, the traditional method of lacZ staining using X-gal with potassium ferri- and ferrocyanide (FeCN), which is also used in the TOPGAL study\(^9\), showed no or very weak lacZ expression in the embryos.
Figure 8. Overactivation of Wnt signalling lead to defective germ layer differentiation in the embryos of mutant mice. (A–C) Ecadherin expression present in the endoderm (marked by white arrow) and lateral thin ectoderm (marked by white arrowhead) of 8.5 dpc mouse embryo from control females. (D–I) No specific Ecadherin expression pattern was observed as most of the cells were positive in embryos of mutant females indicating defective embryonic germ layer differentiation during early stages of embryonic development. Nuclei are marked blue by DAPI. Bars: 100 μm.

Figure 9. Normal corpus luteum development in control and mutant females. H and E stained sections of control (A) and mutant (B) ovaries showing normal development of corpus luteum of 8.5 dpc pregnant mice. Normal development of corpora lutea continued at 15.5 dpc in control (C) and mutant ovaries (D). Arrowheads marks corpus luteum. Bars: 100 μm.
collected from BAT-Gal Wnt reporter mice, suggesting lack of WNT signalling activity during early embryonic development. However, combination of Salmon-gal with TNBT (5-bromo-4-chloro-3-indolyl phosphate) or NBTH (4-nitro blue tetrazolium chloride) revealed robust lacZ expression in the same stage BAT-Gal embryos.

In summary, we have shown that the physiological activation of WNT/β-catenin signalling occurs during different stages of oocyte development and maturation. Sustained activation of this signalling pathway in female germ cells has no effect on oocyte biology and function, but leads to defects during foetal development.

Materials and Methods

Mouse breeding and husbandry. Mice used in this study were housed under standard conditions at the University of Newcastle animal facility. All animals were maintained on C57BL/6;129SvEv mixed genetic background. Animal care and experimental procedures were conducted in accordance with the guidelines of the Animal Care and Ethics Committee of the University of Newcastle, and conformed to the New South Wales Animal Research Act, New South Wales Animal Research Regulation, and the Australian code for the care and use of animals for scientific purposes. All the procedures undertaken on mice were approved by the Animal Care and Ethics Committee of the University of Newcastle. TCF/LEF1::H2B::GFP mice used in the present study were obtained from the Jackson Lab (ME, USA) and referred as TCF/GFP. Ddx4cre (also known as Vasacre) mice were crossed with Ctnmb1<sup>mut</sup> mice for developing a mouse model (Ctnmb1<sup>mut</sup> GFP<sup>+</sup>) with germ cell specific overactivation of WNT/β-catenin signalling. Ctnmb1<sup>mut</sup> GFP<sup>+</sup> mice were bred with homozygous ROSA26<sup>H2B::GFP</sup> mice<sup>36</sup> to obtain Ctnmb1<sup>mut</sup> GFP<sup>+</sup> ROSA26<sup>H2B::GFP</sup> (Ddx4cre;Ctnmb1<sup>mut</sup> GFP<sup>+</sup>;ROSA<sup>H2B::GFP</sup>) mice. DNA isolation and genotyping were performed using REDExtract N-Amp Tissue PCR Kit (Sigma, MO, USA). For recombination PCR, DNA was isolated from whole ovaries of adult control and mutant female mice using the same kit. Sequence of primers used is listed in Table S1.

Time mating and fertility analysis. For timed pregnancies, control and mutant females were mated with males of known fertility. Females were checked for the presence of copulatory plugs in the morning and the day of plug was considered as 0.5 dpc. Time pregnant females were euthanized at several time points and gravid uteri were collected. Implantation sites were counted and the uteri were fixed in 4% PFA, overnight at 4 °C. For fertility analysis, adult control (Ctnmb1<sup>mut</sup> GFP<sup>+</sup>) and mutant (Ctnmb1<sup>mut</sup> GFP<sup>+</sup>) females of reproductive age were paired with Ctnmb1<sup>mut</sup> GFP<sup>+</sup> for four months and the number of litters and pups born per litter were recorded.

Mouse oocyte collection and maturation. Oocyte collection and maturation was performed as described by us in. The oocytes after in vitro maturation (IVM) were scored morphologically for different maturation stages that are, germinal vesicle (GV), germinal vesicle breakdown (GVB), MII determined by the first polar body (PBI) extrusion and oocyte degeneration.

In vitro parthenogenetic development. IVM oocytes were activated parthenogenetically using strontium-containing medium as described in our previous study<sup>36</sup>.

Histology and Immunohistochemistry. Histological and IHC protocols used in this study were adopted from our previous study<sup>36</sup>. 5 μm thick deparaffinised tissue sections were incubated with primary antibodies (Table S2), followed by Alexa fluor secondary antibodies (1:250; Jackson Immunoresearch Laboratories, PA, USA) for signal detection. Pictures were obtained using an Olympus DP72 microscope or Olympus FV1000 (Olympus, Tokyo, Japan) with the same gain and exposure for tissues from the control and mutant mice. For the assessment of fluorescence intensity of β-catenin at least 30 oocytes were counted, from three different animals from both control and mutant group, using ImageJ (National Institutes of Health, USA). β-galactosidase staining procedure is described in.

Statistical Analysis. Statistical significance was calculated by Student t-test using GraphPad Prism 6.0 software with P value < 0.05 considered significant. All values are presented as mean ± SEM. Each experiment was performed with N ≥ 3 for both control and mutant mice.

References

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Author Contributions

M.K., N.C. and J.H. performed all the experiments listed in the manuscript. M.K., N.C., I.H. analysed the data. M.K. and P.S.T. prepared the figures. E.M. and J.M.T. provided Ddx4cre mice and intellectual input to the study. M.K. and R.S.T. wrote the main manuscript text. R.S.T. conceived and designed the experiments, analysed the data, supervised the study, provided financial support, editing and final approval of the manuscript. All authors reviewed and commented on the manuscript.
Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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