Quality Control Mechanisms Responsible for the Maintenance of Genomic Integrity in the Female Germline

Jacinta Martin
Bachelor of Biotechnology (Honours Class I)

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DECLARATION

Statement of Originality

I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision. The thesis contains no material which has been accepted, or is being examined, for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968 and any approved embargo.

Statement of Authorship

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.

Thesis by Publication

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

Signed: ….....

Jacinta Hope Martin

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PUBLICATION AND AWARDS ARISING FROM THIS THESIS

1. Publications

Chapter 1*:*

1. **Martin, J.H**., Bromfield, E.G., Aitken, R.J., Nixon, B., (2017). Biochemical alterations in the oocyte in support of early embryonic development. Cellular and Molecular Life Sciences 74: 469-485. DOI: 10.1007/s00018-016-2356-1.

Published | Cellular and Molecular Life Sciences

Chapter 2:

2. **Martin, J.H.,** Nixon, B., Lord, T., Bromfield, E.G., Aitken, R.J., (2016). Identification of a key role for permeability glycoprotein in enhancing the cellular defense mechanisms of fertilized oocytes. Developmental Biology. 1;417(1):63-76. DOI:10.1016/j.ydbio.2016.06.035.

Published | Developmental Biology

3. **Martin, J.H.,** Bromfield, E.G., Aitken, R.J., Lord, T., Nixon, B., (2016). Data on the concentrations of etoposide, PSC833, BAPTA-AM, and cycloheximide that do not compromise the vitality of mature mouse oocytes, parthenogenetically activated and fertilized embryos. Data in Brief; 8: 1215–1220. DOI: 10.1016/j.dib.2016.07.046.

Published | Data in Brief

Chapter 3:

4. **Martin, J.H.,** Bromfield, E.G., Aitken, R.J., Lord, T. and Nixon, B. (2018). Double strand break DNA Repair occurs via Non-Homologous End-Joining in Mouse MII Oocytes. Scientific Reports; 8 (1); 9685. DOI: 10.1038/s41598-018-27892-2

Published | Scientific Reports.

Chapter 4:

5. **Martin, J.H**., Aitken, R.J., Bromfield, E.G., Cafe, S.L., Frost, E.R., Sutherland, J.M, Nixon, B and Lord, T (2018). Investigation into the presence and functional significance of proinsulin C-peptide in the female germline.

Submitted 26/08/18| Biology of Reproduction

Chapter 5:

6. **Martin, J.H.,** Aitken, R.J., Bromfield, E.G. and Nixon, B. (2018). DNA damage and repair in the female germline; contributions to assisted reproductive technologies

Submitted June 2018 | Human Reproduction Update

2. Statements of Contribution

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

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Professor Brett Nixon
Date: 16/08/18

……

Laurate Professor. John Aitken
Date: 19/08/18

…… ……

Dr. Elizabeth Bromfield
Date: 14/08/18

……

Dr. Tessa Lord
Date: 08/08/18

……

Dr Jessie Sutherland
Date: 15/08/18

……

Miss Emily Frost
Date: 09/08/18

……

Miss Shenae Cafe
Date: 14/08/18

……

Professor Frances Martin
Assistant Dean of Research Training
Date: 27/08/2018

3. Conference proceedings relevant to this thesis

2018:

1. **Martin, J.H,** Bromfield, E.G , Lord, T., Aitken, R.J., and Nixon, B. DNA repair and protection in the female germline. 50th Annual Scientific Meeting of the Society for Reproductive Biology, Adelaide, Australia. August 2018.

   Oral Presentation/ Finalist for the Oozoa award

2. **Martin, J.H,** Bromfield, E.G , Lord, T., Aitken, R.J., and Nixon, B. DNA repair and protection in the female germline. Gordon Research Seminar- Mammalian Reproduction, Renaissance Tuscany Il Ciocco, Italy, August 2018.

   Poster presentation

3. **Martin J.H,** Bromfield, E.G, Lord, T., Aitken, R.J and Nixon, B. DNA repair and protection in the female germline. Australian Society for Medical Research (ASMR) Satellite Scientific Meeting, Newcastle, Australia. June 2018.

   Poster presentation

2017:

1. **Martin, J.H,** Bromfield, E.G, Aitken, R. and Nixon, B. Double Strand Break Repair occurs via Non Homologous End Joining in mouse MII oocytes. 49th Annual Scientific Meeting of the Society for Reproductive Biology, Perth, Australia. August 2017.

   Oral presentation

2. **Martin, J. H,** Lord, T., Nixon B., and Aitken, R. J. Identification of a key role for permeability glycoprotein in enhancing the cellular defence mechanisms of fertilized oocytes. The 20th Annual Frontiers in Reproduction Symposia, Marine Biological institute, Woods Hole, Massachusetts, USA. June 2017.

   Oral presentation

3. **Martin, J. H**, Lord, T., Nixon B., Aitken, R. J. Identification of a key role for permeability glycoprotein in enhancing the cellular defence mechanisms of fertilized oocytes. The Australia and New Zealand Society for Cell and Developmental Biology-Cell & Developmental Biology Meeting. The University of New South Wales Sydney, Australia, USA. April 2017

   Oral presentation

2016:

1. **Martin, J. H**, Lord, T., Nixon B., Aitken, R. J. Identification of a key role for permeability glycoprotein in enhancing the cellular defence mechanisms of fertilized oocytes. 48th Annual Scientific Meeting of the Society for Reproductive Biology, Gold Coast, Australia. August 2016.

   Oral presentation

2. **Martin, J. H**, Lord, T., Nixon B., Aitken, R. J. Identification of a key role for permeability glycoprotein in enhancing the cellular defence mechanisms of fertilized oocytes. NSW Reproduction Forum, Newcastle, Australia. Invited speaker: December 2016.

   Invited speaker | *Oral presentation*

3. **Martin, J. H,** Lord, T., Nixon B., Aitken, R. J. Identification of a key role for permeability glycoprotein in enhancing the cellular defence mechanisms of fertilized oocytes. Australian Society for Medical Research (ASMR) Satellite Scientific Meeting, Newcastle, Australia. April 2016.

   Poster presentation

2015:

1. **Martin, J. H.,** Lord, T., Nixon B., Aitken, R. J. Permeability glycoprotein enhances cellular drug exclusion in the fertilised oocyte; upholding DNA integrity. 20th Annual Newcastle University Research Higher Degree conference. Newcastle, Australia. December 2015.

   Oral presentation | *Winner of the 'Best presentation prize'*

2. **Martin, J. H.,** Lord, T., Nixon B., Aitken, R. J. Permeability glycoprotein enhances cellular drug exclusion in the early embryo, upholding DNA integrity. 47th Annual Scientific Meeting of the Society for Reproductive Biology. Adelaide, Australia. August 2015.

   Oral presentation | *Finalist in the Oozoa award*

3. **Martin, J. H.,** Lord, T., Nixon B., Aitken, R. J. Permeability glycoprotein enhances cellular drug exclusion in the fertilised oocyte; upholding DNA integrity. Australian Society for Medical Research (ASMR) Satellite Scientific Meeting, Newcastle, Australia. April 2015.

   Poster presentation

4. Invited Seminars:

2018

1. **Southhampton University**, South Hampton**, UK|** July 2018

   Seminar title: 'DNA repair and protection in the female germline'

2017

1. **Brown University**, Rhode Island**, USA|** June 2017

   Seminar title: 'How oocytes and embryos protect their genetic integrity'.

2. **Northwestern University,** Chicago, **USA|** June 2017

   Seminar title: 'How oocytes and embryos protect their genetic integrity'.

3. **University of Montreal Hospital Centre**, Montreal, **Canada|** June 2017

   Seminar title: 'How oocytes and embryos protect their genetic integrity'.

4. **McGill University,** Quebec, **Canada|** June 2017

   Seminar title: 'How oocytes and embryos protect their genetic integrity'.

2016

1. **The NSW Reproduction Forum, Hunter Medical Research Institute, Newcastle, Australia |** December 2016

   Seminar title: Seminar title: **'**Identification of a key role for permeability glycoprotein in enhancing the cellular defence mechanisms of fertilized oocytes'.

2. **Newcastle University, Newcastle, Australia |** July 2016

   Seminar title: **'**A PhD in reproductive science: Investigating oocyte and embryo genetic integrity'.

5. Additional Publications

2018

1. Nixon, B., Johnston, S.D., Skerrett-Byrne, D.A., Anderson, A.L., Stanger, S.J., Bromfield, E.G., **Martin, J.H.,** Hansbro, P.M., Dun, M.D. (2018). Proteomic profiling of Australian saltwater crocodile (*Crocodylus porosus*) spermatozoa refutes the tenet that post-testicular maturation is restricted to mammals**|** (2018). **Molecular and Cellular Proteomics**; doi: 10.1074/mcp.RA118.000904

2. Heat exposure induces oxidative stress and DNA damage in the male germ line. Houston B.J, Nixon B, **Martin J.H**, De Iuliis G.N, Bromfield E.G, McEwen K.E, Aitken R.J. **Biology of Reproduction** (2018). Jan 17. doi: 10.1093/biolre/ioy009.

3. Houston, B.J., Nixon, B., **Martin, J. H,** Mcewan, K.E, King, B.V., Aitken, R.J, De Iuliis, G.N. (2018). Whole body exposure to radiofrequency electromagnetic radiation induces DNA fragmentation in mouse spermatozoa. **Manuscript in preparation | PLOS One.**

2017

1. Flourishing follicles: Overview of ovarioles. Kelleher A.M, Khalaj K, **Martin J.H**, Scaia M.F, Wilson R.L. **Molecular Reproduction and Development** (2017). Dec;84(12):1237. doi: 10.1002/mrd.22858.

2015

1. Murine inhibin α-subunit haploinsufficiency causes transient abnormalities in prepubertal testis development followed by adult testicular decline. Itman C., Bielanowicz A., Goh H., Lee Q., Fulcher A.J., Moody S.C., Doery, J.C., **Martin J.,** Eyre S., Hedger M.P., Loveland K.L (2015). **Endocrinology**; 156(6). DOI: 10.1210/en.2014-1555.

2014

1. Accumulation of electrophilic aldehydes during post- ovulatory ageing causes reduced fertility, oxidative stress and apoptosis. Lord, T., **Martin, J. H**., Aitken, R. (2014). **Biology of Reproduction** December; 92(2). DOI: 10.1095/biolreprod.114.122820.

2. Accumulation of electrophilic aldehydes during postovulatory ageing causes reduced fertility, oxidative stress and apoptosis. Lord, T., **Martin, J. H.,** Aitken, R. (2014). **Fertility and Sterility**; 102(3):e330. DOI: 10.1016/j.fertnstert.2014.07.1118.

6. Cover Images

Nature Lab Animal, Volume 46, No. 7, July 2017

Developmental Biology, Volume 417, Issue 1 (1st September 2016).

http://www.journals.elsevier.com/developmental-biology/cover-of-the-week/volume-417-issue-1-1st-september-2016

7. Distinctions and awards

Tuition scholarship| University of Newcastle for the PhD2Postdoc course (2018)| **$180**

Jennie Thomas Medical Research Travel Grant (2017)| HMRI | **$8900**

Greaves Family Postgraduate Scholarship in Medical Research (2017)| HMRI | **$5000**

Frontiers in Reproduction Tuition Scholarship (2017)| Marine Biological Institute and the University of Chicago| **$ 8500**

Higher Degree Research International Conference Scholarship (2017)| UON |**$2500**

Travel Grant (2017)| Faculty of Science, UON to attend Frontiers in Reproduction| **$1500**

Travel Grant (2017)| Australia and New Zealand Society for Cell and Developmental Biology-Cell and 'The company of biologists' |UNSW **$100**

Best Science image (2017)| Australia and New Zealand Society for Cell and Developmental Biology-Cell| UNSW | **$100**

Newcastle University Faculty of Science and IT Best HDR Publication Award (2016)| UON| **$1000**

Newcastle University School of Environmental and Life Sciences 'Excellence Award for First Year Presentation' 20th annual RHD conference| UON| December 2015| **$100**

Newcastle University School of Environmental and Life Sciences Priority Research Centre in Reproductive Science research in the molecular basis of sperm-egg interaction vacation scholarship 2013/2014. Effective date 11/04/2014| UON | **$1500**

Australian Postgraduate PhD Award in the Faculty of Science and Information Technology. 22/02/2015- 22/02/2018

Award Finalist:

Oozoa Award 'Best student presentation' |Society for Reproductive Biology | 2018

Travel Award | Reproductive Biology Journal| 2018

Travel Award | Biology Journal| 2018

HMRI-SA 'Future' Medical Research Travel Grant | 2016

Oozoa Award 'Best student presentation' |Society for Reproductive Biology | 2015

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ABSTRACT

DNA is the genetic repository containing the necessary information for cellular viability, fate decisions and development. In the female germline, genetic integrity also underpins successful conception, embryonic development, pregnancy and the future health of the offspring. In spite of its importance, DNA remains a chemical entity prone to structural alteration. If left unresolved, these structural lesions have the potential to lead to mutation and broader-scale genomic aberrations, which may elevate the predisposition of individuals to non-communicable diseases in later life. While it is therefore likely that female germ cells possess a sophisticated suite of quality control mechanism to defend their genome, the precise nature of these defence systems is not well understood. Given this knowledge gap, the overall aim of the studies described within this thesis was to explore the endogenous DNA protection and repair machinery present in the mammalian oocyte and early embryo.

In completing these studies, we have uncovered several novel protective strategies employed by the oocyte and early preimplantation embryo to safeguard their genomic integrity. These include the first evidence for a critical link between fertilisation and the synthesis of transmembrane transporter molecules belonging to the multidrug resistant protein family. Specifically, we implicate permeability glycoprotein (PGP) in increasing the bi-directional transport capacity of the zygote immediately following fertilisation. We posit that the activity of membrane bound PGP counters the influx of genotoxic agents, shielding the embryonic pronuclei from the induction of DNA damage. Excitingly, we also demonstrate that the preservation of the maternal genome, prior to fertilisation, is enhanced by an endogenous store of DNA repair proteins accumulated during oogenesis, providing the first evidence of an active DNA repair program in the post-ovulatory (MII) oocyte. Accordingly, we

demonstrate a role for non-homologous end joining (NHEJ) as a repair platform for correcting damage of the maternal DNA prior to fertilisation.

Having demonstrated that the oocyte and preimplantation embryo contain a sophisticated suite of defence strategies for the detection, repair or prevention of DNA damage, we hypothesized that the efficacy of these defences may be augmented by pro-survival factors. We therefore explored the capacity of C-peptide, a hormone implicated in the regulation of intracellular signalling pathways, to modulate oocyte and early embryo biology. Through this work, we observed a previously unappreciated abundance of C-peptide within the mouse ovary, oocyte and follicular fluid and uncovered a putative interaction between C-peptide and the DNA repair enzyme, breast cancer type 2 susceptibility protein (BRCA2) following oocyte activation. Collectively, these findings lend support to a novel role for C-peptide in the female germline and raise the prospect that C-peptide may exert direct physiological effects within the female reproductive system.

Taken together, the findings reported in this thesis have enhanced our understanding of the maintenance of genetic stability in the female germline. Importantly, this collection of studies offers a molecular understanding of the endogenous capacity of the oocyte and preimplantation embryo to detect and subsequently respond to DNA damage and, in turn, identifies novel clinical targets to enhance oocyte competence *in vitro* and potentially improve assisted reproductive technologies.

FOREWORD

The uptake of assisted reproductive technologies has exponentially increased since the first successful cycle in 1978, such that 40 years on, 1 in 6 couples routinely seek recourse in assisted reproductive technologies (ART) to achieve pregnancy (Australian Institute of Health and Welfare, 2015). Today, ART accounts for approximately 5.8% of all births in Australia (Australian bureau of Statistics, 2017). Yet, in spite of its routine use, ART remains invasive, expensive and not without risk. Moreover, ART does not address the specific cause of a couple's infertility (Aitken and De Iuliis, 2010). While numerous pathologies can contribute to infertility and its adverse sequelae, mounting evidence suggests that a substantial proportion of assisted reproductive cycles with poor prognoses are associated with reduced genetic integrity of the gamete(s).

Indeed, genomic instability in the germline has the potential to alter embryonic gene expression and drive modified developmental programmes with every subsequent cell cycle (Fleming et al., 2018). In recognition of this, there is an impetus to explore new ways to protect the female germline from genomic damage and thus preserve fertility. Currently however, little is known regarding the innate mechanisms of DNA protection and repair employed by the post-ovulatory oocyte and early embryo. Given this, characterisation of the cellular, metabolic, and physiological mechanisms safeguarding the female germline is key to understanding how to enhance these processes.

In this thesis, we have surveyed the quality control mechanisms utilised by the oocyte and early preimplantation embryo to ensure genomic fidelity. Specifically, the publications arising from this work have focused on the protective strategies of: (i)

efflux transporter proteins (Chapter 2), non-homologous end joining DNA repair pathways (Chapter 3) and novel pro-survival factors, such as C-peptide, which may facilitate the correct localisation of DNA repair machinery in the zygote (Chapter 4). The collective findings described in these publications provide a critical framework for informing novel strategies to preserve oocyte/embryo quality *in vitro* and thus contribute to improvements in contemporary assisted reproductive technologies.

CHAPTER 1: LITERATURE REVIEW

Biochemical alterations in the oocyte in support of early embryonic development

Published: Cellular and Molecular Life Sciences, 74: 469-485

Authors: Martin, J.H[1]., Bromfield, E.G[1]., Aitken, R.J[1]., Nixon, B[1].

Affiliations: [1]Priority Research Centre for Reproductive Biology, School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia.

Correspondence: Jacinta.martin@newcastle.edu.au

CHAPTER OVERVIEW

The aim of the following literature review was to consider the molecular events that occur during oogenesis that are critical for the successful union of the parental gametes. Specifically, we explore the essential role for maternally derived protein and RNA transcripts accumulated during oogenesis, and the post-translational protein modifications required to sustain preimplantation development prior to activation of the zygotic genome. Notably, before the completion of the first meiotic M phase, most transcribed mRNA is immediately translated. However, in a process unique to the oocyte, a significant and developmentally crucial ~30% of mRNA transcripts are translationally repressed and stored alongside protein, in preparation for the transcriptionally inactive meiotic maturation phases and preimplantation development. Notably, this pool includes multiple gene products associated with the DNA damage surveillance and repair responses, which are well positioned to participate in protection of the genetic integrity of the female germline.

This review therefore acts to synthesize the historic and contemporary literature surrounding the biochemical and physiological modifications that endow the oocyte with the capacity to divide and differentiate into the trillions of cells that comprise a new individual. We explore the sequential interaction of elevated intracellular calcium and the post-translational protein modification of the maternally derived proteins products, to bridge the gap between the initiation of development and the maintenance of genomic integrity. Finally we contextualise, novel data from our own research whereby protein phosphorylation enhances membrane bound defence mechanisms to safeguard the genomic integrity of the zygote.

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Cellular and Molecular Life Sciences

REVIEW

Biochemical alterations in the oocyte in support of early embryonic development

Jacinta H. Martin[1] · Elizabeth G. Bromfield[1] · R. John Aitken[1] · Brett Nixon[1]

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Abstract Notwithstanding the enormous reproductive potential encapsulated within a mature mammalian oocyte, these cells present only a limited window for fertilization before defaulting to an apoptotic cascade known as post-ovulatory oocyte aging. The only cell with the capacity to rescue this potential is the fertilizing spermatozoon. Indeed, the union of these cells sets in train a remarkable series of events that endows the oocyte with the capacity to divide and differentiate into the trillions of cells that comprise a new individual. Traditional paradigms hold that, beyond the initial stimulation of fluctuating calcium (Ca^{2+}) required for oocyte activation, the fertilizing spermatozoon plays limited additional roles in the early embryo. While this model has now been drawn into question in view of the recent discovery that spermatozoa deliver developmentally important classes of small non-coding RNAs and other epigenetic modulators to oocytes during fertilization, it is nevertheless apparent that the primary responsibility for oocyte activation rests with a modest store of maternally derived proteins and mRNA accumulated during oogenesis. It is, therefore, not surprising that widespread post-translational modifications, in particular phosphorylation, hold a central role in endowing these proteins with sufficient functional diversity to initiate embryonic development. Indeed, proteins targeted for such modifications have been linked to oocyte activation, recruitment of maternal mRNAs, DNA repair and resumption of the cell cycle. This review, therefore, seeks to explore the intimate relationship between Ca^{2+} release and the suite of molecular modifications that sweep through the oocyte to ensure the successful union of the parental germlines and ensure embryogenic fidelity.

Keywords Zygote · Oocyte activation · Phosphorylation · Protein kinase · DNA repair · DNA protection

Introduction

A defining feature of the mature, ovulated, metaphase II (MII) oocyte is the narrow window of opportunity that it presents to undergo successful fertilization and initiate embryogenesis [1]. Indeed, without the union of an oocyte and a functionally mature spermatozoon, the oocyte will rapidly undergo apoptosis and degradation via a process known as post-ovulatory oocyte aging [2]. Immediately following fertilization, however, the fertilizing sperm cell initiates a series of irreversible biochemical and physiological modifications to the oocyte's cortex and cytoplasm, thus rescuing the cell from its otherwise predestined apoptotic fate and immortalizing its genetic contribution within the conceptus [3, 4]. Accordingly, the molecular basis of the sequential interactions between the fertilizing spermatozoon, the oocyte, and the subsequent events that they set in train has been the subject of considerable attention spanning many decades. Such intense research effort has provided compelling evidence that gamete fusion is followed by a rapid release of intracellular calcium from internal stores [5]. This initial elevation generally occurs within 1–3 min post-fusion [6] and, in turn, stimulates oscillating Ca^{2+} transients that can persist for a further

R. J. Aitken and B. Nixon contributed equally to this work.

✉ Jacinta H. Martin
jacinta.martin@newcastle.edu.au

[1] Discipline of Biological Sciences and Priority Research Center for Reproductive Science, School of Environmental and Life Sciences, The University of Newcastle, University Drive, Callaghan, NSW 2308, Australia

3–4 h [7, 8]. It is during this period of fluctuating Ca^{2+} transients that oocyte activation is initiated (reviewed in [9]).

Coinciding with pronuclear formation and syngamy, these Ca^{2+} oscillations briefly pause, only to transiently resume during the first phase of mitosis [7]. Recent work has provided evidence that the pause in calcium ion fluxes may also occur synchronously with a critical round of DNA repair and a concomitant upregulation of protective machinery [10, 11]. Such mechanisms have been postulated to ensure the zygote is in optimal condition to undergo embryogenesis [12] and appear to be causally linked to the post-translational modification, and hence activation, of an impressive suite of reparative and protective enzymes [10]. Notwithstanding these exciting data, the identity of a majority of the targets, the complex signaling pathways that underpin their activation, and their diverse roles in preparing the oocyte for embryogenesis remain to be fully investigated and, in some instances, are the subject of considerable controversy [13, 14]. In this review, we seek to integrate the leading theories and emerging data to provide a comprehensive appraisal of the role of fertilization and oocyte activation in extending the viability of the oocyte with a focus on highlighting the prominent role of post translation modifications (PTMs), in particular phosphorylation, in these events.

Post-ovulatory oocyte aging

Immediately following ovulation into the female reproductive tract, the mature oocyte remains viable for a relatively short period of time ($\sim$16 h) before defaulting to a terminal pathway of post-ovulatory aging [4, 15]. Oocytes progressing through this degradative process bear the well-characterized hallmarks of apoptosis and recent literature has implicated mitochondrial dysfunction and a consequential generation of reactive oxygen species (ROS) as a key mediator of this process [2, 4, 15, 16]. Produced as an intermediary product of normal cellular metabolism, ROS are well known to play fundamental roles in physiological signaling [17–20]. However, imbalances created by elevated levels of ROS that overwhelm the inherent antioxidant defenses within a cell can lead to the oxidation and alkylation of cellular components, such as DNA, lipids, and proteins with dire consequences for cell viability [21, 22]. Post-ovulatory oocyte aging appears to be orchestrated, at least in part, by these oxidative processes that progress through a cascade of events encompassing elevated ROS and the peroxidation of lipids comprising the cellular membranes (not excluding the mitochondrial membrane) [2, 23]. The ensuing production of electrophilic lipid aldehydes leads to widespread adduction of vulnerable proteins and DNA causing extensive cellular damage [21]. Even prior to the final induction of apoptosis, these profound insults prevent, or severely reduce the capacity of the oocyte to participate in fertilization and subsequently support embryonic development [1, 2, 4, 23, 24]. Significant biochemical abnormalities result in the reduced activity of critical proteins [e.g., maturation-promoting factor (MPF) and mitogen-activating protein kinase (MAPK)], impaired Ca^{2+} homeostasis, increased autophagy-related activity, mitochondrial dysfunction and disruptions to cell-cycle and stress response pathways [2, 15, 16, 24–28].

In this context, recent work in the mouse has shown that the onset of post-ovulatory oocyte aging is precipitated by an imbalance in cellular antioxidants and ROS levels [4]. Furthermore, in vitro studies have shown that mouse oocytes can be induced to display an aging phenotype following exposure to electrophilic aldehydes such as acrolein and 4-hydroxynonenal (4HNE) resulting in a dramatic decline in the fertilizability of these cells [2, 28]. Such consequences are not surprising, considering the integral contribution electrophilic aldehydes play in a number of oxidative stress-associated diseases, including: diabetes, cancer, atherosclerosis, acute lung injury, chronic alcohol exposure, and in neurodegenerative diseases, such as Alzheimer's and Parkinson's disease (reviewed in [29–31]). In addition, this model may also account for reduced pregnancy rates and higher incidences of cytogenetic abnormalities in humans associated with the use of in vitro aged oocytes following 'rescue ICSI' strategies [32, 33]. Although not specifically reviewed here, it is also important to note that oocytes from individuals of increasing maternal age also display many of the characteristic hallmarks of oxidative stress and the corresponding loss in fertility of those which undergo post ovulatory aging (for review, see [34–38]).

In the search for strategies to prevent oxidative threat to female fertility, sirtuins [silent information regulator 2 (Sir2) proteins] and antioxidant supplementation have emerged as candidates to modulate oxidative assault. Sirtuins appear to play a role in sensing and modulating cellular redox status as well as directly deacetylating key proteins involved in the cellular stress response, thus having been shown to provide protective effects in cells and tissues exposed to oxidative stressors in vitro and in vivo [39, 40]. Supplementation of antioxidants, such as melatonin or caffeine, also appears to provide a valuable, yet temporary, solution to oxidative insult in oocytes. However, while such interventions can delay oocyte aging, they do not appear to be able to prevent this phenomenon entirely [2, 4, 23, 27, 41]. While it is clear that ROS can precipitate a number of negative consequences within oocytes, other factors, such as in vitro handling techniques

and oocyte age (as a result of maternal age), have also been correlated with degradation of oocyte quality. This not only encompasses the consequences listed previously, but also manifests in a reduction in abundance of maternal effect proteins, loss of RNA-binding proteins, critical alterations of pericentromeric proteins, aneuploidy, and epigenetic changes inherited by the derivative generation(s) [42–44]. Undeniably, it is only the act of fertilization that can effectively truncate the post-ovulatory aging phenotype and the inevitable induction of apoptosis [4, 15]. Thus, understanding the precise biochemical and physiological modifications that are initiated upon gamete fusion may hold the key to our attempts to prolong the viability of oocytes and the developmental competence of early embryos.

The role of ions as post-fertilization signaling molecule

At the moment of fertilization, the spermatozoon is responsible for activating embryonic development by virtue of its ability to promote a transient elevation and subsequent oscillating waves of intracellular Ca^{2+} levels within the oocyte [5, 6]. Such events release the oocyte from its MII stage arrest and drive it toward embryogenesis via stimulation of meiotic resumption, cortical granule exocytosis, decondensation of the sperm nucleus, recruitment of maternal mRNAs, and pronuclear development [45].

In mammals, the initial burst of Ca^{2+} is of a longer duration and amplitude than that of the subsequent transients [8, 46]. More importantly, while Ca^{2+} has distinctive short-term effects on the initiation and completion of oocyte activation events, it has also been implicated in the downstream events encompassed by peri-implantation development and gene expression [47–49]. Support for the central role of Ca^{2+} in stimulating embryonic development rests with a series of elegant studies incorporating intracellular Ca^{2+} chelating agents [such as 1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA-BA)], which prevents cellular depolarization, metaphase II exit, cortical granule exocytosis, and pronuclear formation [50, 51]. Additional supporting evidence has been secured from experiments involving the judicious use of chemicals such as strontium chloride ($SrCl_2$) and ethanol (EtOH) to artificially stimulate an increase in intracellular Ca^{2+} concentration, and thus drive the chemical or parthenogenetic activation of an oocyte in the absence of a fertilizing spermatozoon [15, 52–55]. The utility of such an approach is recognized by the routine use of artificial activators, together with a spermatozoon, as a supplement in ART settings when the male gamete is unable to activate the oocyte [15, 52–55]. Despite recognition of the importance of Ca^{2+} in these events, the specific sperm factor(s) and signal transduction pathways responsible for triggering its initial release after sperm–oocyte fusion remain unclear and the subject of considerable controversy [13, 14, 56, 57].

What is clear, is that at the moment of fertilization, the hydrolysis of the phosphatidylinositol 4,5-bisphosphate (PIP_2) phospholipid is initiated resulting in the release of cleaved inositol trisphosphate (IP_3) and diacylglycerol (DAG), the former of which binds to IP_3 receptors (IP_3Rs) located in the endoplasmic reticulum (ER), thereby stimulating the release of stored Ca^{2+} [58, 59]. Indeed, the injection of native IP_3 or IP_3 analogs is sufficient to induce Ca^{2+} release in mammalian oocytes [8, 60]. Conversely, inhibitory antibodies and pharmacological reagents that prevent IP_3 binding to IP_3Rs are able to elicit a potent suppression of fertilization induced Ca^{2+} oscillations and subsequently arrest fertilization and downstream embryonic development [58, 61]. The factor(s) that link these signaling phenomena to upstream sperm fusion may be expected to take the form of either oolemmal receptor(s) and/or soluble factor(s) delivered by the fertilizing spermatozoon [62, 63]. Indeed, numerous hypotheses have been put forward to account for the origin of the signal that stimulates the early events of fertilization [13, 14, 56].

Presently, the most widely accepted model centers on sperm specific factor(s) (SSF) that rouse the oocyte by promoting the initial surge in $[Ca^{2+}]_i$. Two purported sperm borne factors that putatively fulfill this role are phospholipase C zeta (PLCζ) and post-acrosomal WW-domain binding protein (PAWP) (Fig. 1) [8, 64, 65]. Indeed, experiments in which either purified PLCζ or PAWP has been injected into an MII stage oocyte have successfully stimulated the production of Ca^{2+} transients that are akin to those that ensue after sperm fusion [60, 64]. Since their initial identification however, numerous research groups have independently reported evidence conferring support for the role of PLCζ in oocyte activation and release of stored Ca^{2+} [8, 66–68]. In this regard, a considerable evidence base has now been established supporting a strong correlation between abnormalities in the structure, expression, and localization pattern of human PLCζ with that of oocyte activation deficiency (OAD) and total fertilisation failure (TFF) [69]. In contrast, since its original identification in 2007, independent research groups have yet to corroborate the ability of PAWP to successfully activate oocytes and/or induce Ca^{2+} oscillations (reviewed in [13]). Indeed, with the generation of a PAWP knockout mouse, it now appears that depletion of PAWP does not elicit the anticipated quantitative change in Ca^{2+} oscillations or in the subsequent rates of embryo development [57].

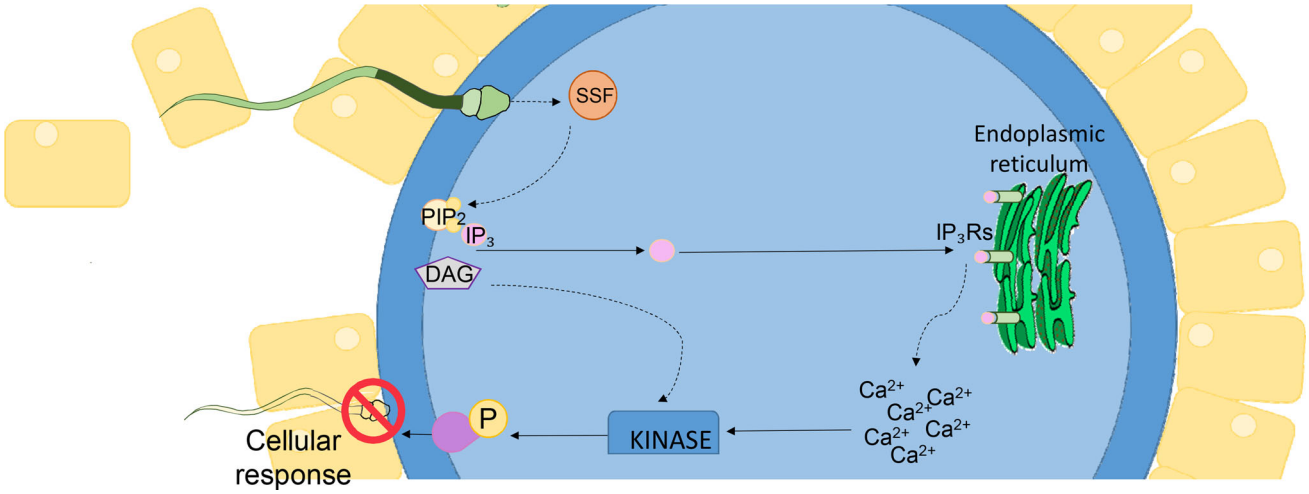

Fig. 1 Fertilization from fusion to activation. The signaling phenomena necessary for oocyte activation and embryonic development encompasses the hydrolysis of the phosphatidylinositol 4,5-bisphosphate (PIP_2) phospholipid anchored in the plasma membrane of the oocyte triggering the subsequent release of cleaved inositol trisphosphate (IP_3) and diacylglycerol (DAG). The released IP_3 is then able to bind IP_3 receptors (IP_3Rs) embedded within the endoplasmic reticulum (ER) and stimulate the release of stored Ca^{2+} to initiate the cellular responses required for oocyte activation. Among the putative sperm-specific factor(s) (SSF) that link sperm fusion to PIP_2 hydrolysis, phospholipase C zeta (PLCζ) and/or post-acrosomal WW-domain binding protein (PAWP) have emerged as key contenders (Adapted from [13])

Ultimately, the definitive identification of the key sperm factor(s) and their mode of action may be crucial for the development of therapeutic intervention strategies to extend the viability of the oocyte, and may also hold value as a prognostic biomarker for the diagnosis of male factor infertility [56, 68, 70, 71]. In this context, strong correlations have been drawn between PLCζ and PAWP protein levels and the success of assisted reproductive cycles, with many instances of infertile human spermatozoa having been found to be deficient in their ability to stimulate the Ca^{2+} oscillations necessary for successful fertilization [72–75]. This work would also benefit from further analysis of the putative synergistic roles that have recently been assigned to dynamic fluxes in alternative ions such as zinc (Zn^{2+}) [76–78]. While still in their relative infancy, the study of the newly coined 'zinc-sparks' has revealed a striking redistribution of Zn^{2+} loaded vesicles immediately at fertilization and demonstrated an inverse relationship between declining Zn^{2+} levels and the all-important increase in Ca^{2+} that is required for successful fertilization, oocyte activation and egg–embryo transition [76–78].

Indeed, during the final hours of meiotic maturation, the mouse oocyte accumulates an impressive twenty billion Zn^{2+} ions (representing an approximate 50 % increase in total Zn^{2+} content) [79, 80], via the two maternally derived and cortically distributed zinc transporters, ZIP6 and ZIP10 [77, 80–82]. Despite this, it is now widely accepted that release from MII arrest requires a dramatic decrease in intracellular Zn^{2+} content. Thus, the act of fertilization must trigger the coordinated release of billions of Zn^{2+} ions, and appears to do so via a novel exocytotic event referred to as a 'zinc-spark.' This process is necessary to re-establish cell cycle progression, oocyte activation and induce the egg-to-embryo transition [76–78, 81]. Accordingly, zinc-sparks appear to be evolutionarily conserved in all mammalian species studied to date, including humans, rodents, and nonhuman primates [77, 79]. The importance of Zn^{2+} homeostasis for oocyte biology is further emphasized by recent studies in which the sequestration of zinc using the heavy metal chelator N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) [83], or the targeted disruption of Zn^{2+} transporters (ZIP6 and ZIP10), both led to immature telophase I-like cell cycle arrest; a response that could be reversed by Zn^{2+} supplementation [77]. Similarly, oocyte zinc-spark profiles have been positively correlated with mouse embryonic development and embryo quality. Thus, those oocytes that released higher concentrations of Zn^{2+} immediately following fertilization displayed the greatest embryonic development potential [84]. In view of such information, zinc-spark profiles hold considerable promise as a novel extracellular physicochemical biomarker of embryonic developmental potential [84].

Despite the clear biological and clinical importance of Ca^{2+}, and now Zn^{2+}, a rapid induction of transient fluxes in the intracellular concentration of either ion, would not in themselves be sufficient to support conception. Rather, it is likely that these ion(s) act in either an indirect and/or direct manner to promote widespread post-translational modifications (PTMs) across a suite of key enzymes (e.g., protein kinases (PK), phosphatases, and acetyltransferases) that themselves are responsible for promoting the changes in

cellular physiology necessary for oocyte activation and embryonic development [85].

Phosphorylation and fertilization

Protein post-translational modifications (PTMs) increase the functional diversity of the cellular proteome via the covalent addition of functional groups, proteolytic cleavage of regulatory subunits or degradation of entire proteins. These chemical modifications influence almost all aspects of normal cell biology and pathogenesis [86], and their requirement during fertilization is driven at least in part, by the unique dependence of the early embryo on a modest store of maternally derived proteins and mRNA to support all of the early events during embryogenesis [87, 88]. Indeed, until recently, the leading paradigms have held that beyond the initial stimulation of oocyte activation, the fertilizing spermatozoon plays limited additional roles in the cleavage stage embryo [88]. Instead, this role is believed to rest predominantly with maternal factors that accumulate during oogenesis and are responsible for directing zygotic genome activation, the cleavage stages of embryogenesis, as well as the establishment of the initial cell lineages [87, 88]. In keeping with this notion, an autonomous transcription program is not established until the 2 (mouse) or 4 cell stages of embryonic development (bovine, ovine, and human) [89–91].

Among a complement of some 300 forms of PTMs, those involving the selective activation and inactivation of substrates via phosphorylation appear to hold a central position in coordinating the regulation of early embryo development (reviewed in [85]). Protein phosphorylation is a dynamic PTM that is mediated by kinases and phosphatases, which selectively phosphorylate and dephosphorylate substrates, respectively. Principally targeting serine, threonine or tyrosine residues, phosphorylation is one of the most important and well-studied PTMs with estimates suggesting that as many as one-third of the proteins in the human proteome are substrates for phosphorylation [92]. Such substrates extend to the fertilized oocyte, where they have been implicated in a diverse suite of physiological responses that encompass: a rapid block to polyspermy, cortical granule exocytosis, polar body extrusion, pronuclear development, plasma membrane reorganization, recruitment of maternal mRNAs, DNA repair, and resumption of the cell cycle (Fig. 2, Table 1).

The vital role of protein kinases in oocyte activation has been eloquently described in *Drosophila melanogaster*, where 311 proteins were shown to exhibit a change in phosphorylation status between mature and activated oocytes [93], suggesting that phosphorylation might simultaneously and rapidly modulate the activity of many proteins. Chief among these protein targets were those integral to Ca^{2+} binding, proteolysis, and protein translation, as well as those required for general oocyte activation and post-fertilization developmental stages. A number of kinases and their regulatory subunits were also identified amongst the candidates, including extracellular signal-regulated kinases (ERK) [also known as MAPK (mitogen-activated protein kinases)] and A-kinase anchor protein 200 (a regulatory subunit required for PKA localization) [93].

Like that of *D. melanogaster*, the mouse oocyte experiences a swift and dramatic alteration to its global phosphorylation status following both in vivo and in vitro activation [94]. Of particular interest, protein kinase C (PKC) activity has been linked to fertilization-induced Ca^{2+} oscillations (Table 1) using fluorescent C-kinase activity reporter (CKAR) probes in tandem with the selective PKC inhibitor, Gö6976. Interestingly, in vitro manipulation of PKC has revealed an additional role for this enzyme in the stimulation and maintenance of the Ca^{2+} oscillations that drive its activation, suggesting that it forms part of an important regulatory feedback loop [95]. As an extension of this model, Gonzalez-Garcia et al. [96] also showed that PKC-induced phosphorylation outlasts each Ca^{2+} transient, thus raising the possibility that it has a prolonged influence over such downstream events as pronuclear formation, spindle dynamics, cytoskeletal reorganization, cell cycle resumption, and DNA repair [97, 98]. In addition to PKC, oscillating Ca^{2+} has also been implicated in stimulation of calmodulin-dependent protein kinase II (CAMII), a response that triggers the phosphorylation and systematic degradation of non-essential proteins through ubiquitination [85, 99], resumption of meiosis and cortical granule exocytosis [100] (Table 1). Accordingly, CAMII activity was also found to spike almost immediately prior to, and remain elevated following, the extrusion of the second polar body (1.5 h after insemination of oocytes with spermatozoa) [100]. In a similar manner, additional kinases, such as myosin light chain kinases (MLCK), zipper-interacting protein kinase (ZIP), and Rho-kinase (ROCK), have each been implicated as playing integral roles in cytoskeletal arrangement during the fertilization cascade (Table 1). Such activity appears to be mediated, at least in part, via a conserved mechanism involving the phosphorylation of serine 19 (Ser19) on myosin regulatory light chains [101]. This phosphorylation event stimulates actin-mediated ATPase activity and the assembly of myosin II into filaments, thereby promoting cytoskeletal remodeling, cortical granule exocytosis, cytokinesis, polar body extrusion, and cleavage [85, 101]. In support of such functions, it has been demonstrated that selective pharmacological inhibition of MLCK, ZIP, and ROCK (by blebbistatin, ML-7 or Y-27632, respectively) is able to ablate the formation of the second polar body and the correct spindle rotation required for normal cytokinesis

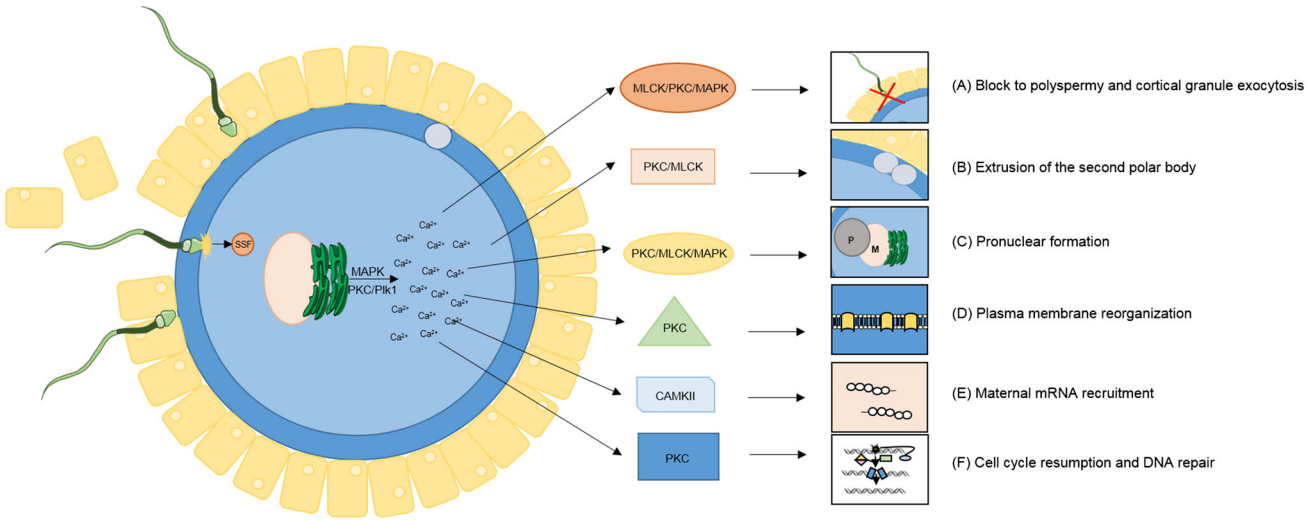

Fig. 2 Protein kinases are intimately tied to each essential event of oocyte activation. Protein kinases and phosphorylation events have been directly implicated in transducing the calcium (Ca^{2+}) signal into many of the necessary activation events required for successful fertilization. Such events encompass a swift block to polyspermy, cortical granule exocytosis, polar body extrusion, pronuclear development, plasma membrane reorganization, recruitment of maternal mRNAs, DNA repair and resumption of the cell cycle (See Table 1) (Adapted from [85])

[102–107]. The role of myosin phosphorylation during fertilization is further underscored by studies in which mouse oocytes were microinjected with nonphosphorylatable myosin regulatory light chain peptides [108]. Such a strategy has been shown to effectively block sperm incorporation cone disassembly and obstruct cell cycle progression with pronounced consequences for successful fertilization [108].

Extending beyond the induction of fertilization, anomalous kinase activity and thus the fidelity of downstream phosphorylation events have been implicated in the etiology of infertility as well as gross phenotypic and developmental abnormalities in offspring [108–110]. Of particular concern, disruption of global phosphorylation by pharmacological or antibody inhibition has been shown to lead to abnormal cortical granule exocytosis, redistribution of the ER and IP_3R1s, disruption of the second polar body formation and extrusion, as well as aberrant cytoskeletal reorganization and cleavage during embryogenesis [100]. For instance, mutation of MAPK signaling pathways abrogates normal IP_3R1 phosphorylation required for the optimal release of Ca^{2+} ions at fertilization; those oocytes deprived of the MAPK signaling pathway during maturation fail to mount normal Ca^{2+} oscillations and show compromised IP_3R1 function leading to compromised or arrested development [111]. Not surprisingly, phosphorylation of IP_3R1 by Polo-like kinase1 (PLK1) also appears to underlie the spatial and temporal regulation of intracellular Ca^{2+} signals required for oocyte maturation. In fact, PLK1 has been shown to co-localize with MAPK and its activity is reduced in the absence of MAPK/ERK activity [112] with devastating consequences for fertilization. Similarly, inhibition of CAM kinase II by pharmacological means [myristoylated-AIP (autocamtide-2-related inhibitory peptide)] disrupted the inactivation of MPF (maturing promoting factor), preventing cell cycle resumption and cortical granule exocytosis in both fertilized and ethanol-activated oocytes [100].

Collectively, these data highlight the importance of phosphorylation cascades in several pivotal aspects of oocyte activation. First, this form of PTM directly affects the activity of a diverse suite of protein targets with dominant roles in transducing the initial Ca^{2+} signal into embryonic activation. Second, even subtle disruptions to these integral pathways can cause dramatic and irreversible consequences for future offspring. Moreover, when these disruptions occur on a broad scale, they can elicit gross biochemical, phenotypic and genomic abnormalities culminating in arrested embryonic development. Such a situation arises, at least in part, because a majority of the enzymes involved in intrinsic cellular repair pathways, such as base excision repair (BER) and homologous recombination (HR), require activation via phosphorylation (or other forms of PTMs) prior to being able to engage in the detection and mitigation of DNA damage (reviewed in [113–115]).

DNA repair within the oocyte

At the moment of fertilization, the majority of oocyte activation events either occur in parallel with, or in quick succession after, the initiation of Ca^{2+} oscillations.

Table 1 Phosphorylation directs key physiological events required for successful fertilization and early embryonic development. Widespread post-translational modifications, in particular phosphorylation has been implicated in directing a diverse suite of physiological responses required for oocyte activation and early embryonic development. A remarkable degree of redundancy exists between kinases at fertilization suggesting the highly integrated nature of these processes for the successful activation of development. A number of key kinases, their regulators, and proposed functions are listed in this table

Kinase	Regulated by	Proposed function(s)	References
Conventional protein kinase C (cPKC)	Intracellular Ca^{2+} DAG Phosphatidylserine	Stimulation/maintenance of Ca^{2+} oscillations Extrusion of the second PB Cell cycle resumption/MII exit PN formation Spindle dynamics/organization Cytoskeletal reorganization DNA repair Block to polyspermy Cortical granule translocation/exocytosis Differential activation of kinases	[85, 95–98, 172–174]
Novel protein kinase C (nPKC)	DAG Phosphatidylserine	Meiotic spindle dynamics Formation/extrusion of the second PN Cortical granule translocation	[95, 175, 176]
Atypical protein kinase C (aPKC)	Phosphatidylserine Negatively charged phospholipids	Regulation of nuclear activity Cortical granule exocytosis	[177]
Calmodulin-dependent protein kinase II (CAMKII)	Intracellular Ca^{2+} bound to calmodulin (CAM)	Protein degradation by ubiquitination Decrease in MPF/resumption of meiosis Cortical granule translocation/exocytosis Maternal mRNA recruitment MII exit Cyclin B1 and securin destruction Decreases in the activity of MAPK Second PB extrusion Formation of maternal PN Phosphorylation of IP_3R1s Regulation of apoptosis Sister chromatid segregation/ decondensation	[85, 99, 174, 178–182]
Polo-like kinase1 (PLK1)	Intracellular Ca^{2+}	Redistribution of the ER Phosphorylation of IP_3R1s Regulation of Ca^{2+} Enhance receptor function on ER Removing an APC/C inhibitor	[85, 112, 183, 184]
Myosin light chain kinases (MLCK)	Intracellular Ca^{2+} phosphorylation of myosin II	Block to polyspermy PN formation Cytoskeletal remodeling PB formation/extrusion Cleavage/cytokinesis Redistribution of the ER and IP_3R1s Calcium oscillations Regulator of myosin II in non-muscle cells Spindle rotation Morula-to-blastocyst transition	[85, 102–107, 185]

Table 1 continued

Kinase	Regulated by	Proposed function(s)	References
Mitogen-activating protein kinase (MAPK) and Extracellular signal-regulated kinases (ERK)	Intracellular Ca^{2+} CSF activity	Activation and regulation of Ca^{2+} release Formation of PN Disintegration of the pronuclear envelope Cortical re-organization prior to fertilization Translocation of the meiotic spindle Redistribution of the ER and IP_3R1s Formation of ER and IP_3R1 cortical clusters phosphorylation of IP_3R1	[85, 93, 102, 106, 111, 186, 187]
M-phase kinases or Maturation promoting factor (MPF)	Intracellular Ca^{2+} ATP Cyclin	Stimulation/maintenance of Ca^{2+} oscillations promoting resumption of meiosis and cell-cycle transitions Nuclear membrane breakdown	[85, 186]
Rho-kinase (ROCK)	Intracellular Ca^{2+}	Rho-furrowing phase of cytokinesis Spindle rotation Polar body formation Phosphorylation of IP_3R1 Cytoskeletal rearrangement Ooplasmic segregation Spindle rotation	[86, 112, 186, 188–190, 107]
Zipper-interacting protein kinase (ZIP kinase)	Intracellular Ca^{2+}	Regulator of myosin II required for cytokinesis	[101]
Protein kinase A (PKA)	Intracellular Ca^{2+}	Phosphorylate IP_3R1	[175, 191]
Protein kinase G (PKG)	Intracellular Ca^{2+}	Phosphorylate IP_3R1	[192]
Tyrosine kinases (FYN and LYN)	Intracellular Ca^{2+}	Phosphorylate IP_3R1	[193, 194]
Protein kinase B (PKB)	Intracellular Ca^{2+}	Phosphorylate IP_3R1	[195]

However, at the time of pronuclear formation, the conceptus experiences a transient arrest of Ca^{2+} oscillations [7]. Recent evidence has raised the possibility that this phenomenon is staged to allow a critical round of DNA repair to occur prior to DNA replication during the first mitotic division [12]. In addition, this temporary suspension of Ca^{2+} oscillations enables the reconfiguration of the sperm chromatin prior to pronuclear formation and syngamy [116]. However, the capacity of preimplantation stage embryos to repair damaged DNA remains to be fully characterized, and it seems that phosphorylation of existing repair proteins and maternal (and more even recently paternal) mRNAs capable of damage detection and cell cycle control are likely responsible for directing DNA repair [117–121]. Fittingly, the effects of paternal miRNAs seem to be mediated, at least in part, by post-transcriptional regulation over maternal and early zygotic mRNAs (e.g., miRNA mediated mRNA stability control; refer to section 'Epigenetics and DNA repair') [117, 122]. The quality of the paternal and maternal genetic contributions at fertilization greatly influences the developmental competency of the derivative organism [123]. It follows that the absence of efficient DNA repair at this critical developmental phase can either result in complete embryonic arrest or poor embryo development associated with increased risks of immunodeficiency, neurological disorders, and cancer within offspring and a concomitant reduction in life expectancy [124–127].

While many somatic cells experience a consequential increase in the synthesis of DNA repair enzymes and protective proteins in response to DNA damage [128, 129], the ovulatory stage oocyte is, in contrast, transcriptionally silent and incapable of mounting such a response [130]. Instead, these cells depend on stores of pre-synthesized proteins and/or mRNA transcripts to drive repair pathways during fertilization and early embryo development [131], which additionally may work in combination with paternal epigenetic modulators [118] (see Section 'Epigenetics and DNA repair'). During these transcriptionally inert stages of development, PTM of pre-synthesized and maternally stored repair enzymes are likely to facilitate the activation of the fundamental machinery necessary for mitigating DNA and/or cellular damage. In this context, an impressive array of PTMs (including acetylation, phosphorylation,

methylation, ubiquitination, and sumoylation, as well as histone modification) have been implicated in cellular repair capacity and have been found to dramatically influence the kinetics of DNA repair [23, 127, 132]. Recent research within our own laboratory has provided evidence for the induction of a dramatic series of post-fertilization reparative (and protective) events involved in ensuring oocyte viability prior to embryogenesis [10, 133, 134]. Furthermore, we have also provided evidence for DNA repair and protection pathways with stage-dependent activity in the mammalian oocyte [10, 133, 134]. As a culmination of this work, it has been suggested that oocytes experience accelerated repair of oxidative DNA damage following fertilization driven by post-translational modifications of proteins that participate in the BER pathway [10] (Fig. 3a). This pathway, which includes the key elements of oxoguanine glycosylase (OGG1), apurinic/apyrimidinic endonuclease (APE1), and X-ray repair cross complementing protein 1 (XRCC1), is capable of repairing the common oxidative DNA base adduct, 8-hydroxyguanosine.

While the BER pathway is considered the primary orchestrator of oxidative damage repair in the zygotic genome, a host of other enzymes have the capacity to contribute to these and other repair processes [134]. A recent proteomic comparison of murine MII oocytes and zygotes identified a total of 53, and in primate oocytes, approximately 37 proteins involved in DNA damage and DNA repair related processes [121, 130]. In the MII oocyte, enzymes required for nucleotide excision repair (NER), single strand break repair (SSB), double strand break repair (DSB), and BER are all highly expressed [121, 130], thus highlighting their potential for activation and use immediately following fertilization. In the same manner, a proteomic analysis of high- and low-quality oocytes in a porcine model indicated a positive correlation between the abundance of DNA repair proteins and the quality of the oocyte [135]. Microarray and bioinformatic approaches have also confirmed the expression of a similar profile of proteins in human oocytes and early embryos [136].

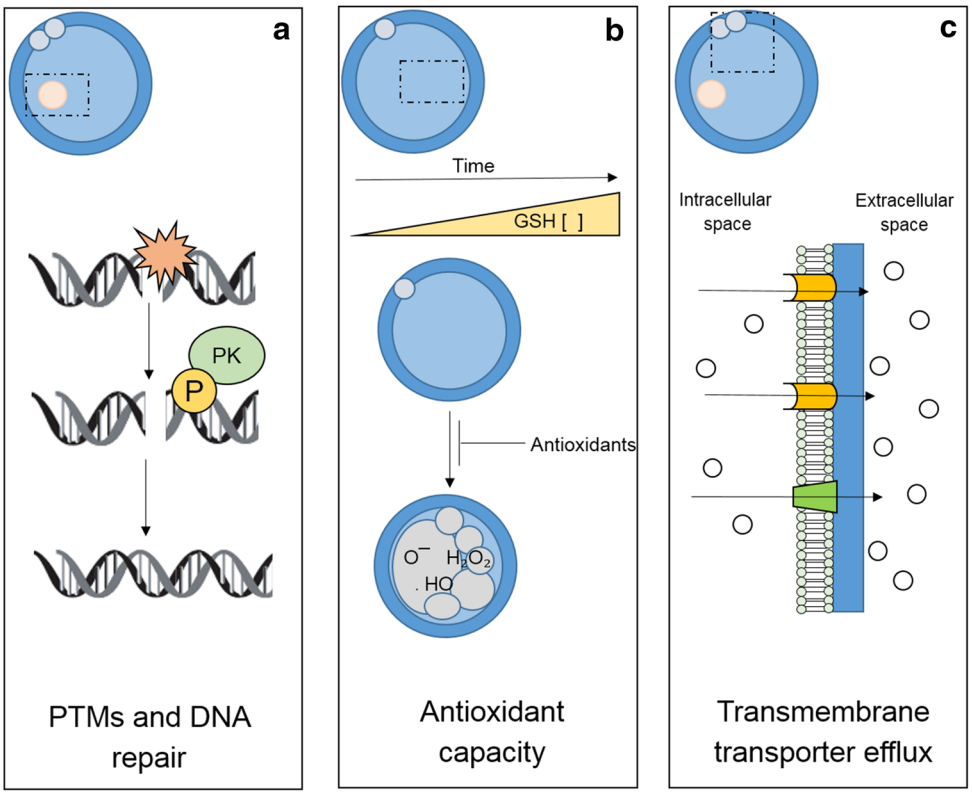

Fig. 3 Fertilization induces a dynamic suite of protective and reparative events to ensure successful egg–embryo transition. These include: **a** post-translational modifications (PTMs) of pre-synthesized maternally derived repair enzymes that provide the fundamental machinery necessary for mitigating DNA and/or cellular damage in the early embryo; **b** significant alterations in glutathione (GSH) and glutathione peroxidase activity, thus truncating the effects of post-ovulatory oocyte aging and defending the embryo against the accumulation of oxidative DNA damage; and **c** a complex assortment of transmembrane transporter molecules implicated in facilitating the removal of genotoxic agents from the cytosol (Adapted from [2, 10, 126, 127])

In addition to direct DNA repair, the oocyte is endowed with a host of alternative protective systems that may serve as the first line of defense against various forms of cellular damage including oxidative insult [10, 126, 127, 137]. In some instances, these protective systems are differentially expressed and/or activated in peri- and post-fertilized oocytes, generally rendering these cells less susceptible after fertilization has commenced. Examples include transmembrane transporter proteins involved in drug exclusion and antioxidant defenses that act to resolve oxidative DNA base adducts and prevent protein alkylation and mitochondrial disruption [10, 131, 133, 134]. In this context, accumulating evidence indicates that immediately prior to fertilization, concentrations of the antioxidant glutathione (GSH) are significantly increased in the oocyte, and remain elevated during the zygote stage, before precipitously falling by the 2-cell embryo stage [138]. Consistent with these data, it has also been shown that glutathione peroxidase activity is increased in zygotes compared to MII stage oocytes, thus providing additional protection to the zygote against hydrogen peroxide-induced DNA strand breakage [10; Fig. 3b] in conjunction with its essential role in paternal pronucleus formation and successful preimplantation development [139].

Epigenetics and DNA repair

Perhaps not surprisingly, embryonic quality and the effective maintenance of genomic integrity are influenced by the epigenetic signatures of both parental gametes [140]. Indeed, it appears that the correct establishment of epigenetic modifications and subsequent chromatin scaffolding are crucial steps in regulating the fidelity of the first mitotic cleavages with their absence leading to pronounced consequences for the development of a preimplantation embryo [127]. In this context, it has been shown that the inhibition of H3K4 demethylation can inhibit cleavage to the 4-cell stage embryo [127], while the inactivation of histone lysine methyltransferase (KMT5A) induces early embryonic lethality prior to the 8-cell stage [141]. Similarly, the deletion of Jumonji C domain-containing demethylase (JMJD2C) arrests embryo development prior to the formation of a blastocyst [142]. Recent evidence also suggests that the propensity of epigenetic modifications to modulate contact between nucleosomes and chromatin may serve to influence the compaction and/or relaxation state of the DNA fiber [143, 127]. In this capacity, epigenetic modifications may not only 'open' and 'close' regions of the DNA for transcription, but they may also act as important molecular gatekeepers during DNA decondensation and repair by providing the requisite enzymes with appropriate access to the DNA template [143–145]. In this respect, an enrichment of proteins responsible for epigenetic modification and chromatin remodeling (e.g., SMARCA5, CHD3, and CHD4) has been found in the mouse MII oocyte and early embryo, a particularly important consideration given the transcriptionally inert nature of immature oocytes prior to fertilization [123]. In further support of this notion, a dramatic loss in DNA demethylation [as evidenced by a reduction in 5-methylcytosine (5mC) content] has been recorded shortly after the protamine-histone exchange that occurs within the paternal genome following fertilization [146, 147]. This demethylation phenomenon appears to be closely associated with the appearance of DNA DSBs (γH2A.X) and DNA repair markers [Poly(ADP-Ribose) Polymerase 1 (PARP-1)] [148]. Furthermore, the distinctive co-localization of γH2A.X foci formation with the sites of demethylation within the paternal pronucleus during the pre-replicative stages of development, has led some to postulate that DNA demethylation may be regulated by indirect DNA repair-induced mechanisms such as the BER or NER pathways [149]. Such a model is consistent with the fact that the paternal gamete must decondense before syngamy [116], and that the maternal gamete is responsible for ensuring the genetic integrity of both nuclear contributions [87, 88].

Interest is also beginning to focus on the epigenetic regulation imposed by the fertilizing spermatozoon [150, 151]. In this regard, it is now recognized that, in addition to delivering the signal(s) that initiate the fertilization cascade, the male gamete may also contribute developmentally important epigenetic modulators (e.g., DNA methylation, sperm-specific histones, and other chromatin-associated proteins), as well as a number of small noncoding RNAs (sncRNAs), to the oocyte during fertilization that may all participate in successful embryogenesis [122, 151–153]. A strong case for this form of regulation has been mounted on the basis of experiments involving the use of conditional germline specific, *Dicer* and *Drosha* knockout mouse models. These studies have revealed that an embryos developmental potential, transcriptomic homeostasis and early zygotic gene activation are each dependent on paternally derived sncRNA cargo [117, 122, 151]. It has also been suggested that a specific sub-class of sncRNAs, known as the small interfering RNAs (siRNAs), may participate in key developmental processes encompassing pronuclear formation, DNA repair, orchestrating oocyte activation, the transition from maternal to embryonic gene control, and the establishment of imprints in early embryos [154]. Such findings offer many exciting avenues for future research, not the least of which will be to determine how epigenetic mechanisms of regulation are seamlessly integrated with maternally mediated PTM of signaling pathways to support early embryonic development.

Additional protective mechanisms of the oocyte and early embryo

In recent years, a variety of additional mechanisms have been identified that may prevent the propagation of damage in the fertilized oocyte. In marine invertebrates as in some mammalian species, post-fertilization activation has been implicated in promoting the synthesis of transmembrane transporter molecules, such as the ABCB protein, P-glycoprotein (PGP), and an ABCC protein similar to the multidrug resistant (MDR)-associated protein (MRP)-like transporter [154]. Though poorly understood, these proteins appear to be trafficked to the plasma membrane where they become functionally active and thereafter increase the bi-directional transport capacity of the cell [133, 134, 154]. Such proteins have been implicated in the transport of genotoxic agents from the intracellular environment and away from the vulnerable genomic material [133, 134, 155], as well as shuttling hormones and amino acids from the surrounding environment to the zygote to facilitate growth and development [156]. In somatic and cancer cell models, a similar complement of transmembrane transporter proteins has been implicated in multidrug resistance [157]. In these cells, the transport activity of the proteins appears to be intimately tied to their activation by post-translational phosphorylation events driven by Ca^{2+} and the serine/threonine kinases of PKC and/or PKA [133, 134, 158–160]. These results are particularly interesting given the notable increase in PKC activity that flows from the elevated levels of intracellular Ca^{2+} as well as a secondary stimulus of DAG, which are present at the time of fertilization. Taken together, it is tempting to speculate that PKC may hold a key role in the increased activation of transmembrane transporters to help protect mammalian oocytes and early embryos (Fig. 3c).

If this hypothesis was correct, it may afford unique opportunities to increase the protection of maturing oocytes with important implications for assisted reproductive strategies. In this regard, the 'drugability' of kinases [161] and their protein targets could provide avenues for the improvement of oocyte and early embryonic quality in vitro. The merits of this approach have been emphasized by recent proof-of-concept studies. For instance, artificial upregulation of transmembrane transporter molecules in bovine and porcine oocytes has been shown to have a dramatic positive impact on the post-cryopreservation viability of late stage embryos [162, 163]. In addition, recent literature indicates that numerous pharmacological (bovine embryos: rifampicin and forskolin) and hormonal (porcine GV stage oocytes: progesterone) signals can significantly elevate membrane transporter protein expression and/or activity, respectively [162, 163]. This paradigm provides an exciting prospect in clinical IVF settings whereby culture medium could be supplemented with compounds to increase the efflux activity of transmembrane transport proteins and thus reduce the intracellular availability of potentially damaging agents. This may also prove to be a valuable mechanism for the protection of the ovary and immature oocytes from both fresh and frozen sources in clinical fertility management.

In the event that the innate prevention and repair systems are inadequate or overwhelmed by a particular insult, mechanisms that are responsible for avoiding the propagation of damage take center stage. This is particularly important when considering the unique role that the oocyte plays in the continuation of a species when, in extreme cases, significant DNA damage could lead to chromosomal loss, translocation or duplication. Cumulatively, such damage could also significantly increase the mutational load borne by the embryo and thus heighten the risk of carcinogenesis in the offspring [164]. Correspondingly, a variety of mechanisms have been identified as being responsible for the removal of severely damaged oocytes or early embryos. These are usually characterized by significant cellular senescence, DNA fragmentation and degradation, and eventually either cell atresia or apoptosis (reviewed in [165–167]). Atresia dominates the mechanisms involved in removal of immature oocytes from the ovarian reservoir and involves ligand–receptor complex systems, including tumor necrosis factor alpha (TNFα), TNF-related apoptosis-inducing ligand (TRAIL or APO-2), Fas ligand, APO-3 ligand, PFG-5 ligand, and associated receptors; on the other hand, mature oocyte and early embryo loss are primarily mediated by Bcl-2 family members, Apaf-1 and caspase activation leading to apoptosis [154, 167–171].

The endowment of such elaborate systems for the detection, prevention, repair, or as a last resort, removal of a compromised cell clearly has evolutionarily benefits for a species. However, the increasing instance of delayed childbirth in our own species means that these mechanisms are working against us, particularly considering the expression of several key repair proteins has been found to be drastically reduced between 'young' and 'aged' oocytes [172] leading to a further potential increase in the demand for assisted reproductive procedures. While the advent of increasingly sophisticated assisted reproductive technologies to bypass such biological obstacles has been remarkably beneficial to many couples wishing to conceive, the reliance on this technology carries the very real risk of exacerbating genetic and cellular damage through gamete handling and culturing procedures in cells that would otherwise fail to participate in fertilization events [36].

Concluding remarks

The process of fertilization elicits a complex suite of biochemical and physiological modifications that prepare the oocyte for sustained embryonic development. These events are intimately tied to a prominent rise in intracellular Ca^{2+} at the moment of fertilization and the concomitant stimulation of an extensive range of oocyte activation events. Chief among these appears to be the activation of kinases that mediate the post-translational modification of a suite of key developmental proteins. It is now known that such events are likely to work in unison with paternally derived small non-coding RNA species, which regulate the stability/translation of the limited pool of maternally stored mRNA transcripts. This review contextualizes the role of fertilization and oocyte activation in extending the viability of the oocyte, initiating a series of biochemical alterations required for embryonic development and highlighting the prominent role that protein PTMs, and in particular phosphorylation, hold in their execution.

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Compliance with ethical standards

Conflict of interest The authors have nothing to declare.

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CHAPTER 2: ORIGINAL RESEARCH ARTICLE

Identification of a key role for permeability glycoprotein in enhancing the cellular defence mechanisms of fertilized oocytes

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Authors: Martin, J.H[1]., Nixon B[1]., Lord T[1]., Bromfield E.G[1]., Aitken R.J[1].

Affiliations: [1]Priority Research Centre for Reproductive Biology, School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia.

Correspondence: Jacinta.martin@newcastle.edu.au

CHAPTER OVERVIEW

As discussed in Chapter 1, DNA integrity and stability are critical determinants of cell viability. This is especially true in the female germline, where genome integrity underpins not only successful conception, but the developmental trajectory of the offspring. The oocyte must therefore be equipped to counteract and/or ameliorate damage that could otherwise be inherited by the embryo. Little however, is known regarding the manner by which the oocyte safeguards its genomic integrity. In this studies described in this manuscript we therefore sought to investigate the response of the post-ovulatory (MII) oocyte and the preimplantation zygote to challenge with etoposide (ETP), a genotoxic agent capable of eliciting double strand break (DSB) DNA damage.

In this manuscript, and its accompanying 'Data in Brief' supplementary article, our collective data demonstrate a significant developmental change in ETP vulnerability associated with fertilization of the oocyte. Specifically, permeability glycoprotein (PGP) undergoes considerable spatial and temporal changes in expression driven by the surge in intracellular calcium following oocyte activation. Belonging to a superfamily of transmembrane transporter proteins, PGP increases the bi-directional transport capacity of the embryo, thereby precluding the intracellular accumulation of ETP. Thus, PGP fulfils an important first line of defence against the induction of DNA damage following fertilisation of the oocyte. These new findings hold promise in informing novel strategies to fortify the genomic integrity, and overall health, of the oocyte and early embryo. Foreseeably, such strategies could provide substantial benefit in a clinical assisted conception setting where chemical stresses (as a product of cell culture) may manifest in, or further exacerbate, DNA damage.

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Identification of a key role for permeability glycoprotein in enhancing the cellular defense mechanisms of fertilized oocytes

Jacinta H. Martin *, Brett Nixon, Tessa Lord, Elizabeth G. Bromfield, R. John Aitken

Priority Research Centre for Reproductive Science, Discipline of Biological Sciences and Hunter Medical Research Institute, University of Newcastle, Callaghan, NSW 2308, Australia

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ABSTRACT

Double strand breaks (DSBs) are highly damaging DNA lesions that can destabilize the genome and generate a suite of adverse physiological outcomes in the oocyte and early embryo. While it is therefore likely that these cells possess a sophisticated suite of protective mechanisms to ameliorate such damage, the precise nature of these defense systems are yet to be fully elucidated. This study characterizes the sensitivity of the oocyte to etoposide, a chemotherapeutic agent with the ability to elicit DSBs. We demonstrate significant developmental changes in etoposide vulnerability, with fertilization of the oocyte leading to an enhancement of its cellular defense machinery. Using a parthenogenic model we show that this response is mediated, at least in part, by permeability glycoprotein (PGP), an endogenous multidrug efflux transporter that is up-regulated, translocated to the oolemma and phosphorylated upon oocyte activation. Moreover, evidence from dye exclusion assays in the presence of a specific PGP pharmacological inhibitor (PSC833), illustrates that these events effectively increase oocyte efflux activity, thereby enhancing the ability of these cells to exclude genotoxicants capable of eliciting DSB formation.

1. Introduction

It is well established that the integrity of the genome underlies the overall functioning of a cell, yet nuclear DNA is highly vulnerable to damage from both endogenous and exogenous sources (Takata et al., 1998). When structural DNA damage occurs, it poses a significant threat to cell viability. Indeed, individual cells within a compromised population can harbor highly potent DNA lesions that enable the propagation of additional damage or lead to premature apoptosis (Jurisicova et al., 1996). In extreme cases, significant DNA damage could see parts of chromosomes lost, translocated or duplicated leading to the acquisition of further damage, mutations and heightening the risk of carcinogenesis (Degtyareva et al., 2008). This is particularly concerning when considered in the context of the germ cells, owing to their unique role in the propagation of a species. In terms of the oocyte, DNA quality not only influences the gamete's fertilization potential but also the developmental competency of the resulting embryo (Lord et al., 2015; Takahashi, 2012; Wang and Sun, 2006). Such adverse outcomes reflect the central role that the maternal gamete plays following its union with a fertilizing spermatozoon in ensuring the genetic integrity of the embryo (Schultz and Heyner, 1992; Wang and Sun, 2006). In this context, it is the oocyte that holds sole responsibility for remodeling the maternal and paternal genomes (Yoshida et al., 2007), attempting the repair of any DNA damage (Harrouk et al., 2000), orchestrating the early events of embryogenesis (Minami et al., 2007), and furnishing the embryo with undamaged functional mitochondria (Sato and Sato, 2011; Wang et al., 2009). It is therefore not surprising that genetic abnormalities in an oocyte predispose the descendant embryo to developmental arrest, malformations and/or potential growth retardation (Walker et al., 2000).

The term DNA damage encompasses a broad suite of structural disruptions including single strand breaks (SSBs), double strand breaks (DSBs), base modifications or losses, the formation of adducts and base-pair mismatches (Menezo et al., 2010). Of these, DSBs are considered as one of the principal forms of deleterious DNA lesion, having been directly associated with a range of pathologic sequelae including both male and female infertility (Aitken and Curry, 2011; Hunt and Hassold, 2008; Jacquet et al., 2005). This form of damage can arise due to errors in DNA replication, or exposure to radiation and genotoxic agents (Xu and Price, 2011). Despite posing considerable risk, DSBs also hold considerable importance in natural biological programing and creating the genetic diversity required for adaptive evolution (Kim and Suh, 2014). As a consequence, DSBs are relatively common events in

* Corresponding author.
E-mail addresses: jacinta.martin@newcastle.edu.au (J.H. Martin), brett.nixon@newcastle.edu.au (B. Nixon), tlord@vetmed.wsu.edu (T. Lord), elizabeth.bromfield@newcastle.edu.au (E.G. Bromfield), john.aitken@newcastle.edu.au (R.J. Aitken).

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eukaryotic cells and a majority of somatic cells are endowed with a succession of coordinated cell cycle checkpoints in addition to repair pathways that provide stringent protection of DNA integrity (Jackson, 2002). This also appears to hold true for immature (germinal vesicle stage) oocytes, which possess a majority of the key enzymes required for DSB surveillance and repair (Jaroudi et al., 2009; Wang et al., 2010).

Notwithstanding these data, recent investigations have cast doubt on whether the female gamete is capable of mediating a robust repair response (Ma et al., 2013; Marangos and Carroll, 2012). Rather, it has been suggested that a majority of immature oocytes possessing DSB lesions will undergo developmental arrest mediated by the spindle assembly checkpoint (Marangos et al., 2015). Alternatively, if the oocytes escape this checkpoint and proceed to extrusion of the first polar body, they become capable of participating in fertilization and potentially forwarding chromosomal abnormalities to the resultant offspring (Ma et al., 2013; Yuen et al., 2012). Indeed, comparatively little is known about the vulnerability of the mature MII oocyte to DSB DNA damage after they have left the relative protection afforded by the ovarian environment (Hennet and Combelles, 2012). In particular, it is not known whether these cells are endowed with defense mechanisms to mitigate the risk of DSB DNA damage and thus secure their developmental competence. This study was therefore undertaken with the goal of exploring the nature of the DSB response in the MII oocyte and to determine whether the efficacy is enhanced in preparation for, or immediately after, fertilization. For this purpose, we elected to investigate the response of the mature (MII) mouse oocytes and their activated (parthenogenic and fertilized) counterparts following challenge with etoposide, a potent chemotherapeutic (and genotoxic) agent with the ability to elicit widespread DSB DNA damage.

2. Materials and methods

2.1. Materials

The reagents used during this study were purchased from Sigma Aldrich (St Louis, MO, USA) unless otherwise stated. Anti-γH2A.X antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA) (SC101696), while anti-topoisomerase (DNA) II alpha (TOP2A) antibodies were purchased from Proteintech (Sydney, NSW, Australia) (24641-1-AP). Anti-permeability glycoprotein (PGP) antibodies for use in immunolocalization studies were from Merck Millipore (Darmstadt, Hesse, Germany) (517310) and those used for immunoblotting and immunoprecipitation were from Abcam (Cambridge, England, UK) (ab170904). Fluorescent probes used for assessment of oocyte efflux activity were obtained from Thermo Fisher Scientific (Carlsbad, CA, USA).

2.2. Gamete retrieval

Oocytes and spermatozoa were harvested as described previously (Lord et al., 2015). Briefly, an intraperitoneal injection regime comprising 7.5 IU equine chorionic gonadotropin and 7.5 IU human chorionic gonadotropin (Intervet, Sydney NSW, Australia) was administered to juvenile (3–5 week old) C57/BL6/CBA F1 female mice to induce superovulation. Once primed, the mice were culled using CO_2 asphyxiation then immediately dissected before cumulus mass retrieval from the ampullae. Oocytes were then denuded in 300 μg/ml hyaluronidase at 37 °C and the remaining cumulus cells removed by 3–5 washes in M2 medium. Spermatozoa were recovered from the cauda epididymides of mature (≥ 8 weeks) male C57/BL6/CBA F1 mice by retrograde perfusion via the vas deferens (Reid et al., 2012). Following collection, spermatozoa were diluted to a concentration of approximately 80–100 × 10^6 and capacitated by incubation in modified Biggers, Whitten and Whittingham (BWW) medium supplemented with 1 mg/ml polyvinyl alcohol and 1 mg/ml methyl-beta cyclodextrin for 1 h at 37 °C under an atmosphere of 5% O_2, 6% CO_2 in N_2 (Lord et al., 2013). The use of animals in this project was approved by the University of Newcastle Animal Care and Ethics Committee, and all the animals were obtained from breeding programs run in the University of Newcastle Central Animal House.

2.3. Strontium activation and in vitro fertilization (IVF)

To promote parthenogenic activation, denuded oocytes were incubated in calcium free KSOM medium containing 10 mM strontium chloride ($SrCl_2$) for 4 h at 37 °C under an atmosphere of 5% O_2, 6% CO_2 in N_2 (Lord and Aitken, 2015). For IVF, oocytes and spermatozoa were harvested as described above, with the exception that oocytes retained their cumulus masses. Cumulus-oocyte complexes were washed in human tubal fluid (HTF) medium 3 times prior to being placed in a droplet of HTF supplemented with 1 mM reduced glutathione (GSH). The oocytes were then co-incubated with 2 × 10^5 capacitated spermatozoa for 4 h at 37 °C. Following incubation, the oocytes were washed to remove unbound and loosely adherent spermatozoa and successful fertilization (or activation in the case of parthenotes) assessed by recording the extrusion of the second polar body and/or pronucleus formation.

2.4. Cumulus cell retrieval

Cumulus cells from denuded oocytes were collected and suspended in M2 medium, before centrifugation (500*g* for 5 min). Subsequent to this, cells were re-suspended in 3.7% (v/v) paraformaldehyde for 15 min at room temperature (RT) and immunocytochemistry carried out as described below.

2.5. Etoposide treatment

To examine whether fertilization conferred any changes in oocyte defense against double strand DNA breaks (DSBs), populations of unfertilized (MII) oocytes, parthenotes and pronuclear stage zygotes, were exposed to varying concentrations of etoposide. This reagent is capable of eliciting DSBs on the basis of its potent inhibition of topoisomerase II α (TOP2A), a property that has led to its adoption as a common chemotherapeutic agent in the treatment of childhood leukemia, testicular tumors, breast cancer, Hodgkin's disease, large cell lymphomas and small cell lung cancer lung cancer (Rezonja et al., 2013). In this study, oocytes, parthenotes and pronuclear stage zygotes were incubated in different concentrations of etoposide (0–100 μg/ml) for 15 min at 37 °C under an atmosphere of 5% O_2, 6% CO_2 in N_2. Following treatment, cells were washed in phosphate buffered saline (PBS) containing 3 mg/ml polyvinylpyrrolidone (PBS/PVP) and prepared for assessment of DNA damage as described below.

2.6. Cellular vitality

The cytotoxicity of etoposide, PSC833, BAPTA (1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester)) and cycloheximide were evaluated using as a standard far red detectable live/dead vitality reagent (Thermo Fisher Scientific). Prior to fixation, oocytes and embryos were incubated with the vitality reagent for 15 min at 37 °C before 3 washes in PVP/PBS after which immunocytochemistry was carried out.

2.7. Immunocytochemistry with γH2A.X and permeability glycoprotein

Following retrieval and appropriate treatment, oocytes were washed in PBS/PVP before being fixed in 3.7% (v/v) paraformaldehyde for 1 h at RT. Subsequently, oocytes were permeabilized with a solution of 0.25% Triton X-100 in PBS for 10 min at RT and blocked in a solution of 3% BSA/PBS supplemented with (or without in the case of PGP and TOP2A) 10% normal goat serum for 1 h at 37 °C. Once blocked, the oocytes were incubated in anti-γH2A.X, anti-PGP, or anti-TOP2A (each diluted 1:100 in 1% BSA/PBS) overnight at 4 °C. The oocytes were then washed in 1% BSA/PBS prior to being incubated with the appropriate Alexa Fluor 488 conjugated secondary antibodies (Thermo Fisher Scientific) (1:1000 in 1% BSA/PBS) at 37 °C for 1 h. Cells were then counterstained with the nuclear marker, 4′,6-diamindino-2-phenylindole (DAPI). Finally, oocytes were mounted on Menzel Gläser microscope slides (Thermo Fisher Scientific) in Mowiol containing 1,4-diazabicyclo[2.2.2]octane (DABCO) and fluorescence intensity assessed against a secondary antibody only control using an AXIO Imager.A1 fluorescence microscope (Carl Zeiss Micro Imaging GmbH, Jena, Thuringia, Germany).

2.8. SDS PAGE and immunoblotting

Oocyte protein extracts were prepared by solubilization of the cells in sodium dodecyl sulfate (SDS) extraction buffer (10% w/v SDS, 10% w/v sucrose in 0.375 M Tris, pH 6.8) containing a protease inhibitor cocktail (Roche Diagnostics, Castle Hill, NSW, Australia) at 100 °C for 5 min. The samples were then centrifuged (500 g for 5 min) and the supernatant stored at −80 °C prior to use. An equivalent amount of protein lysate (100 oocytes per lane) was then resolved on pre-cast gels (4–12% NuPAGE BIS-Tris, Thermo Fisher Scientific). Resolved proteins were transferred to a nitrocellulose membrane before being blocked with 3% BSA for 1 h. Blocked membranes were washed in Tris-buffered saline (TBS) containing 0.1% Tween (TBST) and then sequentially incubated in anti-PGP primary antibody (1:500 in 3% BSA/TBST) overnight at 4 °C and HRP-conjugated secondary antibodies (1:1000 in 1% BSA/TBST) for 1 h at RT. Membranes were subsequently stripped (western reprobe buffer in dH_2O as per manufacturer's instructions; Astral Scientific, Sydney, NSW, Australia) before being reprobed with anti-α-tubulin (1:4000 in 1% BSA/TBST) to ensure equal loading. Membranes were developed using enhanced chemiluminescence reagents (GE Healthcare, Buckinghamshire, England UK) according to the manufacturer's instructions.

2.9. Immunoprecipitation

Immunoprecipitation as described by Bromfield et al. (2015) was used to identify post-translational modification(s)to permeability glycoprotein in oocytes, parthenontes and early embryos. Briefly, oocyte and embryo protein extractions were prepared following incubation in CHAPS lysis buffer (10% glycerine, 10 mM CHAPS, 10 mM HEPES in distilled water; for 2 h at 4 °C with constant rotation) to conserve protein-protein interactions. Simultaneously 10 μg of anti-PGP antibody was cross-linked to Protein G Dynabeads (Thermo Fisher Scientific) using 3,3-dithiobis-sulfosuccinimidyl propionate (DTSSP- 2 mM as per manufacturer's instructions). Following this, dual incubation of anti-PGP-bound Dynabeads with 3 μg of protein lysate (extracted from approximately 120 cells) from MII, parthenote or zygote (previously precleared of nonspecific binding through incubation with unbound Protein G Dynabeads at 4 °C for 1 h) was conducted overnight as above. Finally antibody-antigen bound beads were washed in PBS before protein elution was achieved by boiling in SDS loading buffer at 100 °C for 5 min. Eluted protein and the following controls were loaded onto NuSep 4–20% Tris–glycine gel (NuSep, Bogart, GA, USA): 5 μl of anti-PGP in H_2O (antibody only), protein lysate prior to preclearing (lysate control), precleared elution and Dynabeads-only (see Fig. S1). Following resolution, immunoprecipitated proteins were prepared for immunoblotting with either anti-PGP antibody (to confirm the efficacy of the immunoprecipitation protocol) or anti phosphothreonine and anti-phosphoserine antibodies as described above.

2.10. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

In order to assess DNA damage in treated cells, a TUNEL assay (Roche Diagnostics) was employed. Oocytes, parthenotes or zygotes were initially prepared as indicated for immunocytochemistry. Following permeabilization, a subset of the cells were treated with 3 μl DNase solution (Promega, Madison, WI, USA) as a positive control, while additional subsets served as minus label and minus enzyme negative controls. All cells were then incubated with the TUNEL stain master mix as per manufacturer's instructions, washed and mounted immediately for assessment by fluorescence microscopy. Cells were scored as either TUNEL positive or negative, based on the presence/absence of immunofluorescence with a minimum of 10 cells assessed per treatment.

2.11. Assessment of efflux activity in oocytes, parthenotes and zygotes

Functional assessment of efflux transporter activity was conducted in MII stage oocytes, parthenotes and zygotes through measurement of calcein acetoxymethyester (calcein AM; C1430) dye transport efficiency. These assays were performed 4 h following the addition of spermatozoa or $SrCl_2$ required as described above. In the case of MII oocytes, these cells were incubated in M2 media alone for an equivalent period of time. These conditions were selected to reflect those employed in the analysis of γH2A.X labeling of DSBs.

Calcein AM (5 nM) was supplemented into the incubation medium for 15 min before the cells were washed in M2 medium and mounted for assessment by fluorescence microscopy. Calcein AM is a nonfluorescent, membrane permeant dye that emits a green-fluorescent signal after hydrolysis to calcein AM by intracellular esterases. Thus, cells that accumulate significant amounts of calcein fluorescence represent those that possess little or no efflux transporter activity for this dye. In contrast, cells that actively export calcein AM are characterized by low levels of calcein fluorescence. To ensure that these findings were a true representation of cellular transport activity, as opposed to variations in either esterase activity or membrane permeability, MII, activated and fertilized oocytes were incubated in the cell-permeant esterase substrate, fluorescein diacetate (FDA: F1303). Oocytes were exposed to 0.12 μM FDA for 15 min before 3 washes in M2 media then mounted for microscopy. Importantly, owing to a lack of charge both FDA and the fluorescent product it yields upon cleavage by intracellular esterases are able to freely cross the cellular membrane independent of PGP activity (Jakubikova et al., 2005).

To confirm the role of PGP in mediation of oocyte and early embryo efflux activity, inhibitory studies were conducted in the presence of valsodpar (PSC833). This analog of cyclosporine A has been shown to possess the ability to selectively inhibit the efflux activity of PGP (Aszalos et al., 1999; Tai, 2000). For the purpose of these studies, cells were treated with 5 μM PSC833, or an equivalent dose of the dimethyl sulfoxide (DMSO) vehicle control,

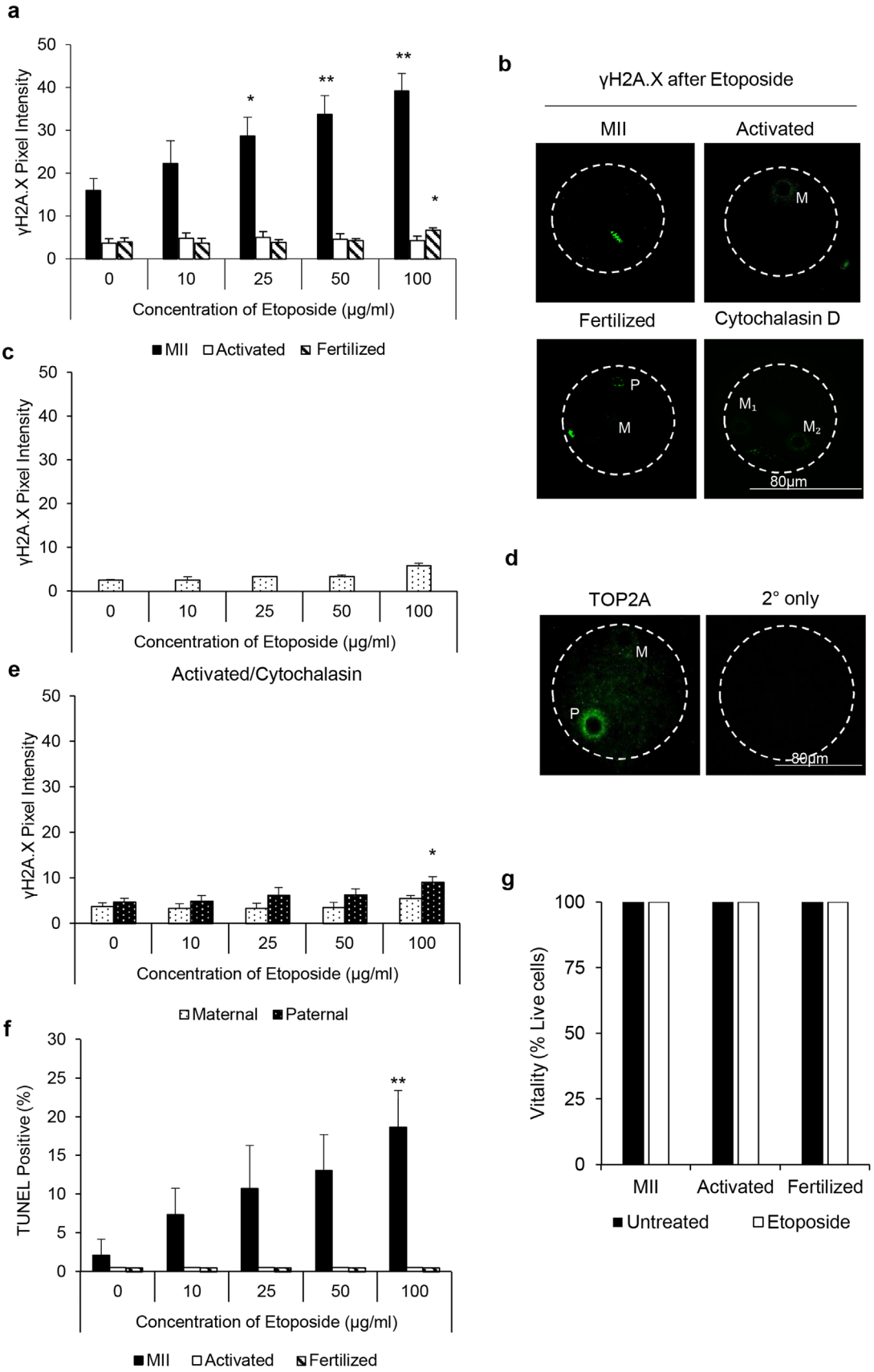

Fig. 1. Maturation and fertilization influences oocyte sensitivity to toxicants. (a,b) MII oocytes demonstrated a dose-dependent increase in γH2A.X labeling localized to the metaphase plate following treatment with 100 μg/ml etoposide. Following fertilization and chemical activation however, the sensitivity of oocytes to etoposide was significantly decreased, with the cells exhibiting only modest γH2A.X staining within the vicinity of the paternal pronuclei. (c–e) The increase in γH2A.X fluorescent signal detected in fertilized oocytes following etoposide treatment was determined to be exclusively attributed to the male pronuclei as shown by supplementation of the plant alkaloid cytochalasin D into the activation media, which inhibits actin polymerization leading to the formation of two pronuclei of maternal origin. This observation may reflect an increased content of the cellular target of etoposide (TOP2a) within the paternal pronucleus. (f,g) TUNEL analysis confirmed that MII oocytes displayed a pronounced, dose-dependent increase in their sensitivity to etoposide treatment while both the activated or fertilized oocytes did not display any such DNA fragmentation, in no instance was there an associated decrease in oocyte vitality following etoposide exposure. Abbreviations: M – maternal pronucleus, P – paternal pronucleus, M_{1}- maternal pronuclei 1 and M_{2}- maternal pronuclei 2. n=3, $^{*}P<0.05$,$^{**}P<0.01$.

in tandem with 100 μg/ml etoposide for 15 min at 37 °C. Following incubation, the cells were assessed for levels of DNA DSBs using γH2A.X labeling as described above.

2.12. Assessment of the PGP expression in oocytes

To investigate if the notable increase in PGP labeling observed in oocytes subjected to parthenogenic activation or fertilization reflected an up-regulation in PGP protein translation, cells were fertilized/activated in modified HTF (or KSOM) media supplemented with cycloheximide (20 μg/ml), an antibiotic with the ability to block protein synthesis. Following activation, cells were both fixed and stained for immunocytochemistry with anti-PGP as described above. Alternatively, to assess the role of activation-associated cytosolic calcium oscillations in terms of promoting the up-regulation of PGP, oocytes were activated or fertilized in the presence of the cell-permeant calcium chelating agent BAPTA (1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester)) (5 μM). They were then fixed and stained for immunocytochemistry as appropriate.

2.13. Statistical analysis

Densitometric analysis and quantification of fluorescence levels within oocytes, parthenotes and pronuclear stage zygotes was achieved using the public sector image processing program, Image J (National Institute of Health, Bethesda, MD, USA). Statistical significance was determined using ANOVA, Tukey–Kramer HSD and *t*-tests employing JMP software (version 10.0.0, SAS Institute, NC, USA). Differences with a value of $P < 0.05$ were considered to be statistically significant. Each experiment was conducted on a minimum of three biological replicates. All data are expressed as means ± s.e.m.

3. Results

3.1. Oocyte activation influences their sensitivity to genotoxicant induced DNA damage

In order to investigate the influence of maturation and fertilization on oocyte sensitivity to exogenous genotoxic insult, unfertilized oocytes (MII stage), pronuclear stage zygotes and chemically-activated parthenotes were exposed to etoposide and subsequently assessed for the presence of DSBs via examination of the relative labeling intensity of phosphorylated histone H2A (γH2A.X) (Fig. 1a). This analysis revealed only low basal levels of γH2A.X labeling in populations of untreated control oocytes. In marked contrast, etoposide treatment led to a significant, dose-dependent increase in γH2A.X labeling (Fig. 1a) ($p=0.02$) that was primarily restricted to the metaphase plate of MII oocytes (Fig. 1b). Interestingly, etoposide failed to elicit a similar response in either fertilized embryos or chemically activated parthenotes thus identifying a substantial difference in the vulnerability of these cells to genotoxicant induced DNA damage (Fig. 1a and b). Pronuclear stage zygotes did exhibit a modest, albeit statistically significant, increase in γH2A.X foci in the presence of the highest dose of etoposide (100 μg/ml; $p=0.03$). In this instance, γH2A.X labeling appeared to be primarily located in the immediate vicinity of the paternal pronucleus (Fig. 1b); this entity being distinguished on the basis of its larger size and position relative to that of the maternal pronucleus (Yamauchi et al., 2009). To investigate whether this increase in γH2A.X signal could be accounted for by the additional genomic material associated with the presence of two pronuclei in the fertilized oocytes, the plant alkaloid cytochalasin D was used to supplement the activation media (Fig. 1c). Cytochalasin D is a potent inhibitor of actin polymerization that is capable of disrupting cytokinesis and hence, in an activated oocyte its addition prevents the extrusion of the second polar body and leads instead to the formation of two pronuclei of maternal origin (Cuthbertson, 1983). As demonstrated in Fig. 1c, no significant increase in overall γH2A.X labeling was observed in the presence of cytochalasin D, a result which is consistent with the immunocytochemical analysis which revealed no substantive difference in the γH2A.X staining of maternal pronuclei in activated oocytes regardless of whether or not they had been treated with cytochalasin D (Fig. 1b).

From these data we infer that the pronuclei may display a differing sensitivity to etoposide depending on their origin. This notion was supported by quantitative analysis of γH2A.X labeling, which demonstrated that the paternal pronucleus consistently exhibited higher levels of γH2A.X staining than the equivalent maternal structure (Fig. 1e) (100 μg/ml: $p=0.03$). These findings also accord with the elevated expression levels of topoisomerase 2α (TOP2A), the molecular target of etoposide action (Baldwin and Osheroff, 2005), that were detected within the paternal pronuclei compared to the corresponding pronuclei of maternal origin (Fig. 1d).

To further investigate the overall cytotoxicity of etoposide on the MII, activated and fertilized oocytes, a TUNEL assay was employed in conjunction with that of a standard live/dead vitality reagent (Fig. 1f and g). By providing an indication of the proportion of cells containing DNA damage (both SSBs and DSBs), TUNEL confirmed that MII oocytes displayed a pronounced, dose-dependent increase in their sensitivity to etoposide treatment ($p=0.01$) compared to that of either activated or fertilized oocytes (Fig. 1f). Indeed, we observed no significant increase in TUNEL positivity in response to etoposide treatment of either of the two latter populations of oocytes. Interestingly, these effects were not accompanied by a decline in oocyte vitality, which remained unaffected across all populations of oocytes during the recovery time course of 3 h employed after etoposide exposure (Fig. 1g). In view of these data we next aimed to characterize potential mechanism(s) by which parthenogenic activation and fertilization provide enhanced resistance to the induction of DNA damage by etoposide, compared with MII oocytes.

3.2. Oocyte activation enhances cellular efflux activity

While we cannot exclude the contribution of upregulated DNA repair pathway(s) following oocyte activation, this scenario was considered unlikely due to the protracted timeframe (~2–6 h) in which DNA repair is usually established after the initial insult (Kinner et al., 2008). Instead, we focused our analysis on the contribution of a mechanism whose timing is compatible with that of the protection we documented (15 min following treatment), namely drug exclusion resulting from enhanced efflux activity in activated and fertilized oocytes. For this purpose, functional assessment of global efflux transporter activity was conducted through measurement of calcein acetoxymethyester (calcein AM) dye accumulation (Roepke et al., 2006). Quantitative analysis of fluorescent intensity revealed that the MII oocyte accumulated the hydrolyzed calcein product at a level that was approximately two- and three-fold higher than that of the activated ($p=0.03$) or fertilized oocytes ($p=0.02$), respectively (Fig. 2a and b). To ensure that these findings were a true representation of cellular transport activity, as opposed to variations in either esterase activity or membrane permeability, the latter were directly assessed in MII, activated and fertilized oocytes using the cell-permeant esterase substrate, fluorescein diacetate (FDA). For these studies, FDA was supplemented into the culture media of the target cell populations and the intensity of intracellular fluorescence measured (Fig. 2c).

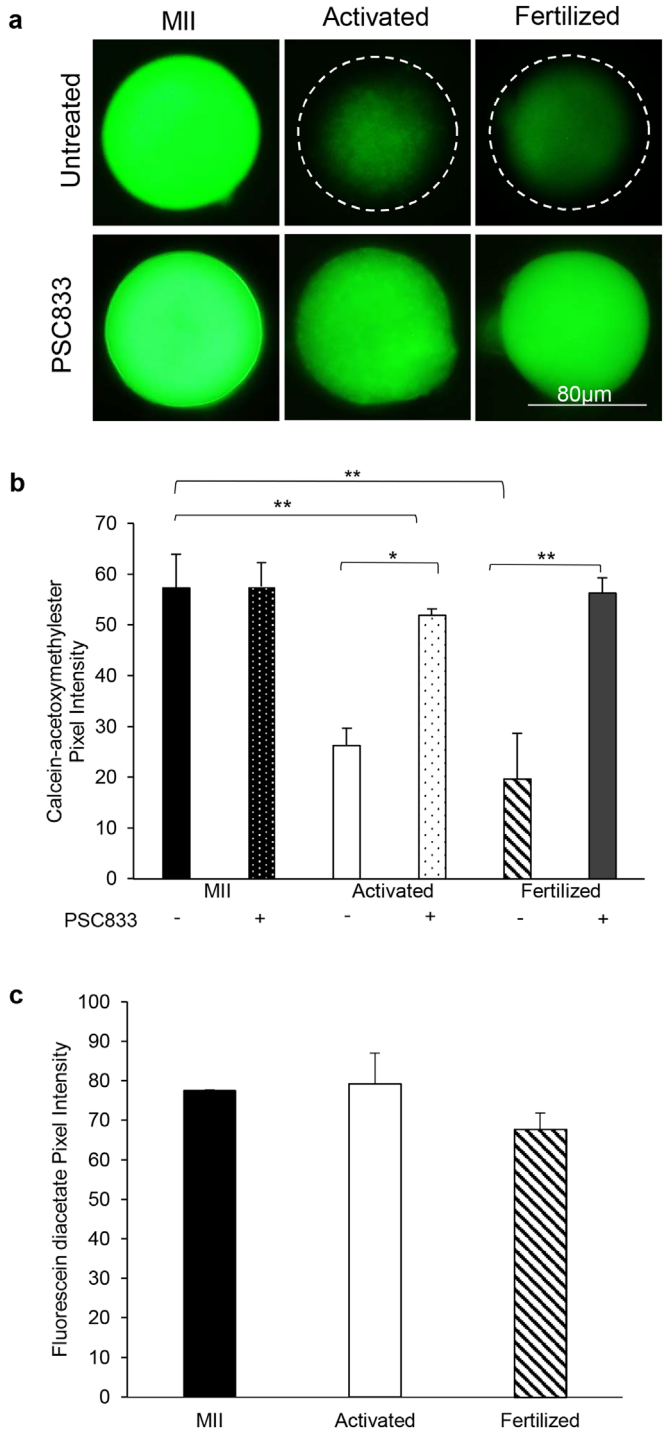

Fig. 2. Maturation and fertilization influences an oocyte's intracellular to extracellular flux activity. (a) Maturation and fertilization dramatically influenced the fluorescent availability of calcein AM, exhibiting greater intensity in the MII oocyte over the activated/fertilized oocytes. This flux was greatly reversed following co-incubation with the permeability glycoprotein inhibitor valsodpar (PSC833). (b) Quantitative analysis of fluorescent intensity revealed that the untreated MII oocyte accumulated calcein AM fluorescence at a level that was approximately two-fold higher than that of activated oocytes and three-fold higher than that of the fertilized oocyte. Addition of PSC833 led to a 2 fold increase in fluorescent calcein in both the activated and fertilized oocytes. (c) Measurement of total esterase content in the MII, activated and fertilized oocyte, using fluorescein diacetate (FDA), determined esterase activity to be consistent between all cell types. n=3, $^{*}P<0.05$, $^{**}P<0.01$.

This assay revealed that equivalent esterase enzymatic activity was present in all oocytes examined irrespective of their development stage.

3.3. Permeability glycoprotein is capable of modulating drug exclusion in the activated and fertilized oocyte

In model species such as those of the marine invertebrates, transmembrane transport proteins have been implicated as playing a primary role in the efflux of drug molecules from the developing oocyte (Hamdoun et al., 2004; Roepke et al., 2006). In view of this evidence, we aimed to charaterize the presence and functional contribution of arguably the most common transporter protein, permeability glycoprotein (PGP), within the mouse oocyte and early embryo. These analyses were complemented with the use of selective pharmacological inhibition of PGP efflux activity (PSC833) (Aszalos et al., 1999; Tai, 2000). As shown in Fig. 2b, PSC833 inhibition of PGP led to a significant increase in the accumulation of calcein AM associated fluorescence in populations of chemically activated and fertilized oocytes ($p=0.0004$). In this context, the level of calcein AM fluorescence in PSC833 treated activated oocytes was 2 fold greater than that of their untreated counterparts ($p=0.04$), while in pronuclear stage zygotes the effect was far more pronounced with a 3 fold increase in calcein AM fluorescence ($p=0.004$). These findings are consistent with PGP acting as a key modulator of the increased resistance to DNA damage we have observed in activated and fertilized oocytes.

To extend these findings, we examined if PSC833 inhibition also led to a concomitant increase in the efficacy of etoposide-induced DNA damage within activated and fertilized oocytes. As noted in Fig. 3, MII oocytes treated with the PSC833 inhibitor experienced a minor increase in γH2A.X fluorescence above that of the etoposide only control. This trend was also observed in activated and fertilized oocytes, both of which demonstrated enhanced sensitivity to etoposide treatment upon co-incubation with PSC833 ($p=0.009$ and $p=0.006$). Immunocytochemical analysis further confirmed the dramatic increases in γH2A.X fluorescence and, as anticipated, demonstrated that this was restricted to the metaphase plate of MII oocytes and to the pronuclei of activated and fertilized oocytes (Fig. 3a). Importantly, PSC833 treatment alone did not induce DNA damage, nor did it influence oocyte vitality, with both parameters remaining unaffected in all cell types examined (Fig. 3a–c and Fig. 1 in Martin et al., (submitted for publication)).

3.4. Permeability glycoprotein is differentially expressed in maturing and activated oocytes

In view of the compelling data from the dye accumulation and inhibitor studies, we next aimed to account for the differing activity of PGP in populations of MII oocytes versus those that had been activated/fertilized. These studies initially focused on determining the spatial and temporal expression patterns of PGP using a pan-antibody that recognizes both the multidrug resistance 1 (MDR1) and MDR3 isoforms of PGP. Complementary immunocytochemical and immunoblotting analyses identified a prominent increase in PGP expression when MII oocytes were compared to parthenogenetically-activated and fertilized eggs (Fig. 4a–d). In terms of the immunoblotting data, anti-PGP antibodies labeled a band of the appropriate molecular weight for PGP (~170 kDa) (Fig. 4a), with densitometric analysis revealing a significant elevation in the staining intensity between the unfertilized (MII) oocytes and their activated ($p=0.008$) and fertilized counterparts ($p=0.004$) (Fig. 4b). Notably, this band appeared as a broad smear as might be expected of a protein as heavily glycosylated as PGP. Quantitative analysis of the overall

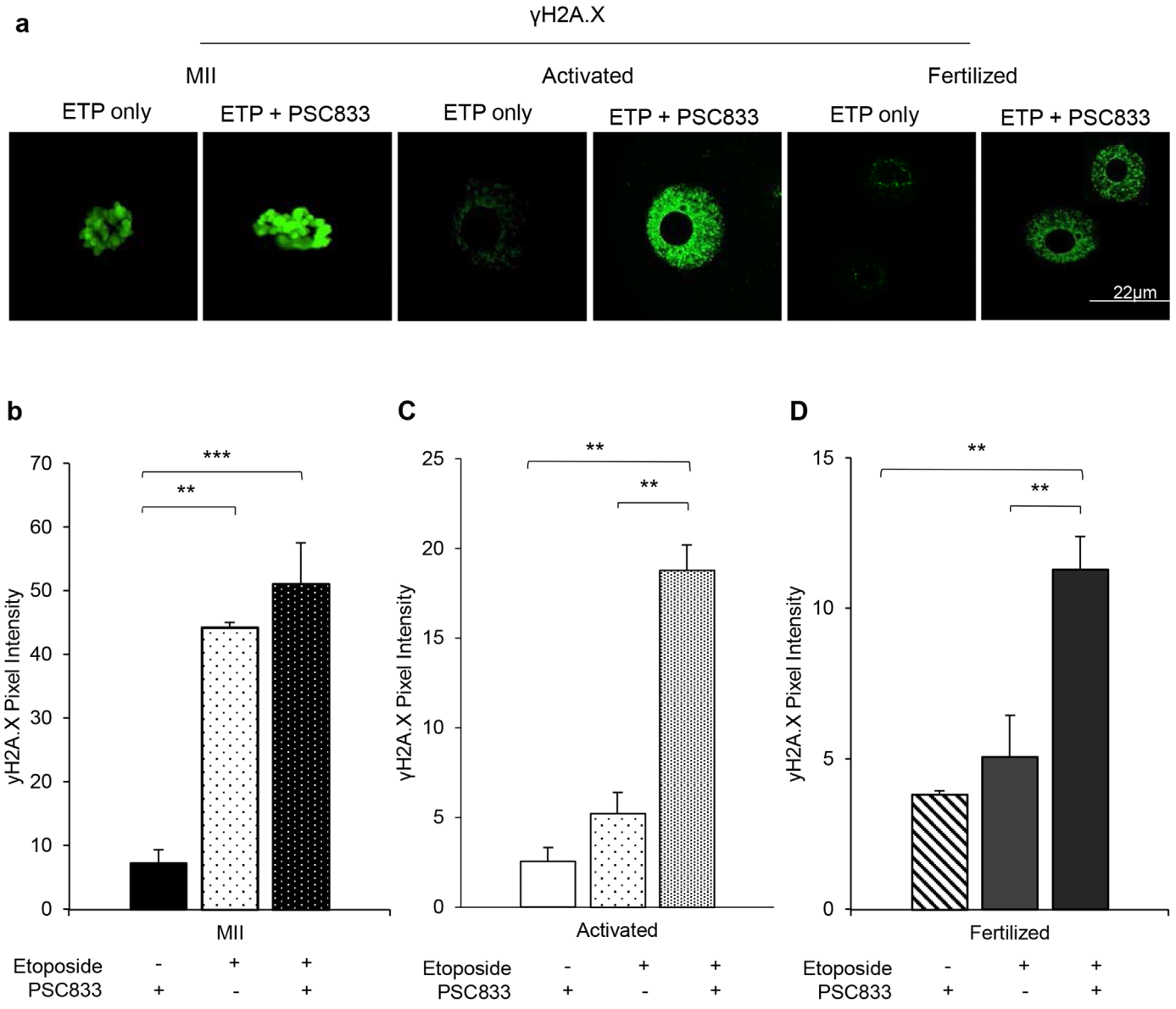

Fig. 3. Permeability glycoprotein is capable of modulating drug exclusion in the activated and fertilized oocyte, thus reducing drug sensitivity. (a–d) PSC833 inhibition of permeability glycoprotein lead to a significant increase in the efficacy of etoposide-induced DSB DNA damage within activated and fertilized oocyte. Immunocytochemical analysis further confirmed the dramatic increases in γH2A.X fluorescence restricted to the metaphase plate of MII oocytes and to the pronuclei of both the activated and fertilized oocytes. n=3, $^{**}P<0.01$, $^{***}P<0.001$.

PGP fluorescence intensity in these cells also revealed a highly significant, (approximately 6 and 7 fold), increase in staining between the MII oocyte and that of activated oocytes ($p=0.008$) and pronuclear stage zygotes ($p=0.004$), respectively (Fig. 4c).

In addition to these findings, immunolocalization data revealed a dramatic translocation of PGP from an initial diffuse localization throughout the ooplasm of MII oocytes, to a pattern of intense labeling primarily associated with the plasma membrane of activated and fertilized populations of oocytes (Fig. 4d). Our use of chemically activated parthenotes demonstrated that elevated PGP expression was driven via maternally derived factor(s) as it occurred in the complete absence of a fertilizing spermatozoon. Consistent with independent evidence (Yokota et al., 2011), we also confirmed that PGP is not present in the mouse sperm proteome (see Fig. 2 in Martin et al., (submitted for publication)), thus discounting the possibility that the increased PGP labeling we documented was an artefact associated with residual spermatozoa bound to the zona pellucida/oolemma following IVF.

Owing to the reduced PGP expression documented within MII oocytes we next sought to examine whether this protein was instead expressed in cumulus cells where it may be able to afford the mature oocyte protection against etoposide treatment (Fig. 5). However, in contrast to what one may expect if cumulus cells were providing protection, the cumulus intact oocytes were characterized by a dose-dependent increase in γH2A.X fluorescence, which proved to be significantly elevated above that of their denuded oocyte counterparts at the highest concentration examined (100 μg/ml; $p=0.05$) (Fig. 5a). Furthermore, immunocytochemical analysis revealed only minimal PGP expression in the cumulus cells (Fig. 5b).

3.5. Upregulation of permeability glycoprotein expression is driven by protein translation, oocyte activation and phosphorylation but not calcium elevation

Investigation of the mechanism(s) responsible for promoting

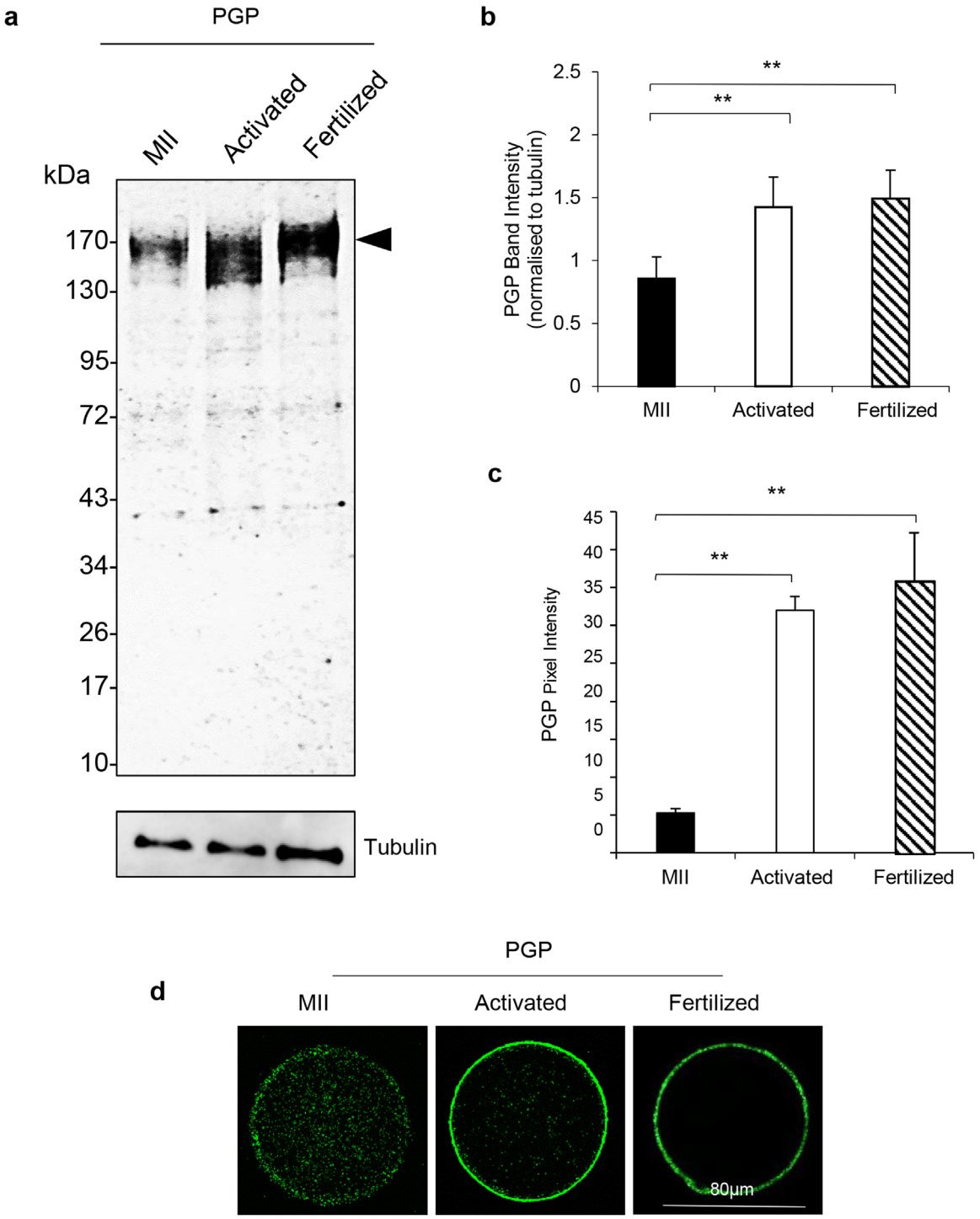

Fig. 4. Permeability glycoprotein is differentially expressed in the activated and fertilized oocyte. (a) Immunoblotting analyses identified a prominent increase in the expression of a single band of the appropriate molecular weight for permeability glycoprotein (170 kDa) as oocytes progressed from the MII stage through to the pronuclear activated and fertilized stages. (b) Complementary quantitative analysis of fluorescence intensity revealed a significant elevation in the staining intensity between the unfertilized MII oocytes and those of the activated and fertilized counterparts (c,d) Immunolocalization data revealed a dramatic 6–7 fold upregulation and translocation of PGP from an initial diffuse localization throughout the ooplasm of MII oocytes, to intense peripheral labeling primarily associated with the plasma membrane of the activated and fertilized oocytes. n=3, $^{**}P < 0.01$,*** $P < 0.001$.

the activation-associated elevation of PGP levels revealed at least three tiers of regulation (Fig. 6). Firstly, through inhibition of the egg activation and calcium oscillations that normally ensue after strontium activation and/or fertilization, we were able to potently suppress the expression of PGP in response to either parthenogenic activation or fertilization. In this context, BAPTA-AM, a membrane permeant calcium chelating agent, was able to elicit an approximate six fold decrease in the expression of PGP in activated/fertilized oocytes ($p=0.0001$ and $p=0.003$, respectively; Fig. 6b and c), thereby reducing its expression to levels reminiscent of the MII oocyte. Similarly, co-incubation of oocytes with cycloheximide, a broad-spectrum inhibitor of protein translation, was also found to dramatically dampen the activation and fertilization-associated upregulation of PGP ($p=0.0001$ and

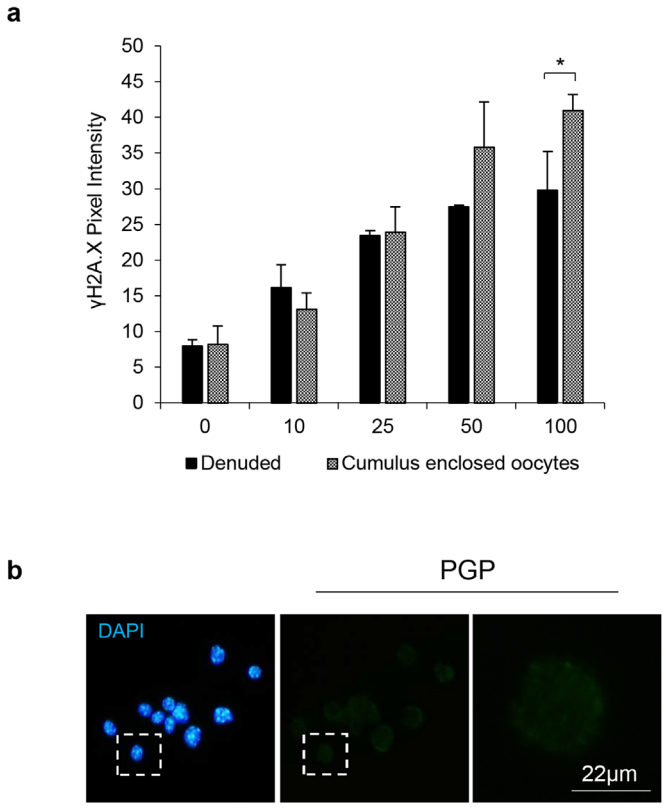

Fig. 5. Permeability glycoprotein does not afford a protective advantage in cumulus enclosed MII oocytes. (a) In an effort to determine if cumulus enclosed oocytes experienced a decreased susceptibility to etoposide induced DSB DNA damage, populations of cumulus-oocyte complexes and denuded MII oocytes were exposed to increasing concentrations of etoposide prior to assessment of γH2A.X fluorescence. As shown, cumulus retention did not provide the MII oocyte with additional protection against the genotoxic insult. (b) Accordingly, immunocytochemical analysis revealed only minimal PGP expression in the cumulus cells. n=3, $^{*}P < 0.05$.

$p=0.003$, respectively; Fig. 6a and b). Importantly, these treatments did not elicit non-specific effects on cell viability as oocytes remained viable following exposure to both BAPTA-AM and cycloheximide (see Fig. 3 in Martin et al., (submitted for publication)). Additionally our data has also identified a prominent role for post-translational modification of the PGP protein through phosphorylation induced by oocyte activation (Fig. 6d and S1). Indeed, immunoprecipitation of PGP revealed that it undergoes substantial phosphorylation of threonine, and to a lesser extent serine, residues following fertilization and parthenogenic activation. Notably, the immunoprecipitated PGP protein resolved as a doublet of ~170 and 172 kDa in all samples possibly reflecting differentially glycosylated forms of the parent protein. In contrast, only a single band of appropriate molecular weight (~170 kDa) was evident in immunoblots probed with either anti-phosphothreonine or anti-phosphoserine antibodies.

4. Discussion

The formation of DSBs is biologically important in providing a source of genetic variation for adaptive evolutionary processes. However, the potentially deleterious impact of this form of cytotoxic lesion (Kim and Suh, 2014) has meant that a majority of somatic cell types have developed sensitive innate defense mechanisms, encompassing both surveillance and reparative machinery, to ameliorate such damage. It is therefore of considerable interest that the immature female gamete, which holds such a prominent position in the propagation of species and in effecting the repair of DNA damage contributed by both the oocyte and fertilizing spermatozoon (Harrouk et al., 2000; Matsuda et al., 1989), appears to possess a limited capacity for DSB repair (Ma et al., 2013; Marangos and Carroll, 2012; Yuen et al., 2012). Indeed, recent evidence suggests that these cells may lack critical regulatory elements of the cell cycle checkpoints and/or key repair enzymes that are required for resolving DSBs (Ma et al., 2013; Marangos and Carroll, 2012). These findings agree with those of the present study in which we revealed two key observations concerning the capacity of oocytes to manage genotoxicant-induced DSBs. Firstly, through investigation of the chemotherapeutic agent, etoposide, we have demonstrated pronounced developmental changes in oocyte sensitivity to chemically-induced DSBs. Secondly, we have identified a novel mechanism involving enhanced efflux activity that, at least in part, accounts for the increased protection afforded to fertilized oocytes in the face of an etoposide attack.

The sensitivity of the post-ovulatory MII stage oocyte to etoposide was reflected in a relatively rapid, dose-dependent increase in γH2A.X foci formation and DNA fragmentation (TUNEL positivity), both of which occurred in the complete absence of any corresponding loss in cell vitality. Interestingly, this response was not ameliorated by the presence of the supporting cumulus cells that surround the ovulated oocyte and have been reported to afford protection against reactive oxygen species and fatty acid induced lipotoxicity in porcine and bovine cumulus-oocyte complexes (Lolicato et al., 2015; Tatemoto et al., 2000). On the contrary, cumulus intact oocytes accumulated levels of γH2A.X fluorescence that were significantly elevated above their denuded-oocyte counterparts. While we do not currently have an explanation for this response, it has been noted when investigating in vitro post-ovulatory oocyte aging that, retaining the cumulus cells can led to a dramatic loss in cellular integrity (Miao et al., 2005; Zhu et al., 2015). Nevertheless, we were able to demonstrate that the negative impact of etoposide was largely abrogated following fertilization of the oocyte. The fact that chemically stimulated parthenotes shared an equivalent level of resistance to DSBs indicates that the protective mechanism(s) associated with oocyte activation are of maternal origin, and not overtly influenced by the presence of a fertilizing spermatozoon. Interestingly, the timeframe in which this response is initiated is not compatible with stimulation of cellular DNA repair pathways that typically occur over a period of several hours following exposure to an insult (Nazarov et al., 2003). Rather, our data highlights an alternative mechanism involving enhanced cellular efflux activity associated with the promiscuous transporter, permeability glycoprotein (PGP). Support for this conclusion rests with the results of our dye exclusions studies in which we identified an impressive 2–3-fold increase in cellular efflux activity in populations of fertilized zygotes and parthenotes above that of MII oocytes. Importantly, this efflux activity was found to be sensitive to selective inhibition with PSC833, a cyclosporine analog that modulates PGP activity, thus rendering the activated and fertilized oocyte susceptible to etoposide induced DSB DNA damage.

PGP is a relatively large highly glycosylated, ATP-dependent efflux pump belonging to the multidrug resistance (MDR) or ATP-binding cassette (ABC) transporter superfamily of proteins, which participate in transport across extra- and intracellular membranes. Such proteins are widely distributed and display broad substrate specificity thus enabling them to fulfil an important first line of cellular defense against a plethora of chemically unrelated toxins (Aller et al., 2009). Owing to their ability to decrease drug accumulation, these proteins, and PGP in particular, have been widely studied in relation to their ability to confer a multidrug-resistant phenotype to cells (Hamdoun et al., 2004; Roepke et al., 2006). The

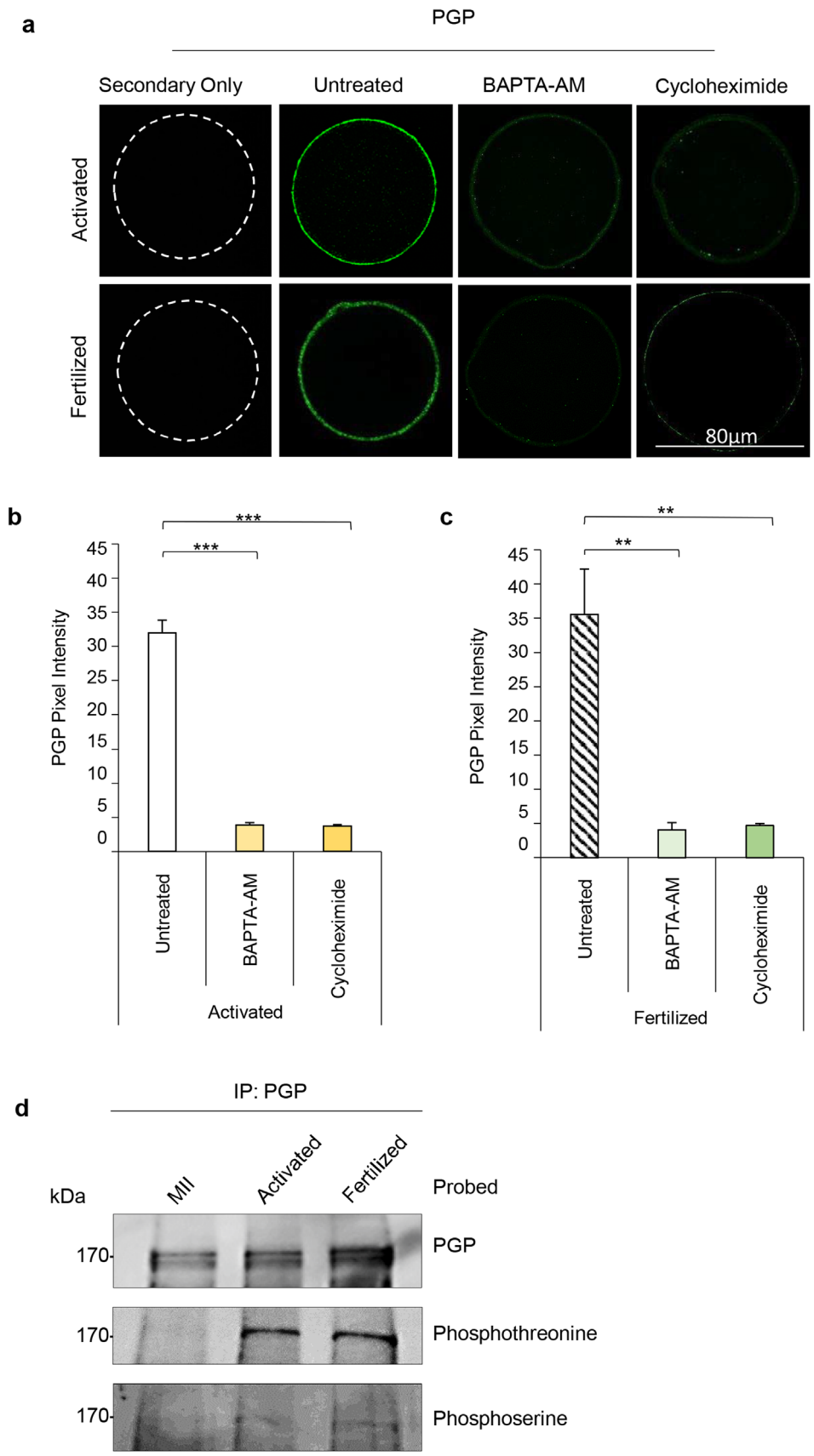

Fig. 6. Upregulation of permeability glycoprotein expression is driven by protein translation, oocyte activation and phosphorylation but not calcium elevation. Several tiers of regulation were determined to be responsible for promoting the activation/fertilization-associated elevation and translocation of PGP. (a–c) Indeed, inhibition of oocyte activation and calcium oscillations through supplementation of in vitro culture media with BAPTA-AM was able to significantly suppress the expression of PGP in response to either parthenogenic activation or spermatozoon mediated fertilization. Similarly, co-incubation of oocytes in activation/fertilization culture media supplemented with cycloheximide, was also able to elicit an equivalent 6–7 fold decrease in the expression of PGP in activated/fertilized oocytes consistent with that documented in MII oocytes. (d) Finally, immunoprecipitation of PGP demonstrated that the protein was targeted for phosphorylation of both threonine and, albeit to a lesser extent, serine residues in fertilized and chemically active oocytes. Thus, a 170 kDa band corresponding to PGP was detected upon probing of PGP immunoprecipitates with both anti-phosphothreonine and phosphoserine antibodies. $n=3$, $^{**}P<0.01$, $^{***}P<0.001$.

fact that this activity extends to many chemotherapeutic agents (including etoposide) (Fahrmayr et al., 2012; Olson et al., 2001) accords with our data implicating PGP as a key agent in limiting the capacity of etoposide to induce DSB DNA damage in the fertilized oocyte. While this is the first report of such activity in the female gamete of a mammalian species, it nevertheless draws interesting analogies with emerging data implicating MDR transporters in the protection of equivalent cells of marine invertebrates such as the sea urchin (*Strongylocentrotus purpuratus*), sea star (*Asterina miniata* and *Pisaster ochraceou*), mussels (*Mytilus edulis*), and oysters (*Crassostrea gigas*) (Hamdoun et al., 2004; Minier et al., 1993; Roepke et al., 2006). Indeed, in these species, PGP has been proposed to reduce the susceptibility of oocytes and early embryos to a range of environmental toxicants including polychlorinated biphenyls (PCB), polycyclic aromatic hydrocarbons (PAH) and dichloro-diphenyls-ethanes (DDT) (Minier et al., 1993). Taken together, these findings suggest that PGP may play an evolutionary conserved defensive role in protection of the female gamete/early embryo against a variety of xenotoxins.

Our data also reveals a previously unappreciated developmental change in both the spatial and temporal expression profile of PGP within the mammalian oocyte. Indeed, the basal levels of PGP documented throughout the cytoplasm of MII oocytes was replaced by a pronounced 6–7 fold increase in expression in both fertilized and chemically activated oocytes. This increased expression was accompanied by an apparent translocation of the protein such that it was only detected within the plasma membrane of fertilized/activated oocytes. A similar phenomenon has been reported for a number of additional oocyte proteins that are also translated in the cytoplasm before being translocated to the plasma membrane during fertilization (Santella et al.,1999) as well as numerous other examples of signal-induced changes in cellular transporter physiology mediated by delivery of transporters to the plasma membrane (Miller et al., 2002; Watson and Pessin, 2001). However, consistent with independent evidence (Roepke et al., 2006) our data confirm that such events are not essential for initiation or progression of the fertilization cascade, which both proceeded normally in the presence PGP inhibition.

The recruitment of PGP to the plasma membrane is consistent with published evidence that the protein possesses a number of transmembrane domains enabling it to adopt an inward facing conformation that exposes the promiscuous substrate binding pocket to the cell cytoplasm (Aller et al., 2009). Alternate cycles of ATP binding and hydrolysis then facilitate substrate binding and transport from the cell (Aller et al., 2009). Herein we have identified three tiers of regulation that may underpin the upregulation of PGP expression and activity. Firstly, we showed that chelation of oscillating intracellular calcium, potently suppressed PGP expression/translocation. Secondly, we demonstrated that a similar level of suppression could also be achieved by treatment of oocytes with cycloheximide, a potent inhibitor of global protein translation. One caveat to this approach is that BAPTA-AM has also been documented to cause protein synthesis inhibition (Lawrence et al., 1998). Thus, to further clarify the requirement for intracellular Ca^{2+} for PGP upregulation and translocation, future experiments using roscovitine alone to activate oocytes (Phillips, 2002) would be required. Additionally we also have identified a prominent role for threonine, and possibly serine, phosphorylation driving the activity and/or translocation of PGP to the plasma membrane in both the chemically activated and fertilized oocytes. These data draw clear parallels with investigations concerning MDR and anion efflux, which have provided extensive evidence for the post-translational modification (by phosphorylation) of PGP whereby ATP (Garman et al., 1983; Mellado and Horwitz, 1987; Posada et al., 1989) or ATP and GTP (Lelong-Rebel and Cardarelli, 2005; Lelong-Rebel et al., 2003) act as phosphate group donors. Phosphorylation of PGP appears to dramatically modulate the efficacy of transporter action by affecting its intrinsic ATPase activity (Fine et al., 1996; Idriss et al., 2000), while phosphorylation of cytoplasmic residues has been implicated in the repositioning of protein within somatic cells (Dusi et al., 1993; Imai et al., 2004) and early embryos (Santella et al., 1999). Notably, increased protein kinase C (PKC) and protein kinase A (PKA) activity in human and mouse MDR cell lines appears to be coupled directly to PGP function (Mellado and Horwitz, 1987; Posada et al., 1989). This aligns with the integral contribution of calcium ions that is required to promote serine/threonine phosphorylation by PKA and PKC at the time of oocyte activation at fertilization (reviewed in Ducibella and Fissore (2008)).

Although it remains to be investigated directly, the capacity for the induction of PGP translation may reflect its presence as part of the pool of maternally derived transcripts that are known to accumulate during oogenesis in preparation for an impressive wave of protein synthesis at the time of fertilization (Derijck et al., 2008). Such a model has been proposed in marine oocytes to account the expedited increase in the levels of transporter molecules associated with MDR (Roepke et al., 2006), and the corresponding protection provided to embryos from environmental toxicants (Hamdoun et al., 2004; Roepke et al., 2006).

Notwithstanding the efficacy through which PGP activity was able to suppress DNA damage in fertilized oocytes, our data also suggest that the capacity of this protective mechanism is not unlimited. Indeed, the detection of a significant, albeit modest, increase in DSBs following exposure 100 μg/ml etoposide suggests that this concentration may have saturated the efflux activity of PGP in fertilized oocytes. These data are consistent with previous reports that indicate the kinetics of PGP with etoposide as a substrate are both saturable and concentration-dependent (Kandimalla and Donovan, 2005). Interestingly, upon exceeding this threshold we were able to reveal differences in the sensitivity of the maternally and paternally derived genomes to the etoposide insult. In this context, elevated levels of γH2A.X labeling appeared to be restricted to the paternal pronuclei. This finding was not entirely unexpected given that etoposide promotes the formation of DSBs through direct inhibition of TOP2A (Baldwin and Osheroff, 2005), an enzyme that was confirmed to be more prevalent in the male pronucleus. However, we cannot exclude the possibility that chromatin decondensation leads to unmasking of pre-existing DNA damage within the germline, particularly given that the fertilizing spermatozoon lacks active DNA repair mechanisms (Agarwal and Said, 2003; Jansen et al., 2001; Van Loon et al., 1991).

In terms of the practical implications of these findings, the ability to enhance the protection afforded to immature oocytes and/or embryos by artificially promoting PGP expression could have considerable benefits in a clinical assisted conception setting. As an important precedent for this work, pharmacological and hormonal regimes have already been developed to successfully elevate the activity of transmembrane transporter molecules in immature oocytes and embryos from the porcine and bovine models (Mori et al., 2013; Yokota et al., 2011). Importantly, this strategy led to significant improvements in both cell quality and the survival of cryostored embryos (Mori et al., 2013; Yokota et al., 2011), thus encouraging us to further explore the utility of PGP manipulation for fertility management.

5. Conclusions

This study has identified a novel role for permeability glycoprotein as an important first line of cellular defense against the genotoxic agent, etoposide. Our collective data has been

incorporated into a model to account for the notable changes in vulnerability to etoposide insult documented in maturing oocytes versus that of fertilized or parthenogenetically-activated cells (Fig. 7). This model seeks to highlight a dynamic upregulation of PGP translation, the translocation of the protein to the oolemma and its modification via phosphorylation. These events coincide with the pronounced elevation of intracellular calcium concentrations that accompany oocyte activation at the moment of fertilization. As a consequence, PGP becomes functionally active and capable of pumping etoposide from the cell cytoplasm thus preventing it from accumulating to the point where it compromises genetic integrity.

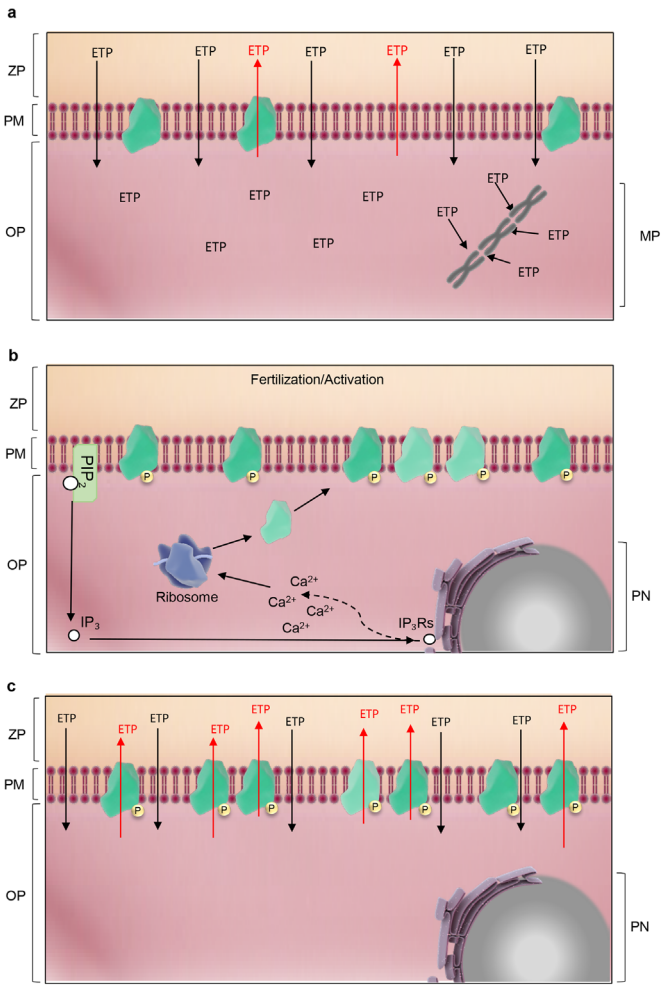

Fig. 7. Permeability glycoprotein provides an important first line of cellular defense against the genotoxic agent, etoposide. (a) A mature ovulatory stage oocyte when subjected to genotoxic insult experiences a significant accumulation of the cytotoxic compound within the ooplasm. A minority of functional multidrug resistant transport channels efflux an minor concentration of etoposide from the cell, but a disparity between active transport channels and the toxin lead to the DNA at the metaphase accumulating significant DNA damage (b) At the moment of fertilization signaling cascades initiate a dramatic upregulation of permeability glycoprotein protein synthesis, whereupon the protein is translocated to the oolemma following serine/threonine phosphorylation, becoming functionally active and capable of (c) pumping etoposide from the cell cytoplasm thus preventing it from compromising genetic integrity. Abbreviations: ETP- etoposide, ZP- zona pellucida, PM- plasma membrane, OP- ooplasm, MP- metaphase plate, PIP_2- phosphatidylinositol 4,5-bisphosphate, IP_3- inositol trisphosphate, Ca^{2+}- Calcium, IP_3Rs- inositol trisphosphate receptor, P- phosphorylation and PN- pronuceli.

Summary statement

This study identifies a novel role for permeability glycoprotein as an important first line of cellular defense against genotoxic agents in fertilized mouse oocytes.

Competing interests

No competing interests declared.

Author contributions

J.H.M., T.L., R.J.A., and B.N. conceived the study and designed the experimental approach. Data analysis, experimentation and manuscript preparation was performed by J.H.M. Protein preparation, immunoblotting and immunoprecipitation procedures were assisted by E.B. Manuscript editing was conducted by T.L., B. N. and R.J.A.

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Data in Brief

Data in Brief Figure 1: Co-incubation with etoposide and valsodpar (PSC833), a selective inhibitor of PGP efflux activity did not see a loss in oocyte vitality. The cytotoxicity of etoposide and PSC833 were evaluated using as a standard far red detectable live/dead vitality reagent. Analysis confirmed that in no instance was there an associated decrease in oocyte vitality following treatment exposure. n=3.

Data in Brief Figure 2: Permeability glycoprotein is absent from mature capacitated mouse spermatozoa. Immunobloting techniques confirmed that permeability glycoprotein is absent from mature capacitated mouse spermatozoa; no bands were recorded over three replicates. n=3

Data in brief Figure 3: Loss of permeability glycoprotein expression is not an artifact of cell death (a-b). Immunofluorescence using a far red detectable live/dead vitality reagent indicated that oocyte and embryo vitality was conserved following treatment with cycloheximide, an antibiotic with the ability to block protein synthesis and the calcium chelator BAPTA (1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester)), in the absence of PGP expression. n=3.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in

the online version at http://dx.doi.org/10.1016/j.ydbio.2016.06.035.

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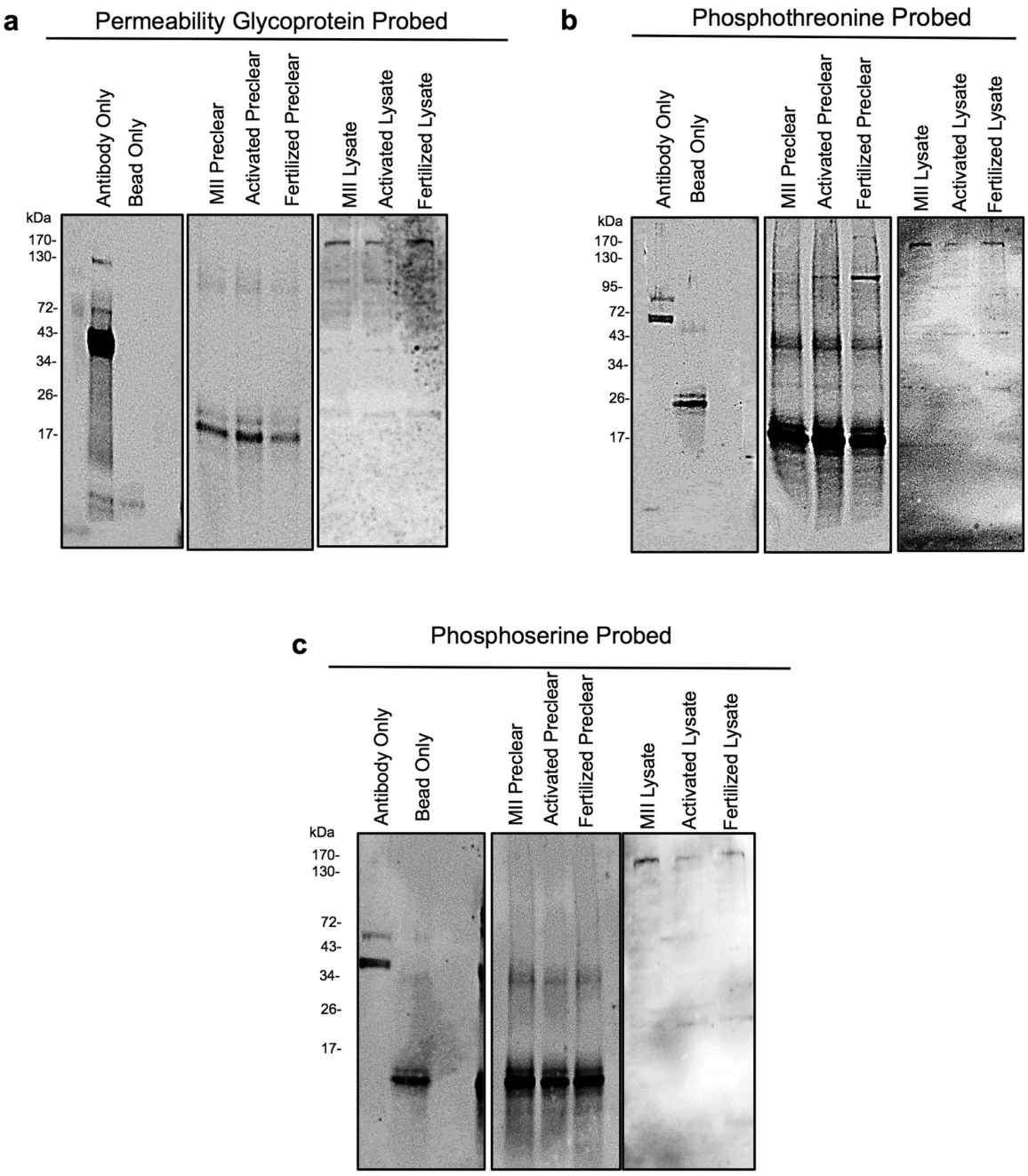

Supplementary Fig. 1: Permeability glycoprotein immunoprecipitation control lanes (a,b). Protein elution from PGP bound Dynabeads were resolved and subjected to immunoblotting in conjunction with the following controls; 5 µl PGP antibody, lysate prior to preclearing step, elution from Dynabeads used for the preclearing step and a Dynabeads-only control. Corresponding 170 kDa bands confirmed that post-translational modification by phosphorylation of threonine, and to a lesser extent serine residues, occurs following fertilization associated with a dramatic increase in PGP activity. n=3.

CHAPTER 2: SUPPLEMENTARY PUBLICATION

Data on the concentrations of etoposide, PSC833, BAPTA-AM, and cycloheximide that do not compromise the vitality of mature mouse oocytes, parthenogenetically activated and fertilized embryos

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Authors*:* Martin, J.H[1]., Bromfield, E.G[1]., Aitken, R.J[1]., Lord T., Nixon, B[1].

Affiliations: [1]Priority Research Centre for Reproductive Biology, School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia.

Correspondence*:* Jacinta.martin@newcastle.edu.au

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Data Article

Data on the concentrations of etoposide, PSC833, BAPTA-AM, and cycloheximide that do not compromise the vitality of mature mouse oocytes, parthenogenetically activated and fertilized embryos

Jacinta H. Martin*, Elizabeth G. Bromfield, R. John Aitken, Tessa Lord, Brett Nixon

Priority Research Centre for Reproductive Science, Discipline of Biological Sciences and Hunter Medical Research Institute, University of Newcastle, Callaghan, NSW 2308, Australia

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ABSTRACT

These data document the vitality of mature mouse oocytes (Metaphase II (MII)) and early stage embryos (zygotes) following exposure to the genotoxic chemotherapeutic agent, etoposide, in combination with PSC833, a selective inhibitor of permeability glycoprotein. They also illustrate the vitality of parthenogenetically activated and fertilized embryos following incubation with the calcium chelator BAPTA-AM (1,2-Bis(2-aminophenoxy)ethane- N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester)), cycloheximide (an antibiotic that is capable of inhibiting protein synthesis), and hydrogen peroxide (a potent reactive oxygen species). Finally, they present evidence that permeability glycoprotein is not represented in the proteome of mouse spermatozoa. Our interpretation and discussion of these data feature in the article "Identification of a key role for permeability glycoprotein in enhancing the cellular defense mechanisms of fertilized oocytes" (Martin et al., in press) [1].

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* Corresponding author.
E-mail address: jacinta.martin@newcastle.edu.au (J.H. Martin).

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Specifications Table

Subject area	*Biology,*
More specific subject area	*Oocyte/zygote protective mechanisms against double strand break DNA damage*
Type of data	*Graph and figures*
How data was acquired	*Immunocytochemistry and immunoblotting*
Data format	*Analyzed*
Experimental factors	*Mouse oocytes and zygotes were treated with etoposide (100 μg/ml) for 15 min when appropriate. Those used in the examination of permeability glycoprotein (PGP) efflux activity were pretreated with either the PGP inhibitor PSC833 for 15 min (5 μM) or with BAPTA-AM (5 μM)), or cycloheximide (20 μg/ml) for 4 h during the allotted activation/fertilization period.*
Experimental features	*Mouse oocytes and spermatozoa were harvested and zygotes or parthenotes produced via IVF or strontium chloride chemical activation, respectively. Oocytes were treated with etoposide and PSC833 in combination (a selective inhibitor of PGP), BAPTA-AM or cycloheximide. The cytotoxicity of these drugs was evaluated by labeling of the cells with a standard vitality reagent for 15 min at 37 °C.*
Data source location	*N/A*
Data accessibility	*All relevant data are presented within this article*

Value of the data

- These data provide valuable insight into the maintenance of mature mouse oocyte and zygote vitality following genotoxic insult with etoposide (100 μg/ml); a chemotherapeutic agent that elicits a potent inhibition of topoisomerase II α action.
- Similarly, these data indicate that selective pharmacological inhibition of permeability glycoprotein (PGP) with PSC833 (5 μM), as well as incubation of oocytes in BAPTA-AM (5 μM) and cycloheximide (20 μg/ml) for periods of up to 4 h following insemination with spermatozoa or activation with strontium, does not adversely affect oocyte or embryo vitality.
- This information is of use to the scientific community as it establishes concentrations of various pharmacological reagents that can be utilized without compromising oocyte and embryo viability.
- Finally, these data provide evidence that mouse spermatozoa do not harbor PGP within their proteome, thus discounting the possibility of a paternal contribution to elevated levels of PGP found in the zygote.

1. Data

The files included in this article comprise vitality profiles of mouse MII stage oocytes, chemically activated and fertilized zygotes following exposure to etoposide (100 μg/ml) in combination with PSC833 (5 μM) (Fig. 1), cycloheximide (20 μg/ml), BAPTA-AM (5 μM) or hydrogen peroxide (1 mM) (Fig. 3). This latter treatment was included as a positive control. Immunoblots of mouse sperm lysates with of anti-PGP antibodies are also included in this article (Fig. 2).

2. Experimental design, materials and methods

2.1. Reagents

Reagents were purchased from Sigma Aldrich (St Louis, MO, USA) unless otherwise stated. Anti-permeability glycoprotein (PGP; ab170904) antibody used for immunoblotting was procured from Abcam (Cambridge, England, UK).

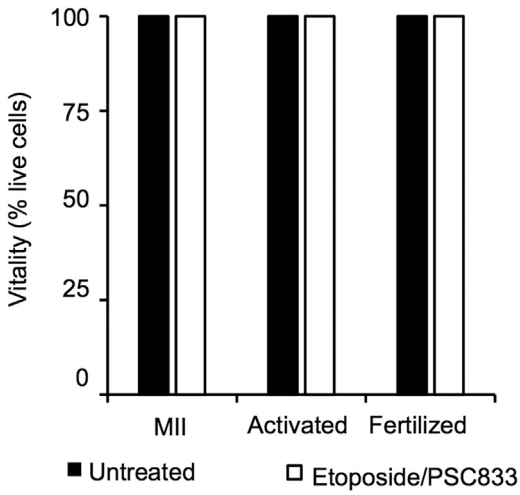

Fig. 1. Co-incubation with etoposide and valspodar (PSC833), a selective inhibitor of PGP efflux activity did not see a loss in oocyte vitality. The cytotoxicity of etoposide and PSC833 were evaluated using as a standard far red detectable live/dead vitality reagent. Analysis confirmed that in no instance was there an associated decrease in oocyte vitality following treatment exposure $n=3$.

2.2. Gamete retrieval

Gamete retrieval was achieved via superovulation of juvenile (3–5 week old) C57/BL6/CBA F1 female mice. Once primed, the mice were culled using CO_2 asphyxiation and the oocytes recovered from the cumulus mass following incubation in hyaluronidase (300 μg/ml, 3–5 min) [2]. Spermatozoa were recovered from the cauda epididymides of mature (≥ 8 weeks) male C57/BL6/CBA F1 mice by retrograde perfusion via the vas deferens [3]. Sperm capacitation was achieved by incubation in modified Biggers, Whitten and Whittingham (BWW) medium supplemented with 1 mg/ml polyvinyl alcohol and 1 mg/ml methyl-beta cyclodextrin [2].

2.3. Strontium activation and in vitro fertilization (IVF)

Parthenogenic activation was stimulated by incubation of cumulus free oocytes in calcium free KSOM medium supplemented with 10 mM strontium chloride for 4 h. *In vitro* fertilization was carried out in human tubal fluid (HTF) medium containing 1 mM reduced glutathione (GSH). Recovered oocytes were co-incubated with 2×10^5 capacitated spermatozoa for 4 h at 37 °C and successful fertilization (or activation in the case of parthenotes) was assessed by recording the extrusion of the second polar body and/or pronucleus formation [4].

2.4. Assessment of factors influencing permeability glycoprotein (PGP) expression

A number of drugs were used to investigate the factors responsible for the notable increase in PGP labeling following fertilization and activation [1]. The relative contribution of protein translation was analyzed by the inclusion of cycloheximide (20 μg/ml), an antibiotic with the ability to block protein synthesis. Alternatively, the role of activation-associated cytosolic calcium oscillations was assessed in the presence of the calcium chelator, BAPTA-AM (5 μM).

2.5. Etoposide treatment and cellular vitality

MII oocytes, parthenotes and pronuclear stage zygotes, were exposed to etoposide (100 μg/ml for 15 min at 37 °C), a topoisomerase II α inhibitor capable of eliciting DSB DNA damage. The cytotoxicity

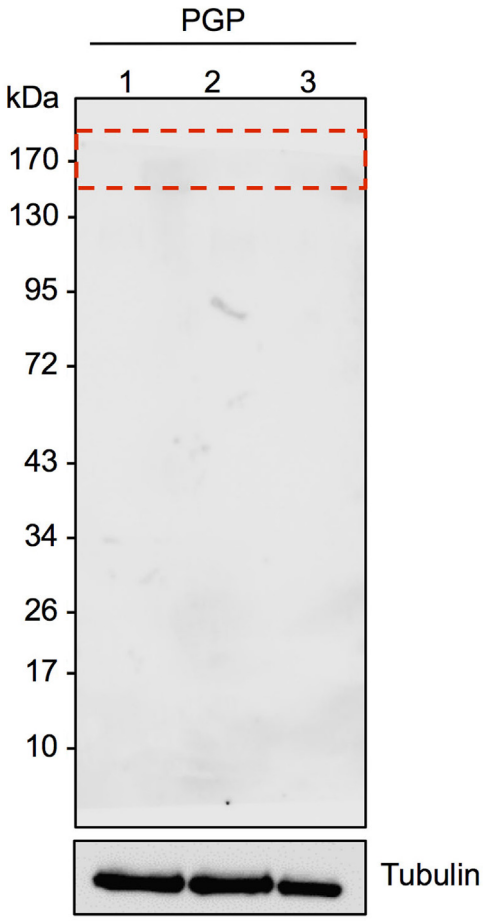

Fig. 2. Permeability glycoprotein is absent from mature capacitated mouse spermatozoa. Immunoblotting techniques confirmed that permeability glycoprotein is absent from mature capacitated mouse spermatozoa; no bands were recorded over three replicates $n=3$.

of etoposide, PSC833 [5,6], BAPTA-AM and cycloheximide at the specific concentrations employed in this investigation were evaluated by labeling of the cells with a far red detectable live/dead vitality reagent (Thermo Fisher Scientific, Waltham, MA, USA).

2.6. *Immunofluorescence, SDS PAGE and immunoblotting*

Following retrieval and appropriate treatment, oocytes were washed in PBS/PVP and fixed by incubation in 3.7% (v/v) paraformaldehyde for 1 h at room temperature (RT). Oocytes were later mounted on Menzel Gläser microscope slides (Thermo Fisher Scientific) in Mowiol containing 1,4-diazabicyclo[2.2.2]octane (DABCO) and fluorescence intensity assessed using an AXIO Imager.A1 fluorescence microscope (Carl Zeiss Micro Imaging GmbH, Jena, Thuringia, Germany). SDS PAGE and immunoblotting was conducted on solubilized (sodium dodecyl sulfate (SDS)) sperm protein extracts. A total of 10 μg of protein was resolved on pre-cast gels (4–12% NuPAGE BIS-Tris, Thermo Fisher Scientific) and transferred to a nitrocellulose membrane. Membranes were blocked in Tris-buffered saline (TBS) containing 0.1% Tween (TBST) with 3% BSA for 1 h) [7]. Blocked membranes were then sequentially incubated in anti-PGP primary antibody (1:500 in 3% BSA/TBST) overnight at 4 °C, HRP-conjugated

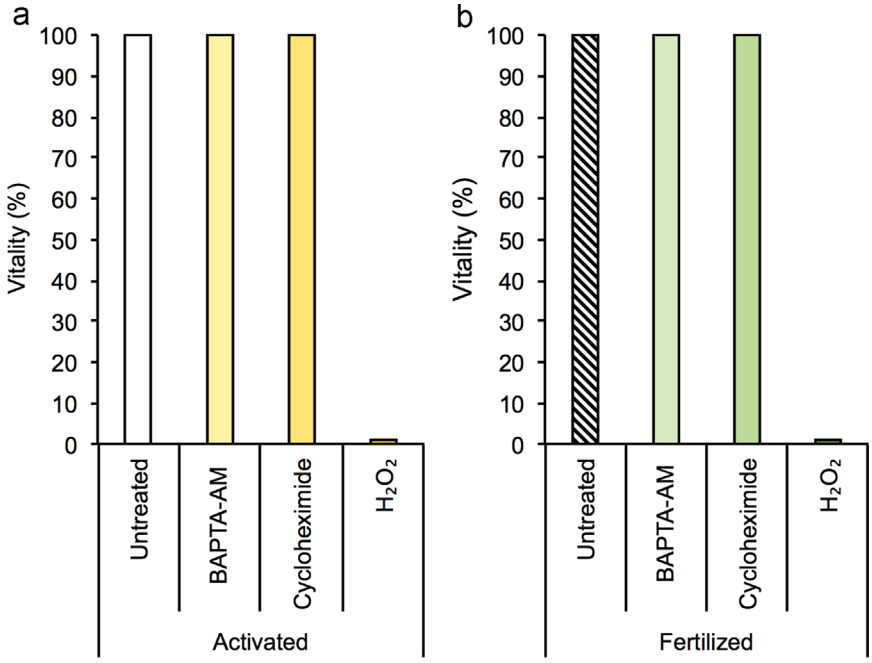

Fig. 3. Loss of permeability glycoprotein expression is not an artifact of cell death (a,b). Immunofluorescence using a far red detectable live/dead vitality reagent indicated that oocyte and embryo vitality was conserved following treatment with cycloheximide, an antibiotic with the ability to block protein synthesis and the calcium chelator BAPTA (1,2-Bis(2-amino-phenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester)), in the absence of PGP expression $n=3$.

secondary antibodies (1:1000 in 1% BSA/TBST) for 1 h at RT, stripped (western reprobe buffer in dH_2O, Astral Scientific, Sydney, NSW, Australia) and reprobed with an anti-α-tubulin antibody (1:4000 in 1% BSA/TBST) to ensure equal protein loading.

2.7. Statistical analysis

Image processing was achieved using the public sector program, Image J (National Institute of Health, Bethesda, MD, USA). Statistical significance was determined using JMP software (version 10.0.0, SAS Institute, NC, USA). Each experiment was conducted on a minimum of three biological replicates and expressed as the mean ± s.e.m. Differences with a value of $P<0.05$ were considered statistically significant.

Transparency document. Supplementary material

Transparency data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.dib.2016.07.046.

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CHAPTER 3: ORIGINAL RESEARCH ARTICLE

Double strand break DNA repair occurs via non-homologous end-joining in mouse MII oocytes

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Authors: Martin, J.H., Bromfield, E.G., Aitken, R.J., Lord, T[2]. and Nixon, B. (2018).

Affiliations: [1]Priority Research Centre for Reproductive Biology, School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia.

[2]School of Molecular Biosciences, Centre for Reproductive Biology, College of Veterinary Medicine, Washington State University, Pullman, WA, 99164, USA.

Correspondence: Jacinta.martin@newcastle.edu.au

CHAPTER OVERVIEW

In view of the finding that mature MII oocytes and zygotes display divergent responses to genotoxic assault, we next sought to assess the ability of the MII oocyte to effect DNA repair. The impetus to study the DNA damage response in MII oocytes rests with the finding that these cells are extremely vulnerable to DSB DNA damage, owing to an inability to engage the membrane defences of PGP (Chapter 2). Instead, we hypothesised that the MII oocyte may be equipped to resolve DNA damage, as a consequence of the significant and, hitherto unappreciated, enrichment of mRNA transcripts and pre-synthesised proteins implicated in DNA surveillance and repair pathways. Thus, the aim of the studies described in this manuscript was to determine if mature MII oocytes are able to utilize these resources to respond, and subsequently mount an effectual DNA repair strategy, to mitigate the impact of genotoxic insult.

The findings of this study support the presence of an active DSB DNA resolution pathway, namely the non-homologous end joining repair pathway (NHEJ). Here we confirm the presence, localization and functional significance of several key enzymes with important roles within the NHEJ pathway; ataxia telangiectasia mutated (ATM), X-ray repair complementing defective repair in Chinese hamster cells (KU80/XRCC5), DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and proliferating cell nuclear antigen (PCNA). Importantly, these data represent the first demonstration of an operational endogenous DNA repair programme during the later stages of oogenesis, encouraging a reappraisal of the long-held notion that oocytes are refractory to DNA repair. The characterisation of an active DNA repair process in the oocyte raises the prospect of novel therapeutic targets likely to have important

implications for *in vitro* fertility management, age-related subfertility and premature ovarian failure, diagnoses which all display a reduced capacity for DNA repair.

Lastly, in this manuscript we also explored the utility of alternative prophylactic interventions to prevent, rather than repair, DNA damage. For this purpose, we assessed the capacity of co-administration of 2 compounds, N-acetylcysteine (NAC) and sodium salicylate (SS). Of these compounds, SS did indeed prove successful in mitigating the deleterious consequences of genotoxic exposure. Such activity is likely attributed to the ability of SS to act as a catalytic inhibitor of topoisomerase Iiα, thereby blocking genotoxic agent induced topoisomerase IIα-DNA cleavable complex formation and DNA damage. These data therefore provide the impetus to further explore the beneficial effects of co-administration of SS, as a means of protecting the female germline following exposure to genotoxic agents as might be seen during chemotherapeutic interventions.

SCIENTIFIC REPORTS

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Double Strand Break DNA Repair occurs via Non-Homologous End-Joining in Mouse MII Oocytes

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Jacinta H. Martin[1,2], Elizabeth G. Bromfield[1,2], R. John Aitken[1,2], Tessa Lord[3] & Brett Nixon[1,2]

The unique biology of the oocyte means that accepted paradigms for DNA repair and protection are not of direct relevance to the female gamete. Instead, preservation of the integrity of the maternal genome depends on endogenous protein stores and/or mRNA transcripts accumulated during oogenesis. The aim of this study was to determine whether mature (MII) oocytes have the capacity to detect DNA damage and subsequently mount effective repair. For this purpose, DNA double strand breaks (DSB) were elicited using the topoisomerase II inhibitor, etoposide (ETP). ETP challenge led to a rapid and significant increase in DSB ($P = 0.0002$) and the consequential incidence of metaphase plate abnormalities ($P = 0.0031$). Despite this, ETP-treated MII oocytes retained their ability to participate in *in vitro* fertilisation, though displayed reduced developmental competence beyond the 2-cell stage ($P = 0.02$). To account for these findings, we analysed the efficacy of DSB resolution, revealing a significant reduction in DSB lesions 4 h post-ETP treatment. Notably, this response was completely abrogated by pharmacological inhibition of key elements (DNA-PKcs and DNA ligase IV) of the canonical non-homologous end joining DNA repair pathway, thus providing the first evidence implicating this reparative cascade in the protection of the maternal genome.

All cells within the human body encounter potentially thousands of DNA lesions on a daily basis owing to their exposure to a variety of internal and environmental factors[1]. If left unresolved, these lesions can potentially lead to mutations and broader-scale genomic aberrations that compromise cell viability and/or elevate predisposition to diseases such as cancer[2,3]. To ameliorate this threat, most cells are capable of mounting stringent DNA damage responses, which incorporate pathways for surveillance of DNA damage and mediation of its immediate repair. In somatic cells, molecular pathways of DNA repair include non-homologous end joining (NHEJ), homologous recombination (HR), mismatch repair (MMR) and nucleotide excision repair (NER)[4]. These pathways are generally very well-characterised, with a defined subset of the proteome devoted to sequentially detecting DNA lesions, remodelling chromatin and effecting DNA repair. In contrast to somatic cells however, there is less known about the DNA damage response in the oocyte beyond the ongoing maintenance of the resting population of primordial follicles by the Tap63 pathway[5–8]. Moreover, unlike somatic cells that can dynamically upregulate the synthesis of DNA damage response machinery following genotoxic insult[9,10], the mature ovulatory stage oocyte is transcriptionally silent and thus incapable of mounting an equivalent response. Instead, these cells must depend on endogenous stores of pre-synthesised proteins and/or mRNA transcripts accumulated during oogenesis to enact DNA repair[11].

The balance of evidence suggests that, beyond the follicular phases of development, immature oocytes and those arrested in the germinal vesical stage (GV), contain relatively inefficient DNA damage response mechanisms and are therefore largely refractory to DNA repair[12,13]. For instance, despite harbouring sufficient DNA damage to delay germinal vesicle breakdown (GVBD), reduce polar body extrusion rates and alter spindle assembly checkpoint dynamics, GV stage oocytes nonetheless retain the ability to progress to the fertilisation competent MII stage of development[14–16]. The absence of efficient DNA damage responses capable of effecting repair, or stalling development, in GV oocytes carries with it the attendant risk of propagating DNA lesions into the embryo. This situation appears to arise, at least in part, from an inability of the GV oocytes to activate the master regulator

[1]Priority Research Centre for Reproductive Science, School of Environmental and Life Sciences, The University of Newcastle, Callaghan, NSW, 2308, Australia. [2]Preganancy and Reproduction Program, Hunter Medical Research Institute, New Lambton Heights, NSW, 2305, Australia. [3]School of Molecular Biosciences, Centre for Reproductive Biology, College of Veterinary Medicine, Washington State University, Pullman, WA, 99164, USA. Correspondence and requests for materials should be addressed to J.H.M. (email: Jacinta.martin@newcastle.edu.au)

of the DNA damage response pathway, ataxia telangiectasia mutated kinase (ATM) unless the levels of DNA damage surpass a particularly high threshold[14]. In the presence of such severe damage, it has been shown that the GV oocyte will arrest due to activation of an alternative, oocyte-specific checkpoint known as the spindle assembly checkpoint (SAC), which prevents exit from M-phase[12,13]. Although still relatively insensitive, the SAC may act to protect against both aneuploidy and the inheritance of DNA damage by preventing the production of abnormal mature oocytes and subsequent embryos[12]. While it is not yet clear why immature oocytes fail to activate major DNA damage response or repair factors, it has yet to be determined whether mature MII phase oocytes are also recalcitrant to DNA repair, or whether this later developmental stage is equipped to resolve DNA lesions and thus mitigate the possibility of DNA damaged oocytes participating in fertilisation[17].

The impetus to study this mature stage stems from our recent findings that metaphase II (MII) oocytes are inherently susceptible to DSB DNA damage, owing to an inability to engage membrane defence proteins, such as those of the permeability glycoprotein family, which would otherwise counter the influx of genotoxic agents into the ooplasm[18,19]. Moreover, comparative proteomic analyses of mouse MII oocytes performed in an independent study have identified a total of 53 proteins putatively involved in the DNA damage response and DNA repair related processes, including: NER, single strand break repair (SSB), double strand break repair (DSB) and base excision repair (BER)[20]. Notably, 35 (66%) of these proteins were upregulated in the MII oocyte when compared to their GV oocyte and zygote counterparts[20]. This previously unappreciated level of enrichment of DNA damage response proteins may reflect their integral role in ensuring the integrity of the maternal genome required for the correct initiation of embryonic transcription. The current study was undertaken to expand our understanding of the extant molecular repair mechanisms in the post-ovulatory oocyte and the cell's capacity for the repair of DSB DNA damage. Such mechanistic understanding is of critical importance in protecting the genetic integrity of the oocyte to allow for its unimpeded transition through embryogenesis.

Results

Exposure to chemotherapeutic agents induces DNA damage and metaphase plate misalignment in MII oocytes. To assess the vulnerability of the ovulatory stage oocyte to genotoxic stressors, mouse MII oocytes were liberated from the ampulla and cultured in the presence of the chemotherapeutic agents etoposide (ETP), phleomycin (PHL) or doxorubicin (DOX), each of which are capable of inducing potent DSB DNA damage. Oocytes were subsequently assessed for the presence of DNA DSBs via measurement of the relative intensity of either γH2A.X labelling (ETP and PHL treatments) or DOX fluorescence within the vicinity of the metaphase plate (DOX treatment; DOX has a fluorescent signature that correlates with DNA damage[21]) (Fig. 1). As anticipated, neither untreated nor vehicle (DMSO) control oocytes presented with any appreciable evidence of DSB DNA damage. However, consistent with previous studies[12,13,19] significant, dose-dependent increases in criteria indicative of DSB DNA damage were recorded following administration of either ETP (Fig. 1a, $P = 0.0002$), PHL (Fig. 1c, $P = 0.0096$) or DOX (Fig. 1e, $P < 0.0001$). The pervasive impact of such treatments also manifested in the form of marked increases in the incidence of metaphase plate alignment abnormalities (Fig. 1i; ETP $P = 0.0031$, PHL $P = 0.0264$ and DOX $P < 0.0001$) and, in the case of ETP and PHL this response was again dose-dependent (Fig. 1b,d). Indeed, approximately half of all oocytes exposed to the highest concentrations of ETP (200 μg/ml; $P = 0.0062$) or PHL (50 μg/ml; $P = 0.0171$) experienced pronounced misalignment of their metaphase plates (Fig. 1b,d). This effect was even more pronounced in the case of DOX treatment, with ~90% of exposed oocytes responding with misalignment of their metaphase plates at the lowest dose tested (Fig. 1f; $P < 0.0001$).

Fertilising potential of etoposide treated MII oocytes. On the basis of the consistent response to each of the three chemotherapeutic agents used (Fig. 1), herein the decision was made to focus on the single insult of ETP treatment in order to begin to investigate: (i) the physiological consequences of this genotoxic agent on the developmental competence of MII oocytes and (ii) whether the MII oocyte is capable of engaging in DNA repair. As an initial line of enquiry, we sought to determine if ETP-induced misalignment of the metaphase plate was associated with additional perturbation of meiotic spindle characteristics. Specifically, we focused our assessment on meiotic spindle length and width (Fig. S1), owing to the utility of spindle morphology as a predictor of embryonic developmental potential[22–27]. Perhaps surprisingly, this analysis revealed that ETP-induced perturbation of the metaphase plate was not accompanied by appreciable alterations in either spindle length (Fig. 1g,j) or spindle width (Fig. 1h,j), thus raising the prospect that ETP treated MII oocytes may retain some developmental capacity. Accordingly, this possibility was directly assessed via *in vitro* fertilisation (IVF) and early embryonic development assays.

IVF assays revealed that ETP-treated MII oocytes retained their competence to participate in fertilisation, with fertilisation rates of >80% documented among populations of ETP-exposed oocytes; rates that proved indistinguishable from those recorded in unexposed or vehicle control oocyte groups (Fig. 2a). We also confirmed that the total number of pronuclei remained unchanged (i.e. 1 of maternal and 1 of paternal origin) in zygotes resulting from fertilisation of ETP-treated oocytes. While such results are consistent with the lack of overt defects in the morphometric characteristics of the meiotic spindle (Fig. 1), they are nonetheless at odds with the elevated levels of DNA damage these treated oocytes harbour (Fig. 1) and the fact that they present with significant misalignment of the metaphase plate. Consequently, our analysis was extended to determine the competency of ETP-treated MII oocytes to support normal embryonic development through to blastocyst formation (i.e. up to 5 days post-fertilisation) (Fig. 2b).

As illustrated in Fig. 2b, as many as 40–60% of embryos arising from ETP-treated MII oocytes failed to progress beyond the two-cell stage of development (100 μg/ml ETP, $P = 0.004$ and 200 μg/ml ETP, $P = 0.02$, compared to the controls). A modest number of the remaining embryos (~13–18%) progressed to the three - four cell stage, whilst only a very small subset (3%) developed beyond this point (Fig. 2b). This latter cohort were all arrested at the morula stage prior to proceeding to blastocyst formation. In stark contrast, >80% of embryos conceived from

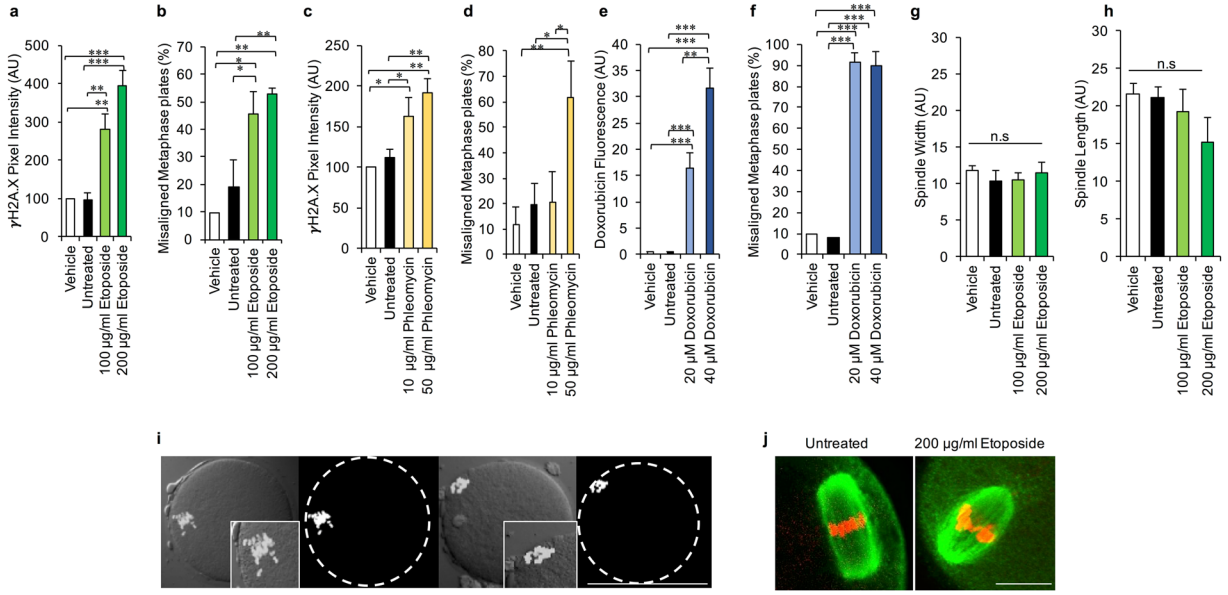

Figure 1. Assessment of the vulnerability of the mouse ovulatory stage oocyte to genotoxic stressors. (**a,c,e**) MII oocytes demonstrated a dose-dependent increase in γH2A.X and doxorubicin labelling (DOX treatment) at the metaphase plate following exposure to ETP, PHL or DOX. (**b,d,f**) γH2A.X labelling was accompanied by a pronounced, concomitant increase in metaphase plate abnormalities. (**g,h**) In addition spindle width and length were analysed as an early predictor of embryonic competency. This analysis revealed that ETP-induced perturbation of the metaphase plate was not associated with overt alterations in either measurement. (**i–j**) Representative images illustrate precocious chromatin segregation in MII oocytes as well as (**j**) confirm the retention of normal spindle width and length (green = tubulin, red = pseudocoloured DAPI). Statistical significance was determined using ANOVA, Tukey–Kramer HSD and Student's t-tests. $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$. Each experiment was conducted on a minimum of three biological replicates, with each replicate comprising a minimum of 30 oocytes. All data are expressed as means ± S.E.M. Scale bars in (**i**) and (**j**) =80 μm and 10 μm, respectively.

untreated or vehicle control oocytes experienced normal development through to the hatching and expanding blastocyst stages (Fig. 2d) (P = 0.0001). Based on these collective data we infer that ETP can dramatically attenuate both the genomic integrity and developmental competency of the mouse MII oocyte and the embryos that arise through the fertilisation of these cells.

Investigation of the physiological characteristics of arrested two cell embryos. To better define the characteristics of ETP-induced restriction of blastomere proliferative capacity, two cell embryos generated from the ETP-treated population of MII oocytes were subjected to morphometric analysis with a particular focus on cytoplasmic partitioning and nuclear integrity. This analysis revealed that embryos originating from ETP-exposed MII oocytes presented with significantly elevated fragmentation and unequal cytoplasm partitioning ($P = 0.0034$) (Fig. 3a). The latter commonly manifested in the form of one 'large' blastomere accompanied by a significantly 'smaller' counterpart (Fig. 3b; denoted as B1 and B2, respectively). Notably, the abnormally large blastomeres were characterised by a significant disparity in diameter ($P = 0.0495$ and $P = 0.016$, respectively) when compared to the blastomeres present within control embryos (Fig. 3b,d). Such findings raise the prospect that the cytoskeletal network responsible for faithful completion of blastomere cytokinesis is compromised by the legacy of oocyte ETP exposure. This interpretation is in keeping with reports that senescence-like phenotypes induced by cytostatic drugs such as ETP (and DOX) are coupled to alterations in the integrity of cytoskeletal elements present in the treated cells[28,29].

Accordingly, we sought to localise both the actin and tubulin networks of the preimplantation zygote and 2 cell embryos derived from these untreated and ETP-treated populations of oocytes. At the resoltuion afforded by confocal microscopy, neither embryonic stage exhibited overt changes in actin or tubulin labelling intensity or distribution patterns irrespective of the treatment they received (Fig. 3f). Notably, in contrast to the unaltered distribution of actin and tubulin in the embryos following ETP exposure, multinucleate structures were exclusively detected in the daughter cells derived from ETP-treated MII oocytes, and were not present in their control counterparts (Fig. 3c,e) ($P = 0.005$).

DNA repair occurs via non-homologous end-joining (NHEJ). Having established the impact of acute ETP exposure in terms of attenuating the developmental competency of the MII oocyte, we next sought to address our second goal of determining whether this cell is capable of mounting any form of DNA damage repair in response to this genotoxic insult. For this purpose, MII oocytes were treated with ETP prior to monitoring the intensity of γH2A.X fluorescent labelling over a recovery period of up to 6 h[30]. Under these experimental

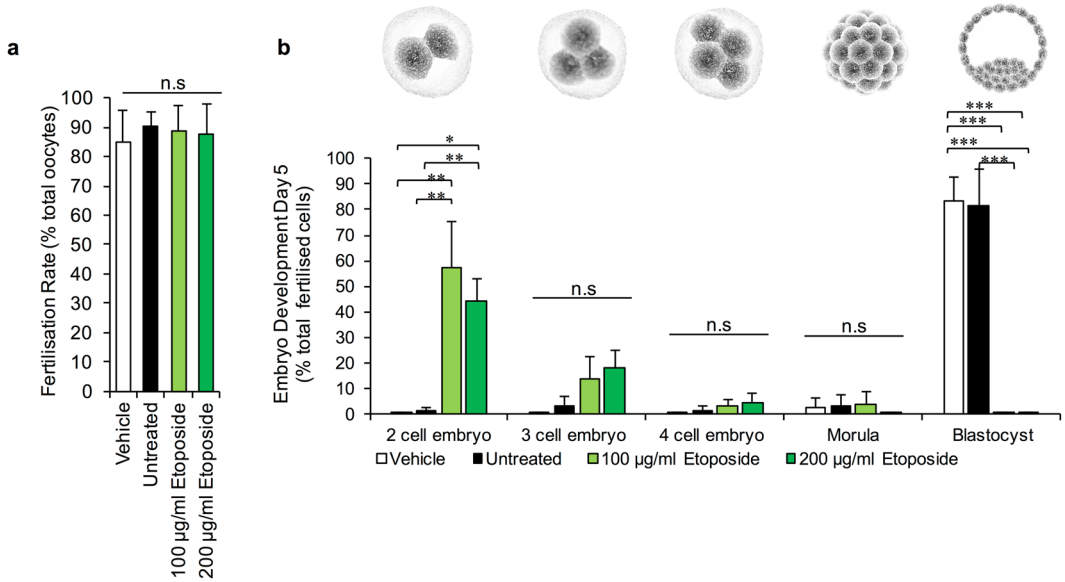

Figure 2. Physiological consequences of etoposide exposure. The developmental competence of ETP exposed MII oocytes was directly assessed via *in vitro* fertilisation and early embryonic development assays. For this purpose oocytes were co-cultured with approximately 2×10^5 capacitated spermatozoa for 4 h at 37 °C. (**a**) ETP-treated MII oocytes retained their competence to participate in fertilisation. (**b**) However, ETP-treated MII oocytes were not able to support embryonic development through to blastocyst formation (i.e. 5 days post-fertilisation) whereby 40–60% of embryos failed to progress beyond the two cell stage of development. Statistical significance was determined using ANOVA, and Tukey–Kramer HSD. $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$. Each experiment was conducted on a minimum of three biological replicates, with the total number of embryos assessed across these replicates being: vehicle = 95; untreated = 93; 100 µg/ml ETP = 95; 200 µg/ml ETP = 86. All data are expressed as means ± S.E.M. Scale bar = 80 µm.

conditions, the intensity of γH2A.X foci was significantly elevated above that of untreated control oocytes as early as 15 min after ETP administration in all oocytes (100%). Thereafter, γH2A.X labelling appeared to plateau at 1 h post-treatment, before gradually decreasing to basal levels comparable to those of the untreated controls by 4 h post-treatment (Fig. 4). No further reduction in the intensity of γH2A.X labelling was observed between 4 and 6 h post-treatment. We also noted that ETP failed to elicit any overt changes in oocyte morphology irrespective of the concentration used in this study. This remained true following the initial insult and subsequent to the recovery period (T = 6 h).

This kinetic profile of γH2A.X resolution in ETP treated oocytes is consistent with that expected following the recruitment of DSB DNA repair pathway(s) (2–6 h[31]). In this context, the repair of DSB lesions is generally mediated through two key repair pathways, homologous recombination (HR) and non-homologous end-joining (NHEJ)[4]. While we cannot specifically exclude the contribution of HR, this scenario was considered unlikely due to it's restricted activity within the late S-G2 phases of the cell cycle[4,32]. Instead, we focused our analysis on the contribution of NHEJ in resolving the ETP induced DSB DNA damage. For this purpose, immunocytochemistry was used to investigate the presence and localisation of several enzymes that hold key functional roles within this pathway, namely: Ataxia telangiectasia mutated (ATM), X-ray repair complementing defective repair in Chinese hamster cells (KU80/XRCC5), DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and Proliferating cell nuclear antigen (PCNA) (Fig. 5). In keeping with data from recent global proteomic analyses[20], our study confirmed the expression of ATM, KU80, DNA-PKcs and PCNA in mouse MII oocytes (Fig. 5). Moreover, we were also able to detect the active, phosphorylated forms of both ATM (p-Ser1981) and DNA-PKcs (p-Ser2056)[33,34] (Fig. 5). In all instances, the fluorescence associated with these enzymes was dispersed throughout the ooplasm and was generally characterised by punctate spatial patterning. The specificity of these labelling patterns was confirmed by the complete absence of labelling in negative controls in which the primary antibody was substituted with buffer alone. In addition to this, immunoblotting with the positive controls HEK293 and UV treated HELA lysates further confirmed the specificity of these antibodies (Fig. S2).

Having confirmed that MII oocytes harbour key elements of the NHEJ pathway, we next examined the functional significance of NHEJ repair activity through the application of selective pharmacological inhibitors (Fig. 6). Specifically, ETP treated oocytes were incubated in culture medium supplemented with either 50 µM NU7441 (a potent and selective agonist of DNA-PKcs)[35] or 20 µM SCR7 (marketed as a selective inhibitor of DNA ligase IV)[36] over a time course of 6 h (Figs 6 and S3). After this recovery period, oocytes were fixed and examined for γH2A.X fluorescent labelling as an indicator of DNA damage. This experimental strategy confirmed that inhibition of either DNA-PKcs (Fig. 6a,b; $P < 0.0001$) or DNA ligase IV (Fig. 6c,d; $P < 0.0001$) completely abrogated the resolution of γH2A.X foci. Indeed, γH2A.X labelling intensity was maintained at a level reminiscent of that achieved in all oocytes immediately following ETP treatment (NU7441: 100 µg/ml $P = 0.3789$ and 200 µg/ml

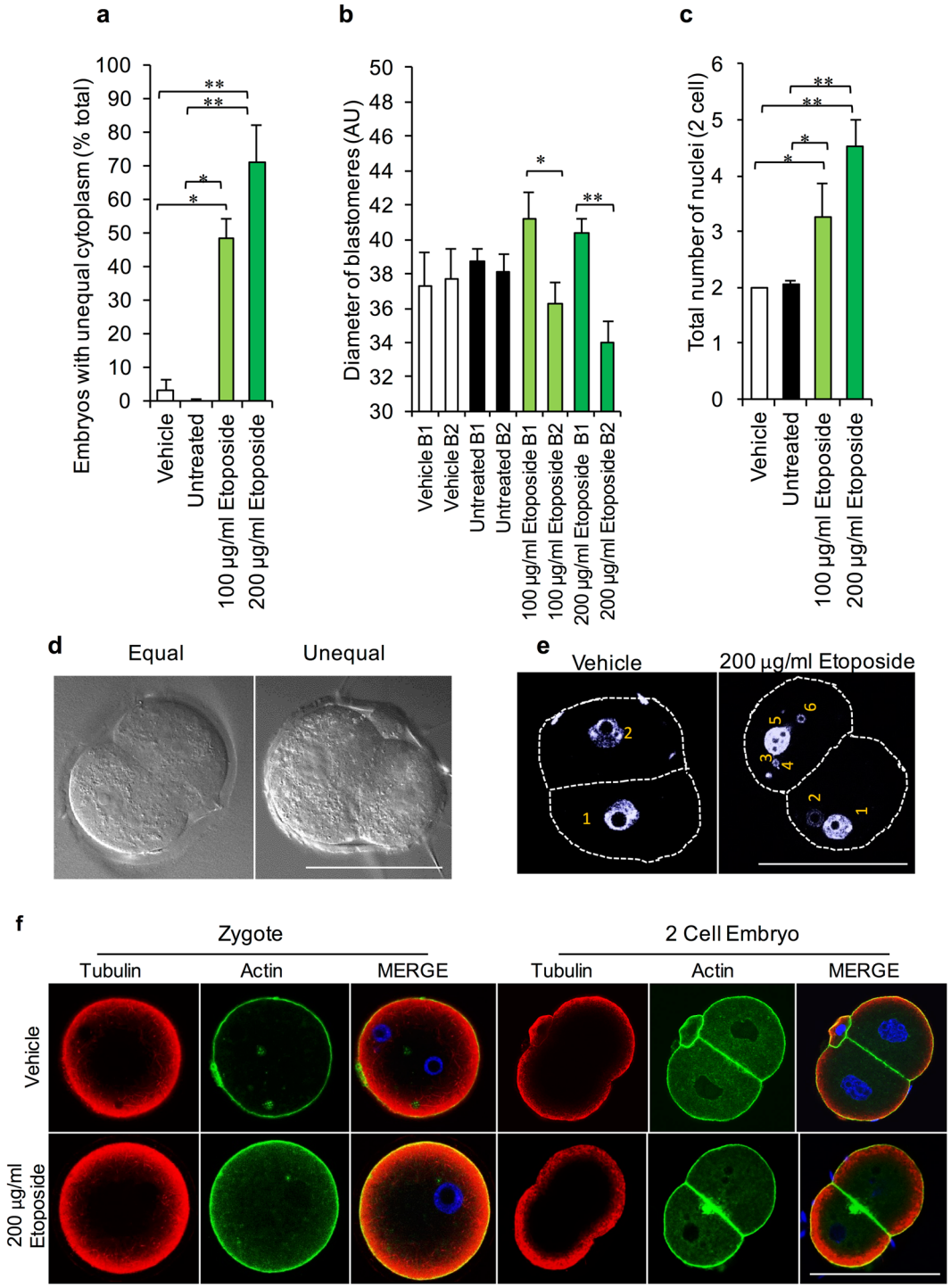

Figure 3. Morphometric analysis of arrested two cell embryos generated from the etoposide-treated MII oocytes. (**a–d**) Embryos borne of ETP-exposed MII oocytes were characterised by significant fragmentation and unequal cytoplasm partitioning, accompanied by an increase in diameter in the notably larger blastomeres (greater than 25% difference). (**c–e**) Accordingly, nuclear integrity was also analysed, revealing that daughter cells derived from ETP-treated MII oocytes contained additional multinucleate structures in addition to the pronucleus. (**f**) Localisation of the actin and tubulin networks of the preimplantation zygote and 2 cell embryos were analysed for their potential contribution to the unequal cytoplasmic partitioning. However, neither embryonic stage exhibited apparent modifications in labelling intensity or distribution patterns of tubulin or actin irrespective of the treatment they received. Statistical significance was determined using ANOVA and Student's t-tests. $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$. Each experiment was conducted on a minimum of three biological replicates, with each replicate comprising a minimum of 30 oocytes. All data are expressed as means ± S.E.M. Scale bar = 80 µm.

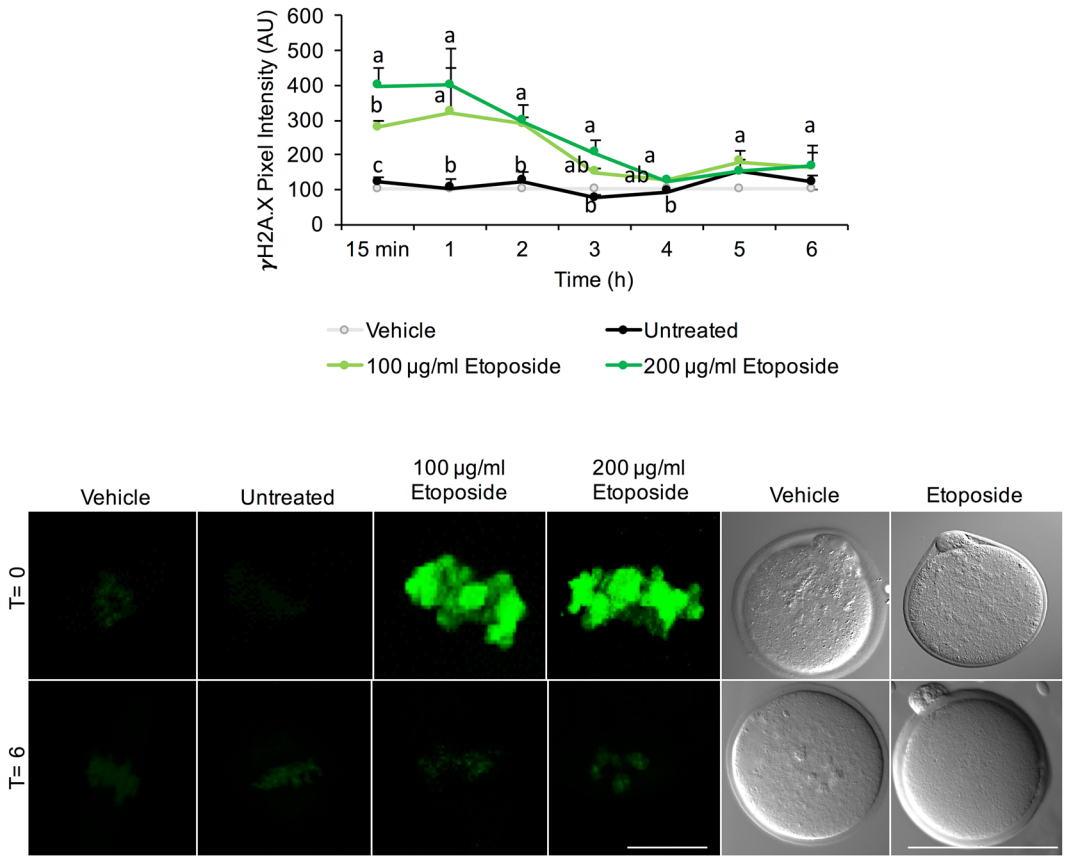

Figure 4. DNA repair in the MII oocyte occurs via non-homologous end-joining (NHEJ). To determine if the MII oocyte was capable of mounting DNA damage repair, γH2A.X fluorescent labelling was utilised to produce a kinetic profile of DNA repair over a recovery period of 6 h. γH2A.X labelling was significantly reduced by 1 h post-treatment; reaching basal levels by 4 h. Representative images illustrate that at 4 h post treatment, γH2A.X fluorescence intensity was comparable to the basal levels documented in untreated control oocytes. Statistical significance was determined using ANOVA and Tukey–Kramer HSD. A connecting letter plot was used to denote which groups differed significantly from each another. Each experiment was conducted on a minimum of three biological replicates, with each replicate comprising a minimum of 30 oocytes. All data are expressed as means ± S.E.M. Scale bar = 20 μm.

$P = 0.8407$; SCR7: 100 μg/ml $P = 0.8885$, and 200 μg/ml $P = 0.6419$), even after the maximal recovery period of 6 h. Over the same time course, those ETP treated oocytes that were not challenged with pharmacological inhibitors experienced the customary decrease of γH2A.X labelling (NU7441: 100 μg/ml $P = 0.0032$ and 200 μg/ml $P = 0.0157$; SCR7: 100 μg/ml $P = 0.0056$ and 200 μg/ml $P = 0.0005$) to basal levels indistinguishable from that of untreated control cells (Fig. 6a,c and insets in Fig. 6b,d).

NHEJ-mediated repair of ETP-induced DNA DSBs is not sufficient to rescue embryonic development. Given that the progression of NHEJ DNA repair is generally considered to be of lower fidelity than that of HR, it was of interest to determine if this repair pathway could rescue the developmental potential of oocytes challenged with an ETP insult. Thus, ETP treated oocytes were allowed the minimum recovery time of 4 h prior to being inseminated via IVF. The embryonic competency of fertilised oocytes was subsequently assessed revealing that the extent of NHEJ taking place within 4 h post-ETP treatment was not able to appreciably alter the number of embryos progressing beyond the two-cell stage of embryogenesis (Fig. 7a). Notably, as a likely consequence of the post-ovulatory oocyte aging process[37,38], the control (vehicle exposed and untreated) oocytes in this experiment also experienced a reduction in both their fertilisability and developmental competency (Fig. 7a,b) when compared to embryos generated from freshly isolated MII oocytes (Fig. 2). Notwithstanding this phenomenon, the deterioration in oocyte quality we encountered was certainly not of sufficient magnitude to account for the restricted proliferative capacity of blastomeres derived from 'repaired' oocytes. In seeking to account for these data, we assessed the integrity of the metaphase plates formed in ETP treated oocytes following 4 and 6 h recovery time points (Fig. 7c,d). Regrettably, neither period of post-treatment recovery proved sufficient to ameliorate the incidence of metaphase plate misalignment (4 h: $P = 0.009$ and 6 h: $P = 0.0088$ compared to untreated controls), with both populations characterised by alignment abnormalities equivalent to those recorded in the absence of any recovery period (Fig. 1b).

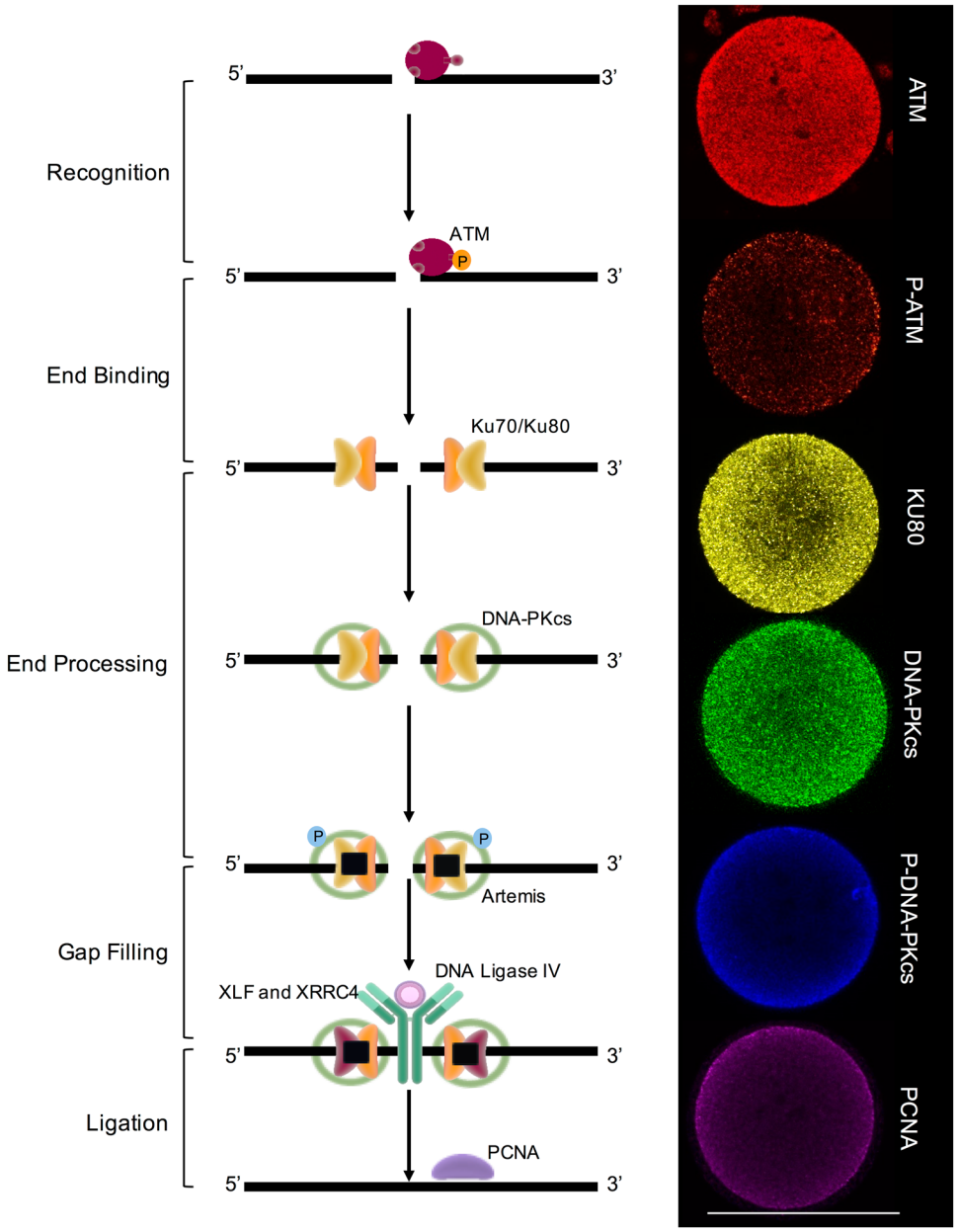

Figure 5. MII oocytes contain the necessary protein machinery for NHEJ. The presence and localisation of several key enzymes within the NHEJ pathway were investigated using immunocytochemistry. Ataxia telangiectasia mutated (ATM), X-ray repair complementing defective repair in Chinese hamster cells (KU80/XRCC5), DNA-dependent protein kinase catalytic subunit (DNA PKcs) and Proliferating cell nuclear antigen (PCNA) were each identified within the MII oocyte. In addition, the active phosphorylated forms of both ATM (p-Ser1981) and DNA PKcs (p-Ser2056) were also detected within these cells. In all instances, punctate fluorescence was disseminated throughout the ooplasm. Scale bar = 80 μm.

DNA damage can be prevented by co-administration of sodium salicylate. Our collective evidence indicates that, despite the presence of an active NHEJ pathway, oocytes are unable to mount a repair pathway of sufficient efficacy to alleviate the impact of genotoxic agents such as ETP. We therefore sought to explore the utility of alternative therapeutic interventions to prevent, rather than repair, ETP damage. Specifically, we explored the potential to ameliorate the effects of ETP on the MII stage oocyte via co-administration of either N-acetylcysteine (NAC) or sodium salicylate (SS), compounds that have been proposed to modulate the effects of ETP in somatic cells[39–41].

The thiol, NAC, has an impressive array of mechanisms by which it can putatively afford protection against DNA damage. These attributes include its capacity to act as a nucleophile (and therefore antioxidant), modulate DNA repair, alter cellular metabolism, influence anti-inflammatory and finally anti-angiogenic activity[42]. Despite these myriad of beneficial actions, supplementation of NAC (2.5 mM[43]) in our culture system failed to significantly improve the response of ETP treated MII oocytes. Thus, γH2A.X related fluorescence remained equivalent

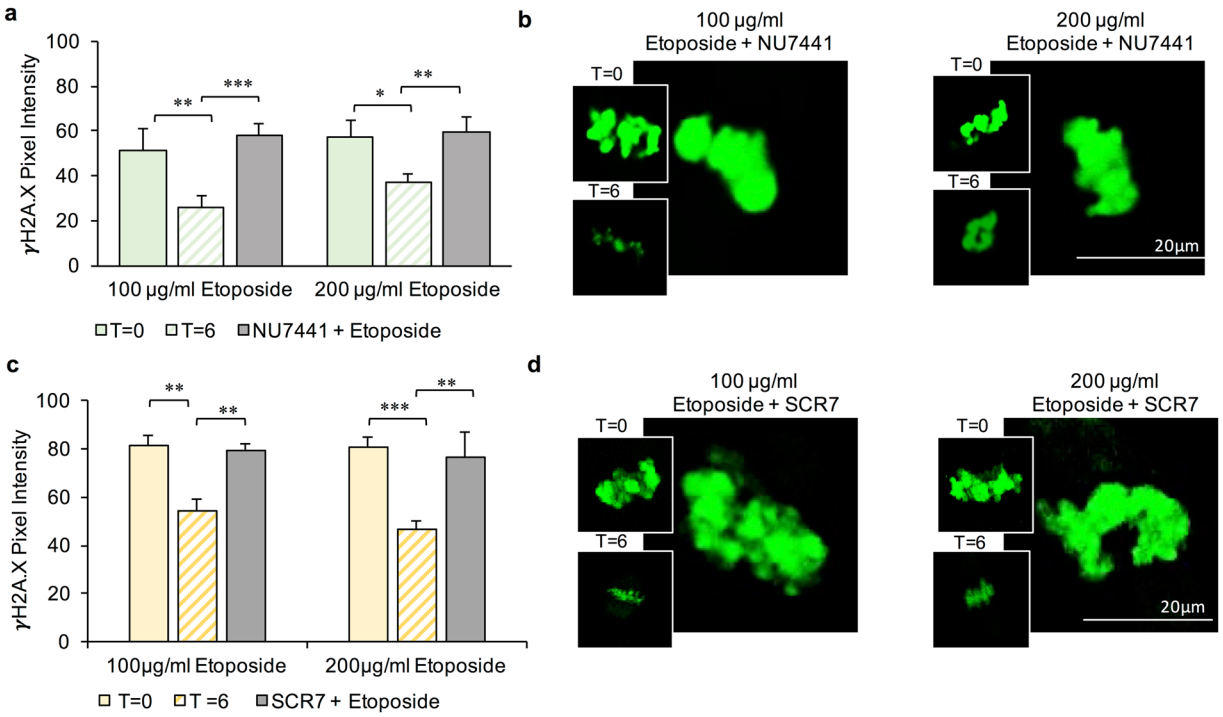

Figure 6. Assessment of the functional significance of NHEJ repair activity by selective pharmacological inhibition. (**a**) NU7441 (50 μM) a potent and selective antagonist of DNA-PKcs and **(c)** SCR7 (20 μM), a selective inhibitor of DNA ligase IV were incubated with ETP-treated oocytes during a recovery time course (6 h). In both instances, inhibition of DNA-PKcs or DNA ligase IV completely prohibited the resolution of γH2A.X foci. In contrast, ETP-treated oocytes not challenged with these pharmacological inhibitors experienced a significant decrease in γH2A.X labelling following an equivalent recovery period. (**b** and **d**) Representative immunofluorescence images of γH2A.X labelling of the metaphase plate are presented, with the main image corresponding to oocytes treated with (**b**) ETP + NU7441 and (**d**) ETP + SCR7. Similarly, the insets in panels (**b**) and (**d**) correspond to γH2A.X labelling of the metaphase plate in ETP treated oocytes at T = 0 (i.e. upper insets) and again at T = 6 h (lower insets). Statistical significance was determined using ANOVA and Student's t-tests. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$. Each experiment was conducted on a minimum of three biological replicates, with each replicate comprising a minimum of 30 oocytes. All data are expressed as means ± S.E.M. Scale bar = 20 μm.

in both ETP/NAC exposed oocytes and those exposed to the ETP insult alone (Fig. 8a) (100 μg/ml $P = 0.2201$ and 200 μg/ml $P = 0.9056$). To confirm this result, analysis of the cytosolic ROS response elicited by ETP exposure was undertaken using DCF-DA, a fluorescent probe that reports total cytosolic ROS activity. As illustrated in Fig. 8b, the concentrations of ETP administered herein were unsuccessful in increasing ROS production above that recorded in control populations of oocytes ($P = 0.935$).

The second compound tested was that of SS, the active ingredient in aspirin. As a catalytic inhibitor of topoisomerase IIα, SS has been documented to inhibit genotoxic agent induced topoisomerase IIα-DNA cleavable complex formation *in vivo*[39]. Since ETP also relies on the formation of ETP stabilised topoisomerase IIα-DNA complexes to propagate DNA damage[44], it was reasoned that pre-treatment of oocytes with SS could mitigate the deleterious impact of ETP treatment. This was precisely the case, with 10 mM SS[39] proving effective in combating ETP genotoxicity as measured by the intensity of γH2A.X fluorescent foci (Fig. 8c; $P = 0.0134$). In addition, co administration of SS with PHL (Fig. 8d) and DOX (Fig. 8e) also proved effective at reducing the genotoxicity of these drugs *in vitro* (PHL: $P = 0.0076$ and DOX: $P < 0.0001$).

Discussion

It is widely held that the mammalian oocyte has limited capacity for DNA repair beyond the imposition of cell cycle checkpoints, which serve to arrest development[12,13]. It is therefore curious that these cells are endowed with an impressive suite of proteins that are capable of enacting DNA repair[20,45]. Such findings have promoted the current study in which we have begun to explore the extant molecular repair mechanisms present in the post-ovulatory oocyte as well as the cell's ability to enact the repair of DSB DNA damage. Our collective data support the presence of an active non-homologous end joining pathway in MII stage mouse oocytes. In addition, we have identified that co-administration of sodium salicylate with the cytotoxic drugs ETP, PHL and DOX, effectively prevents their induction of genomic instability.

Previous reports have established that the oocyte is susceptible to genotoxic assault and the consequential induction of DNA damage[12–16,19,46]. These data agree with the findings of the current study, where we have demonstrated that a range of chemotherapeutic agents, including ETP, PHL and DOX are each capable of eliciting a

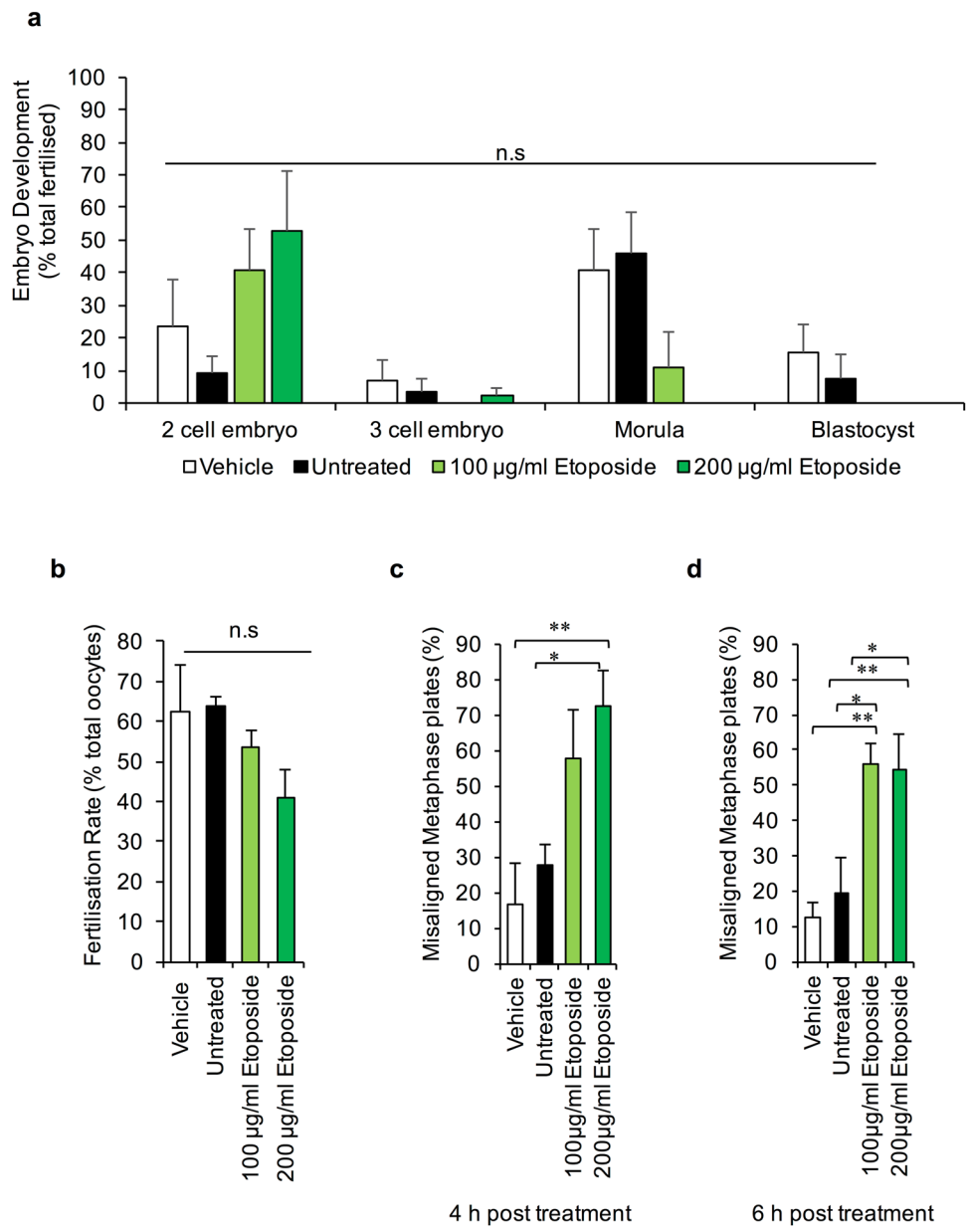

Figure 7. NHEJ-mediated repair of ETP-induced DNA DSBs is not sufficient to rescue embryonic development. The developmental potential of oocytes challenged with an ETP insult was analysed whereby treated oocytes were provided a minimum recovery time of 4 h prior to *in vitro* fertilisation. (**a**) Embryonic competency was not appreciably altered within the 4 h post-ETP recovery window with limited numbers of embryos progressing beyond the two-cell stage of embryogenesis. (**b**) In addition, a notable reduction in fertilisability was evident in all oocytes derived of both the treated and untreated oocytes. (**c**) Analysis of metaphase plate integrity at 4 and 6 h recovery time points indicated that neither period of post-treatment recovery was sufficient to ameliorate the incidence of metaphase plate misalignment. Statistical significance was determined using ANOVA and Tukey–Kramer HSD. $*p \leq 0.05$ and $**p \leq 0.01$. Each experiment was conducted on a minimum of three biological replicates, with the total number of embryos assessed across these replicates being: vehicle = 40; untreated = 50; 100 µg/ml ETP = 40; 200 µg/ml ETP = 27. In regard to oocytes, each experiment was conducted on a minimum of three biological replicates, with each replicate comprising a minimum of 30 oocytes. All data are expressed as means ± S.E.M.

significant, dose-dependent increase in DNA DSB in MII mouse oocytes. Notably, the impact of such treatments extended beyond DNA damage to include a striking increase in metaphase plate alignment abnormalities and chromatin aggregations. Interestingly however, these irregularities were not accompanied by overt changes in meiotic spindle dimensions. A recent study by Marangos *et al.*[12] reported phenotypically similar spindle aberrations without an attendant dysregulation of spindle proportions in response to ETP exposure of MI oocytes. In this instance, as many as 80% of the oocytes succumbed to a SAC mediated cell cycle arrest. In view of these data, it is perhaps surprising that the legacy of ETP treatment did not extend to preventing ETP treated MII oocytes

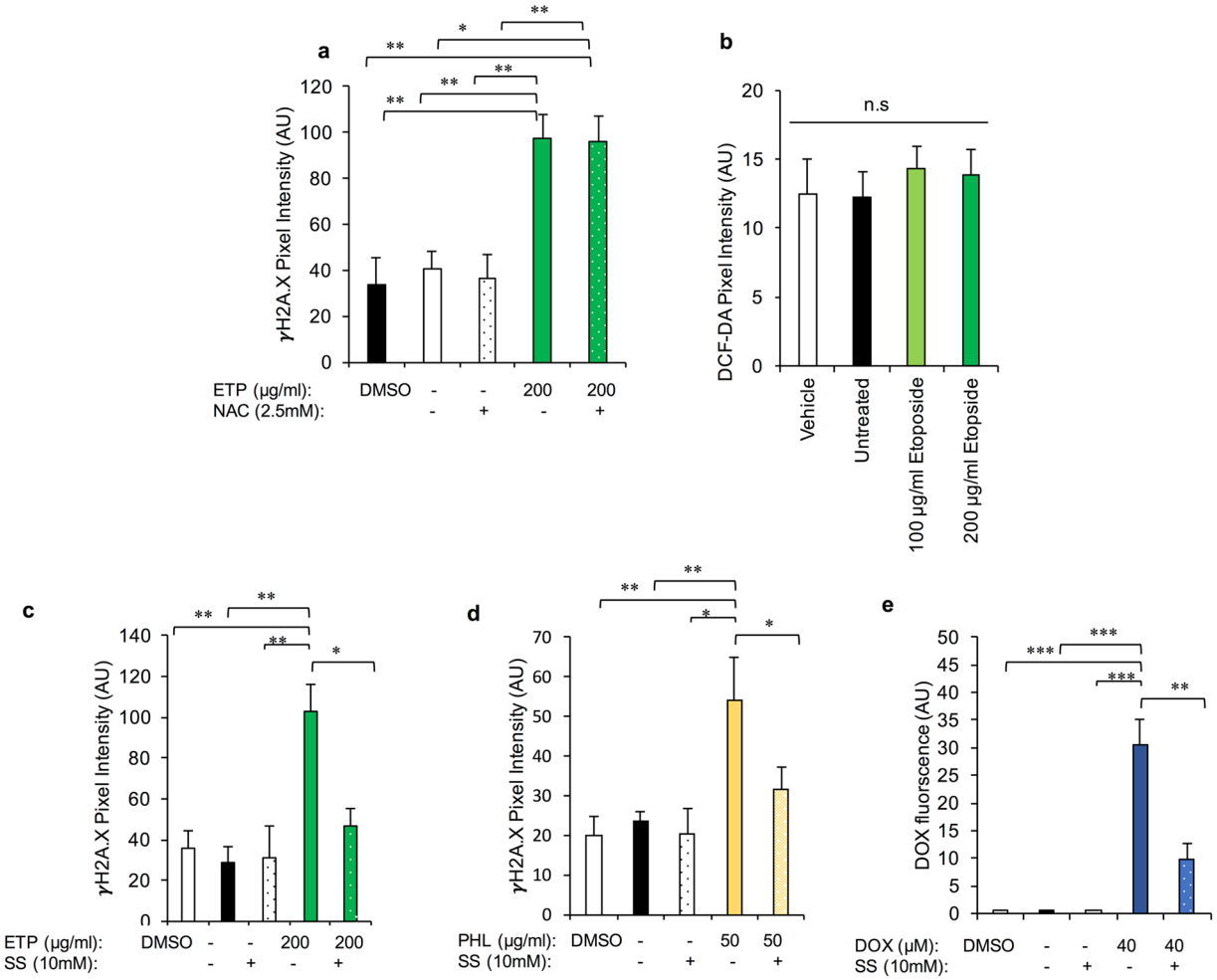

Figure 8. DNA damage can be prevented by co-administration of sodium salicylate. Several compounds were analysed for their ability to prevent DNA damage as a consequence of ETP, PHL, and DOX exposure. (**a**) Co-administration of the thiol and antioxidant N-acetylcysteine (NAC) failed to significantly improve the response of ETP-treated MII oocytes. (**b**) Co-incubation of oocytes with sodium salicylate (an inhibitor of topoisomerase IIα) and ETP was capable of mitigating the deleterious impact of ETP treatment at 10 mM. (**c**) Analysis of cytosolic reactive oxygen species did not reveal an appreciable increase following ETP exposure as assessed by the intensity of DCF-DA probe fluorescence. (**d,e**) In addition, co-incubation of sodium salicylate and PHL and DOX proved effective in decreasing the level of DNA damage (γH2A.X fluorescence) generated by these genotoxic compounds. Statistical significance was determined using ANOVA and Tukey–Kramer HSD. Each experiment was conducted on a minimum of three biological replicates, with each replicate comprising a minimum of 30 oocytes. All data are expressed as means ± S.E.M.

from participating in the fertilisation cascade following *in vitro* fertilisation. In this regard, presumptive zygotes appeared morphometrically normal and did not retain γH2A.X labelling despite being generated from a pool of oocytes with significant chromosomal abnormalities.

Downstream of fertilisation, embryonic development assays revealed that ETP did severely compromise early embryonic competency with ~40–60% of embryos failing to progress beyond the two-cell stage of development. Notably, many of these embryos displayed a permanent senescence-like arrest, with little evidence of apoptosis being detected after 5 days of development[47]. This phenomenon mirrors that reported to occur in approximately 10–15% of all human and bovine IVF embryos, which also experience arrested development at the 2- to 4-cell cleavage stages[47]. It has recently been postulated that this permanently-arrested state may constitute part of a quality control mechanism that prevents further development of low-quality embryos[48]. In the context of our study, it is notable that the 2-cell stage coincides with the timing of embryonic genome activation in murine species[49–51]. It is therefore not surprising that embryos harbouring pronounced morphometric abnormalities such as those documented in this study would be removed from the developmental programme at this stage.

In seeking to account for the unequal cytoplasmic partitioning induced by ETP, it is noteworthy that cell division in the mammalian embryo typically remains symmetrical up until at least the 16–32-cell stage[52]. Thus, the formation of large and small blastomeres during earlier developmental phases may be attributed to ETP-mediated dysregulation of the cytoskeletal network responsible for faithful completion of blastomere cytokinesis. This

interpretation is consistent with reports that senescence-like phenotypes induced by cytostatic drugs such as ETP (and DOX) are coupled to alterations in the integrity of cytoskeletal elements[28,29]. Despite this, at the level of resolution afforded by confocal microscopy, we did not detect any overt changes in either the labelling intensity or distribution patterns of the actin and tubulin networks assembled within the preimplantation zygote or 2 cell embryos irrespective of the treatment they received. As an alternative explanation, it is known that both the initiation and orientation of the first cleavage is tightly coupled with spindle quality and cleavage symmetry[53]. Indeed, embryos displaying significant spindle abnormalities often initiate incorrect cytokinesis leading to premature developmental arrest[54]. This accords with our findings that the spindle suffered significant misalignment during ETP treatment at the MII stage.

Having established the substantial impact of acute ETP exposure in terms of attenuating the developmental competency of the MII oocyte, we next sought to address whether the MII oocyte is capable of mounting an effective DNA damage repair response to protect the female genome against genotoxic insult. In somatic cell models, DNA repair kinetics are relatively slow, with the interval from initiation to completion generally two to six hours after the primary insult[31]. The loss of γH2AX at DSB sites is widely held to reflect the completion of repair of DNA at break sites[30,55,56]. Accordingly, we tracked the resolution of γH2A.X in mouse MII oocytes over a recovery period of up to 6 hours[30,55]. The kinetic profiling of γH2A.X expression was characterised by a gradual decline to basal levels, equivalent to those of the untreated controls by 4 hours post-treatment. Consistent with data from recent global proteomic analyses of both mouse and human oocytes[20,57], immunocytochemistry confirmed the presence and localisation of several enzymes that hold key functional roles within the NHEJ pathway, namely; ATM, KU80/XRCC5, DNA PKcs and PCNA. Notably, the expression of the active, phosphorylated forms of both ATM (p-Ser1981) and DNA PKcs (p-Ser2056) were also detected in the MII oocyte. Moreover, the functional significance of NHEJ repair activity was supported by the application of selective pharmacological inhibitors, NU7441 (a selective agonist of DNA-PKcs) and SCR7 (a potent inhibitor of DNA ligase IV); both of which prevented the resolution of γH2A.X foci. These data encourage a reappraisal of the paradigm that, beyond the early follicular phases of development, oocytes are largely refractory to DNA repair[12,13].

Notwithstanding the potential importance of an active NHEJ in mature oocytes, it was apparent that this reparative pathway did not noticeably rescue the developmental potential of oocytes challenged with an ETP insult. Indeed, embryos generated from ETP-treated oocytes were 6-times more likely to retain significant metaphase plate abnormalities over their untreated counterparts. Moreover, the fact that such irregularities persisted throughout the entire six-hour recovery time suggests that spindle organisation may be refractory to repair and thus contribute to the reduction in developmental capacity of these embryos. An alternative explanation to account for the relative inefficacy of NHEJ in rescuing developmental competence, is that a primary function of the MII oocyte's DNA surveillance/repair proteins (not restricted to NHEJ) may be the resolution of DNA damage harboured by the fertilising spermatozoon. Indeed, this model has recently been advanced to account for the action of the base excision repair (BER) pathway in mouse MII oocytes[45].

Irrespective, the finding that ETP-treated oocytes were unable to progress through embryonic development despite harbouring an active NHEJ pathway, prompted us to explore alternative therapeutic interventions to prevent, rather than repair, the associated DNA damage. For this purpose, we assessed the efficacy of co-administration of 2 compounds, N-acetylcysteine (NAC) and sodium salicylate (SS), both of which mitigate the effect chemotherapeutic agents in somatic cell cancers[39–41], in terms of providing additional protection for the female germline. The thiol NAC has a broad array of activities[42], including that of an antioxidant; a property that reflects its ability to act as a potent nucleophile. Such activity is of interest in the context of the current study owing to evidence that ETP can elicit a reactive oxygen species (ROS) response following introduction to various tumorigenic cells[58]. However, despite its promise, NAC supplementation failed to significantly reduce the sensitivity of ETP treated MII oocytes to DNA damage; a finding that accords with the failure of ETP to elicit an appreciable ROS response in our experimental regimen. The second compound tested, SS is a catalytic inhibitor of topoisomerase IIα with the potential to block genotoxic agent induced topoisomerase IIα-DNA cleavable complex formation[39]. Since ETP, DOX and PHL have been purported to also rely on the formation of drug stabilised topoisomerase IIα-DNA complexes to elicit DNA damage[44], it was reasoned that SS could mitigate the deleterious impact of genotoxic insult on the MII oocyte. Indeed, co-administration of SS with ETP, PHL and DOX proved effective in preventing the induction of genomic instability.

Conclusion

In summary, our data provide the first evidence that the MII oocyte has the potential to conduct DNA repair via NHEJ. Notably, this repair capacity did not prove successful in mitigating the deleterious secondary consequences of genotoxic exposure (i.e. metaphase plate abnormalities), suggesting that there exists a threshold of DNA damage beyond which this pathway becomes insensitive. Nonetheless, our findings provide an intriguing line of inquiry for defining the role of NHEJ as a repair platform for correcting damage of the maternal, and possibly more importantly, the paternal DNA at the moment of fertilisation. Our data also provide the impetus to explore the beneficial effects of co-administration of sodium salicylate, as a means of protecting the female germline during chemotherapeutic interventions.

Materials and Methods

Materials. The reagents used during this study were purchased from Sigma Aldrich (St Louis, MO, USA) and all antibodies were from Abcam (Cambridge, UK) unless otherwise stated. Anti-γH2A.X antibodies (SC101696) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA) and anti-XRCC5/KU80 (16389-1-AP) from Proteintech (Rosemont, IL, USA).

Ethics statement. All research animals in this study were handled, monitored and euthanised in accordance with NSW Animal Research Act 1998, NSW Animal Research Regulation 2010 and the Australian Code for the Care and Use of Animals for Scientific Purposes 8th Ed. as approved by the University of Newcastle Animal Care and Ethics Committee (approval number A-2012-208). C57/BL6/CBA F1 hybrid female mice were bred and held at the institutes' Central Animal House with food and water ad libitum. Animals were housed under a 12 h light/12 h dark cycle at a constant temperature of 21–22 °C and euthanised immediately before use via CO_2 asphyxiation.

Gamete retrieval. Oocytes and spermatozoa were harvested as described previously[19,38]. Briefly, our intraperitoneal injection regimen consisted of the administration of 7.5 IU equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) (Intervet, Sydney, NSW, Australia) to juvenile (3–5 week old) C57/BL6/CBA F1 female mice to induce superovulation. Thirteen to 15 h after the final injection, female mice were sacrificed via CO_2 asphyxiation before the immediate retrieval of the cumulus enclosed MII oocytes from oviductal ampullae. Oocytes were subsequently denuded by incubation in 300 μg/ml hyaluronidase at 37 °C for no more than 5 min. Any remaining cumulus cells were removed by a further 3–5 washes in M2 medium.

Oocyte treatments. To induce DSB DNA damage, MII oocytes were exposed to either 100 or 200 μg/ml concentrations of etoposide (ETP), in parallel with a vehicle (containing the equivalent highest concentration of dimethyl sulfoxide (DMSO)) and an untreated control (M2 media alone) for 15 min at 37 °C as previously described[19]. Alternative treatments included the administration of phleomycin (PLE, 9564) (10 or 50 μg/ml, 1 h), doxorubicin hydrochloride (DOX, PHR1789) (20 or 40 μM, 1 h), N-acetylcysteine (138061) or sodium salicylate (71945), along with their associated vehicle controls supplemented with the equivalent highest concentrations of H_2O and DMSO, respectively. Following treatment, oocytes were washed in M2 media and either prepared for assessment of DNA damage or for fertilisation using standard *in vitro* fertilisation (IVF) protocols, as described below.

***In vitro* fertilisation and embryo culture.** Following appropriate treatment, denuded MII stage oocytes were liberated from the cumulus matrix and washed in human tubal fluid (HTF) medium 3 times to ensure the complete removal of all ETP reagent (see above) prior to fertilisation. The oocytes were then allocated into a droplet of HTF supplemented with 1 mM reduced glutathione (GSH). Spermatozoa were recovered simultaneously from the cauda epididymides of mature (≥8 weeks) male C57/BL6/CBA F1 mice by retrograde perfusion via the vas deferens and capacitated by incubation in modified Biggers, Whitten and Whittingham (BWW) medium containing 1 mg/ml polyvinyl alcohol and 1 mg/ml methyl-beta cyclodextrin for 1 h at 37 °C under an atmosphere of 5% O_2, 6% CO_2 in N_2 as previously described[38].

Oocytes were then co-incubated with 2×10^5 capacitated spermatozoa for 4 h at 37 °C. The resultant zygotes or unfertilised oocytes were washed to remove unbound or loosely adherent spermatozoa before being assessed for markers of successful fertilisation (i.e. extrusion of the second polar body and/or pronucleus formation)[19]. In addition, oocytes which arrested at the one cell stage following IVF were assessed via labelling of the nuclear content with DAPI. This strategy enabled us to differentiate between those cells possessing pronuclei (i.e. arrested zygotes) versus those with an intact metaphase plate (i.e. non-fertilised oocytes) and thus increase the accuracy of the fertilisation rate assessment. To promote embryonic development, zygotes were cultured in GSH free HTF medium overnight, before the 2 cell embryos were transferred to G1 PLUS culture medium (Vitrolife, Göteborg, Sweden). After 4 days of culture, embryos underwent an additional transfer into G2 PLUS medium (Vitrolife)[38]. All embryos were monitored on a daily basis and developmental rates recorded. The percentage of oocytes that fertilised and reached the blastocyst stage was calculated on the morning of day 5 post-fertilisation.

Immunocytochemistry. To ensure access of antibodies to intracellular antigens, oocytes and embryos were first fixed in 3.7% (v/v) paraformaldehyde (1 h) and permeabilised in a solution of 0.25% Triton X-100 diluted in PBS for 10 min at room temperature (RT). Notably, fixation was conducted immediately after the embryos/oocytes had been washed by serial aspiration in fresh M2 medium (i.e. within a maximum of 1–2 min after completion of the appropriate treatment). All cells were then blocked in 3% BSA/PBS for 1 h at 37 °C and incubated in either anti-γH2A.X, anti-α-tubulin (1:400, A11126, Thermo Fisher Scientific, Waltham, MA, USA), anti-ATM (ab82512), anti-p-ATM (phosphor-Ser1981) (ab81292), anti-XRCC5/Ku80, anti-PCNA (ab29), anti-DNA PKcs (ab70250) or anti-p-DNA PKcs (phospho-Ser2056) (ab18192) (each diluted 1:100 in 1% BSA/PBS with the exception of anti-PCNA which was diluted 1:100 in 1% BSA/TBS) antibodies overnight at 4 °C. After primary antibody binding, oocytes and embryos were washed in 1% BSA/PBS prior to incubation with the appropriate Alexa Fluor 488 or 594 conjugated secondary antibodies (Thermo Fisher Scientific) (diluted 1:1000 in 1% BSA/PBS) at 37 °C for 1 h. Dual labelling of oocytes to facilitate morphometric assessment of the meiotic spindle, was achieved by sequential incubation in appropriate primary antibodies prior to the addition of appropriate secondary antibodies in tandem. All cell preparations were counterstained with the nuclear marker, 4′,6-diamindino-2-phenylindole (DAPI) and mounted onto Menzel Gläser microscope slides (Thermo Fisher Scientific) in antifade reagent (Prolong Gold Antifade, Thermo Fisher Scientific). The intensity of fluorescent labelling was assessed against a secondary antibody only control using microscopy images captured an AXIO Imager.A1 fluorescence microscope (Carl Zeiss Micro Imaging GmbH, Jena, Thuringia, Germany), with all imaging parameters being kept consistent between treatment groups. Briefly, microscopy images were imported into the ImageJ software program and, depending on the nature of the analysis, the 'circle selection tool' was used to accurately trace around the entire oocyte or the 'polygon selection tool' was used to restrict the analysis to the metaphase plate (defined by the DAPI counterstain); and the mean fluorescence value recorded. As a complementary approach, confocal laser scanning microscopy was utilised to determine the spatial profile of immunofluorescence signals, with all representative

images captured using the Olympus FV1000 confocal and a 60×/1.2 NA UPLSAPO oil immersion objective lens (Olympus, Australia). To enable spindle and chromosome configuration analyses, these structures were labelled with a combination of anti-α-tubulin antibody, a fluorescent phalloidin conjugate (which labels polymeric actin), and DAPI (20 min at 37 °C)[59]. The confocal Z stacking function was utilised to record the dimensions of the entire spindle from pole to pole. Finally, the images collected were again imputed into Image J and analysed using a custom spindle analysis tool/macro designed by Dr. S. Lane[60], thus facilitating calculation of spindle size and length. To avoid operator bias, these analyses were conducted in a blinded manner.

Embryonic diameter analysis. Confocal microscopy was utilised to capture an optical section through the centre of each blastomere. This image was then imported into Image J in preparation for measurement of cell diameter (as described in the above text). In line with ESHRE recommendations, a threshold of >25% difference in diameter was used to discriminate the percentage of embryos harbouring blastomeres of unequal size[52].

DNA repair. To establish whether DNA repair could occur in MII oocytes, cells were treated with ETP, washed and then provided with a 6 h recovery period in fresh M2 media. A subset of cells were removed at each time point and assessed for the extent of DSB DNA damage (γH2A.X). To provide functional evidence of DNA repair occurring via non-homologous end-joining (NHEJ), a separate subset of oocytes were subjected to co-incubation with both ETP and an agonist of NHEJ for the duration of the recovery period. In these experiments, agonists consisted of either NU7441/KU57788 (50 μM; S2638, Selleckchem, Houston, TX, USA) a potent and selective inhibitor of DNA-PKcs or SCR7 (20 μM; M60082, Xcessbio Biosciences, San Diego, CA, USA) a specific inhibitor of DNA ligase IV. Thereafter, oocytes were fixed and examined for indicators of DNA damage and metaphase plate misalignment (as previously described).

SDS-PAGE and immunoblotting. Ultra violet light treated HELA cell (ab157396) and HEK293 (ab7902) protein lysates were sourced from commercial suppliers and diluted to a working concentration of either 10 μg/ml (anti-XRCC5/Ku80, anti-PCNA and anti-ATM) or 20 μg/ml (anti-ATM, anti-DNA PKcs and anti-p-DNA PKcs (phospho-Ser2056)) with SDS-PAGE loading buffer. The protein lysates were then resolved on pre-cast polyacrylamide gels (4–12% NuPAGE Bis-Tris, Thermo Fisher Scientific), prior to transfer to either nitrocellulose or polyvinylidene difluoride (PVDF: anti-XRCC5/Ku80) membranes. Membranes were blocked with 5% LFDM (low fat dried milk) or 5% BSA (anti-ATM) for 1 h, washed in Tris-buffered saline (TBS) containing 0.1% Tween (TBST) and then sequentially incubated in the appropriate primary antibody (diluted 1:1000 in 1% BSA or LFDM in TBST) overnight at 4 °C and HRP-conjugated secondary antibodies (diluted 1:1000 in 1% BSA or LFDM or TBST) for 1 h at room temperature[61]. Membranes were developed using enhanced chemiluminescence reagents (GE Healthcare, Buckinghamshire, England UK) according to the manufacturer's instructions.

Cytosolic reactive oxygen species (ROS) measurement. Assessment of cytosolic ROS levels were conducted following ETP administration using the 5′-carboxy-2′,7′-difluorodihydrofluorescein diacetate (DCF-DA probe) (Molecular Probes, OR, USA) as previously described[37,62]. Briefly, control and ETP treated MII stage oocytes were incubated in a 10 μM solution of DCF-DA in M2 media for 30 min at 37 °C. Oocytes were then washed 5 times before mounting and analysis by fluorescence microscopy[37,62].

Statistical Analysis. Densitometric analysis and quantification of fluorescence levels within oocytes and pronuclear stage zygotes was achieved using the public-sector image processing program, ImageJ (National Institute of Health, Maryland, USA). Statistical significance was determined using ANOVA, Tukey–Kramer HSD and Student's t-tests employing JMP (version 13.0.0, SAS Institute, NC, USA) and Excel software (Version 15.32, Microsoft, Washington, US). Differences with a value of $P < 0.05$ were considered to be statistically significant. Each experiment was conducted on a minimum of three biological replicates. All data are expressed as mean ± S.E.M.

Data Availability. All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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Author Contributions

J.H.M., B.N., E.G.B. and R.J.A., conceived the study and designed the experimental approach. Data analysis, experimentation and manuscript preparation was performed by J.H.M. Manuscript editing was conducted by E.G.B., B.N., T.L. and R.J.A.

Additional Information

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Competing Interests: The authors declare no competing interests.

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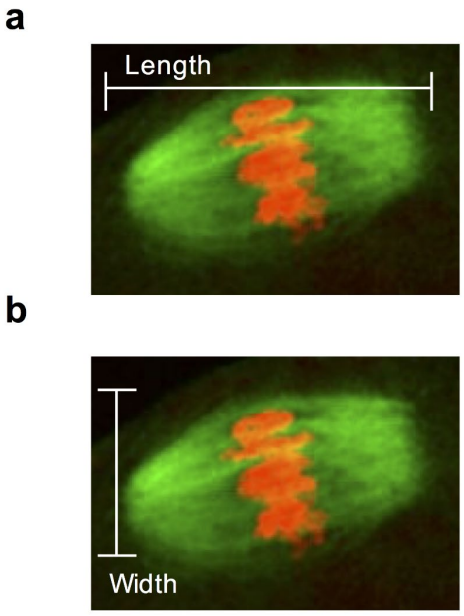
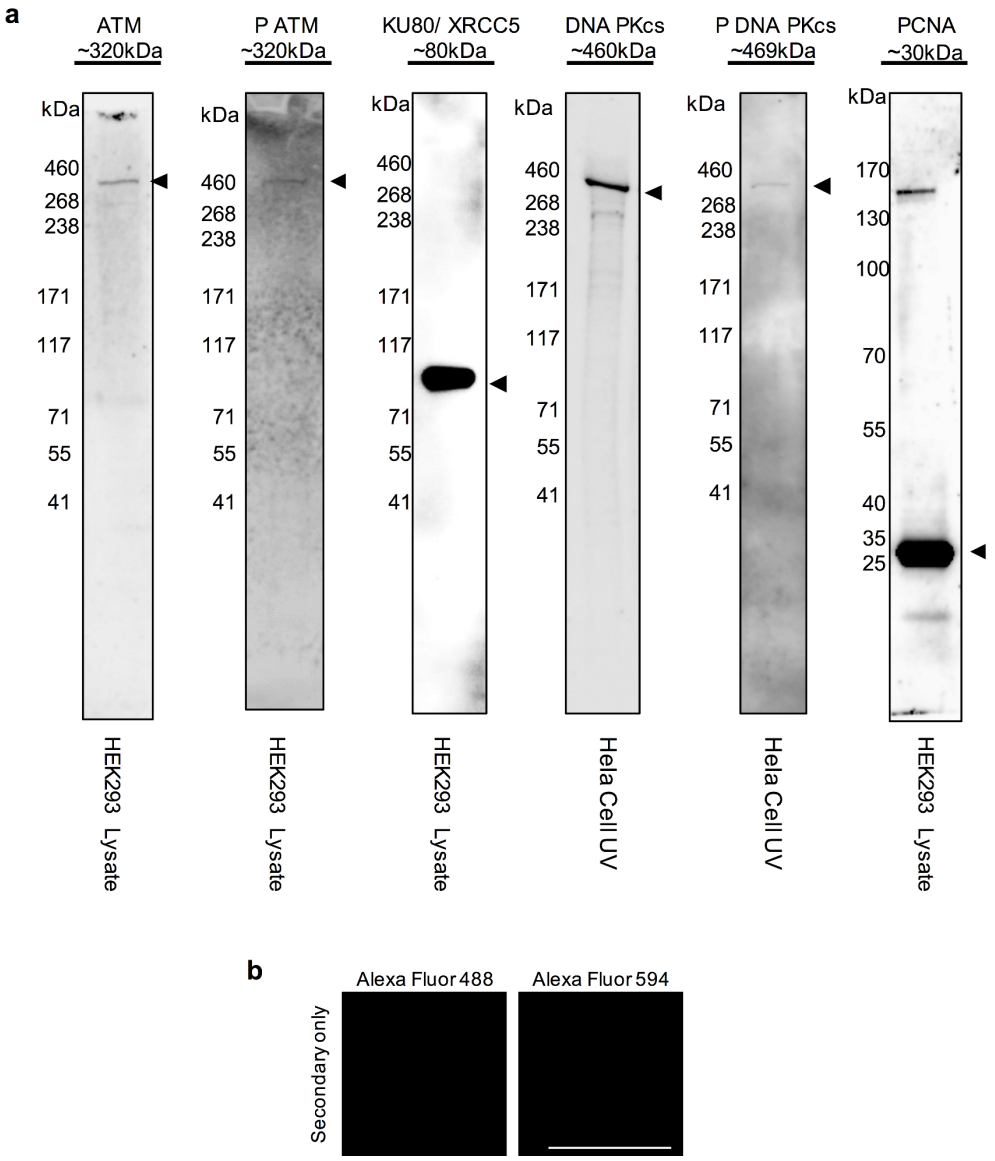
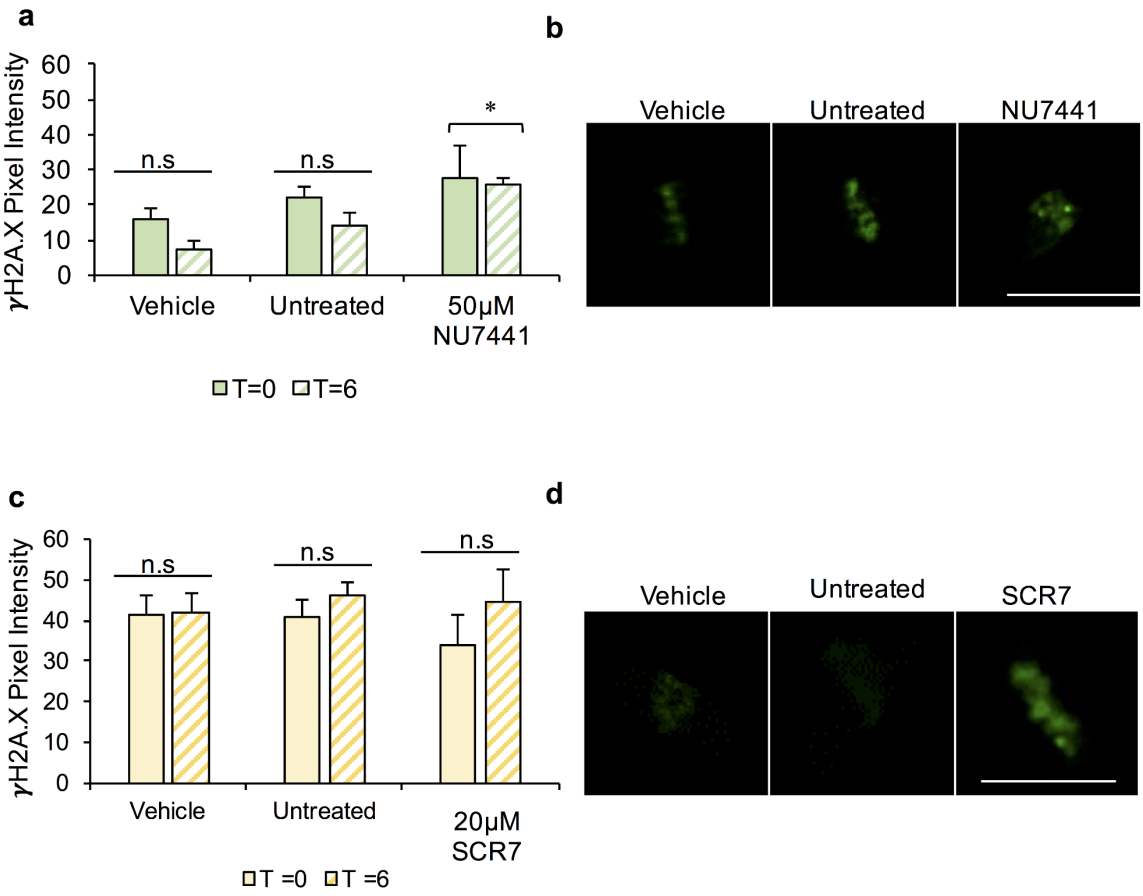


Supplementary Figure. 1: Measurement of the MII spindle barrel. Confocal microscopy, Image J image processing software in conjunction with a macro designed by Dr. Simon Lane (Lane and Jones, 2013) was used to determine the length and width of the MII spindle barrel.



Supplementary Figure 2: Validation of DNA repair antibodies by western blotting and immunocytochemistry. The specificity of the labelling pattern identified in Figure 6 were confirmed first by inclusion of a secondary antibody labelling control which revealed no significant background staining, and secondly by western blotting using the recommended commercially available positive control cell lysates (UV treated Hela cells and HEK293). For each antibody, a single dominant band was evident of appropriate molecular weight (Ataxia Telangiectasia Mutated (ATM; 350kDa), DNA-dependent protein kinase catalytic subunit (DNA PKcs and DNA PKcs (p-Ser2056); ~460 and 469kDa), X-ray repair complementing defective repair in Chinese hamster cells (KU80/XRCC5; 80kDa), and proliferating cell nuclear antigen (PCNA; 29kDa)). Scale bar = 80 μm.



Supplementary Figure 3: Experimental controls for the Pharmacological inhibition of Non Homologous End Joining (NHEJ) repair activity. Selective inhibition of DNA-PKcs by 50 μM NU7441 and DNA ligase IV by 20 μM SCR7 indicated no significant different in γH2A.X fluorescent labelling of the vehicle untreated and SCR7 only controls between the initial time point and 6 hours post treatment. There was a slight, albeit significant, decrease in γH2A.X fluorescent labelling in the NU7441 control treatment group. Statistical significance was determined using ANOVA and students t-tests. $*p \leq 0.05$. Each experiment was conducted on a minimum of three biological replicates, with each replicate comprising a minimum of 30 oocytes. All data are expressed as means ± S.E.M. Scale bar = 80 μm.

CHAPTER 4: ORIGINAL RESEARCH ARTICLE

Investigation into the involvement of proinsulin C-peptide in mammalian oocyte function

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Authors: Martin, J. H., Aitken, R.J., Bromfield, E.G., Cafe, S.L., Frost, E.R., Sutherland, J.M., Nixon, B., and Lord, T[2]. (2018).

Affiliations*:* [1]Priority Research Centre for Reproductive Biology, School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia.

[2]School of Molecular Biosciences, Centre for Reproductive Biology, College of Veterinary Medicine, Washington State University, Pullman, WA, 99164, USA.

Correspondence: Jacinta.martin@newcastle.edu.au

CHAPTER OVERVIEW

In the studies documented in the previous chapters, we have demonstrated that the oocyte and preimplantation embryo contain a sophisticated suite of defense strategies for the detection, repair or prevention of DNA damage. Given these findings, we sought to investigate whether the efficacy of these defenses may be augmented by pro-survival factors (Chapter 4). Specifically, we opted to study the involvement of insulin and proinsulin C-peptide in the context of oocyte biology.

C-peptide is a 31 amino acid 'connecting' peptide responsible for linking the A and B chains of the immature insulin molecule. The impetus to explore such a molecule rests with encouraging findings following therapeutic administration of C-peptide to diabetic patients and experimental animal models. In this manner, C-peptide directs the activation of several transcription factors of importance for cell protection including activation of the pro-survival factors Na+/K+-ATPase, phosphatidylinositol-4,5-bisphosphate 3 (PI 3)- kinases and stimulation of mitogen-activated protein kinases (MAPKs). Such findings represent an important insight when considered in the context of diabetic women who are known to be particularly susceptible to reproductive problems, including an increased risk of miscarriage, congenital malformations and other significant abnormalities in their offspring, which are directly associated with poor oocyte quality.

For the purpose of this final study, we sought to explore the localisation of C-peptide in the female germline and investigated whether C-peptide, or insulin, exert direct physiological effects within the female reproductive system. Through this work we present the first evidence for an abundance of C-peptide within the mouse ovary,

oocyte and follicular fluid. In addition, we report that the oocyte expresses the putative C-peptide receptor, GPR146, and exhibits the capacity to both internalize and utilize exogenous C-peptide upon initiation of the GV to MII transition. Additionally, we observed a pronounced redistribution of the subcellular localization of C-peptide following fertilization, a change that closely mirrored that of the DNA repair enzyme, breast cancer type 2 susceptibility protein (BRCA2). The putative interaction between C-peptide and BRCA2 was strengthened on the basis of co-immunoprecipitation and mass spectrometry analysis. In view of these findings, we propose that C-peptide may warrant consideration as a novel additive to culture media in order to support oocyte quality during *in vitro* manipulation.

TITLE: Investigation into the presence and functional significance of proinsulin C-peptide in the female germline.

RUNNING TITLE: Functional significance of proinsulin C-peptide in the oocyte.

SUMMARY SENTENCE: Proinsulin C-peptide is internalized by the mouse oocyte and thereafter may play a key role in folliculogenesis and embryogenesis.

KEY WORDS: Proinsulin C-peptide; Insulin; Ovary; Oocyte; In vitro fertilization (IVF); In vitro maturation (IVM); BRCA2

AUTHORS: Jacinta. H Martin[1, *], R. John Aitken[1], Elizabeth G. Bromfield[1], Shenae L. Cafe[1], Jessie M. Sutherland[1,], Emily R. Frost[1,], Brett Nixon[1 #] and Tessa Lord[1,2,#]

AUTHOR AFFLIATIONS:

[1]Priority Research Centre for Reproductive Science, Discipline of Biological Sciences. The Hunter Medical Research Institute, New Lambton Heights and the University of Newcastle, Callaghan, NSW 2308, Australia.

[2]School of Molecular Biosciences, Centre for Reproductive Biology, College of Veterinary Medicine, Washington State University, Pullman, WA, USA, 99164.

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[1] Senior authors Tessa Lord and Brett Nixon contributed equally to this manuscript

[2] **Correspondence:** Jacinta H Martin,
Priority Research Centre for Reproductive Science, School of Environmental and Life Sciences, the University of Newcastle, Callaghan, NSW 2308, Australia.
Email: Jacinta.martin@newcastle.edu.au

ABSTRACT

Diabetes is associated with poor oocyte quality and the dysregulation of ovarian function and is thus a leading contributor to the increasing prevalence of female reproductive pathologies. Accordingly, it is well-established that insulin fulfils a key role in the regulation of several facets of female reproduction. What remains less certain is whether proinsulin C-peptide, which has recently been implicated in cellular signaling cascades, holds any functional role in the female germline. In the present study, we examined the expression of insulin, C-peptide and its purported receptor; GPR146, within the mouse ovary and oocyte. Our data establishes the presence of abundant C-peptide within follicular fluid and raise the prospect that this bioactive peptide is internalized by oocytes in a G protein coupled receptor (GPCR)-dependent manner. Further, our data reveal that internalized C-peptide undergoes pronounced subcellular re-localization from the ooplasm to the pronuclei post-fertilization. In exploring potential binding partners for C-peptide in fertilized oocytes, immunoprecipitation analysis and mass spectrometry identified breast cancer type 2 susceptibility protein (BRCA2), the meiotic resumption/DNA repair protein, as a primary binding partner for C-peptide within the oocyte. Collectively, these findings establish a novel accumulation profile for C-peptide in the female germline and provide the first evidence for an interaction between C-peptide and BRCA2. This interaction is particularly intriguing when considering the propensity for oocytes from diabetic women to experience aberrant meiotic resumption and perturbation of traditional DNA repair processes. This therefore provides a clear imperative for further investigation of the implications of dysregulated C-peptide production in these individuals.

INTRODUCTION

Synthesized by the β cells of the pancreatic islets, proinsulin is cleaved into two products; the biologically active insulin and an entity known as C-peptide. The latter is a 31

amino acid peptide with a precursor role in connecting the A and B chains of the immature insulin molecule [1, 2]. Subsequent to sequential cleavage events, equimolar quantities of insulin and C-peptide are secreted from the pancreas into the circulation where insulin is known to enact its essential role in glucose metabolism. Insulin also influences several developmental processes important for female fertility and reproduction, such as folliculogenesis [3-5] and embryonic development [6-8]. By contrast, a role for C-peptide in female reproduction has not yet been established.

Until recently, C-peptide had been considered a biologically inactive by-product of insulin biosynthesis, required only to facilitate insulin folding and formation of interchain disulfide bridges [1, 2]. Contrary to this, it is now known that C-peptide is, in its own right, capable of binding to cell membranes and effecting intracellular signaling via direct internalization or interaction with G protein coupled receptors (GPCR) and Ca^{2+} -dependent pathways [reviewed in [9]]. In this way, C- peptide has been shown to activate and increase the expression of endothelial nitric oxide synthase, Na+, K+ ATPase and several transcription factors of importance for anti-oxidant and alternative cell protective mechanisms [reviewed in [10, 11]]. In this context, the exogenous application of C-peptide has been explored as a potential therapeutic approach for the treatment of diabetes-associated dysfunction [12-16]. Encouragingly, considerable success has been attained with long and short term remedial administration of C-peptide. In these studies, short term administration of C-peptide at physiological concentrations in patients and experimental animal models led to significant improvements in renal function, glucose utilization, blood flow, and autonomic nerve function [16-19]. Further, prolonged C-peptide administration (i.e. greater than 1 month) resulted not only in improved renal function, but also ameliorated the severe autonomic and sensory nerve dysfunction that are symptomatic of diabetes [14, 20].

In the oocyte, insulin signaling and responsiveness is facilitated by the action of gonadotropins [21]. Physiological levels of insulin in the embryo have been shown to have significant positive effects on protein synthesis, cellular proliferation, and nucleic acid synthesis [6, 22-24]. In contrast, prolonged exposure to insulin, as might be experienced during hyperinsulinemia, appears to have detrimental effects on meiotic chromatin remodeling, via the induction of condensation errors, resulting in compromised embryonic developmental competence [5, 25]. It is perhaps not surprising that diabetic women, who may present with dysregulated insulin and C-peptide levels, are known to be particularly susceptible to reproductive problems, including an increased risk of miscarriage, congenital malformations and other significant abnormalities in their offspring [26-28]. This decline in reproductive potential appears to be directly linked to poor oocyte quality [29], as zygotes transferred from diabetic mice to non-diabetic surrogates continue to display impairments in embryogenesis [30, 31]. Accordingly, oocytes from diabetic mouse models exhibit delayed meiotic progression [32], mitochondrial dysfunction, and impaired communication with surrounding cumulus cells [reviewed by Wang and Moley [29]].

In view of the key role of insulin in female reproduction and the breadth of functions C- peptide is now known to mediate, we propose that C-peptide exerts positive physiological effects within the female reproductive system. To address this hypothesis, the current study utilized a C57BL6 × CBA F1 mouse line in tandem with immunocytochemistry, quantitative real time PCR (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA) techniques to establish the presence, and explore the origin of C-peptide and insulin within the ovary, oocyte and follicular fluid. Moreover, to begin to ascertain the role of C-peptide during oocyte maturation we used a combination of immunoprecipitation and proximity ligation assays (PLA) to identify key binding partners for C-peptide in preimplantation embryos.

MATERIALS AND METHODS

Ethics

The research animals used in this study were monitored, handled, and euthanized in accordance with the NSW Animal Research Act 1998, NSW Animal Research Regulation 2010 and the Australian Code for the Care and Use of Animals for Scientific Purposes 8th Edition as approved by the University of Newcastle Animal Care and Ethics Committee (approval number A-2012-208). C57/BL6/CBA F1 hybrid female mice were bred and housed at the institute's Central Animal House under a 12 h light/12 h dark cycle at a constant temperature of 21–22 °C with food and water ad libitum. Animals were euthanized immediately before use via CO_2 asphyxiation.

Materials

All chemicals used within this study were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated. All antibodies were supplied by Abcam (Cambridge, UK), with the exception of anti-insulin H86 (SC-9168) anti-GPR146 (SC-104284) and anti-BRCA2 (SC-1819) which were purchased from Santa Cruz Biotechnology (Dallas, TX, USA), and anti-phosphorylated AKT1 (124001) purchased from Calbiochem (Darmsadt, Germany). Information about antibody optimization is provided in Supplementary Table S1.

Ovary and Oocyte collection

Oocytes were harvested from 3-4 week old C57BL6 × CBA F1 female mice following an intraperitoneal injection regime of equine Chorionic Gonadotropin (eCG) (Intervet, Sydney, Australia), followed, 48 h later, by human Chorionic Gonadotropin (hCG) (Intervet) to induce superovulation. Germinal vesicle (GV) stage oocytes were recovered 48 h following eCG injection and metaphase two (MII) stage oocytes retrieved 12-14 h following hCG injection,

as described previously [33]. MII oocytes were identified by the presence of a single polar body, and the absence of a germinal vesicle. Ovaries collected for immunohistochemistry were also retrieved from 3-4 week old C57BL6 × CBA F1 females and were immediately fixed in Bouins solution, followed by paraffin embedding and serial sectioning [34].

Granulosa cell isolation and purification

Granulosa cells were isolated from the ovaries of 10 × post-natal day 7 mice. Following retrieval, the ovary was dissociated by incubated in DMEM/F12 media containing 0.02% type II collagenase (Roche Diagnostics, Castle Hill, Australia) and 0.02% DNAse I (Roche) for 1 h at 37°C. The cell suspension was then centrifuged (1,500 × g) for 3 min and the media replaced with 1 mL 0.05% trypsin-EDTA (Thermo Fisher Scientific, MA, USA) in PBS for 20 min. Trypsin activity was then quenched with the addition of 100 µL fetal bovine serum (FBS) and the cells centrifuged (1,500 × g) for a further 3 min before resuspension in complete ovarian culture medium (10% FCS, 0.2% BSA, 2% penicillin-streptomycin, 1% insulin transferrin-selenium, 200 mM L-glutamine in DMEM/F12 HAM media). Finally, the cell suspension was seeded into a 6 well culture plate (Sarstedt, Nümbrecht, Germany), granulosa cells were cultured overnight to enable adhesion, after which the cells were harvested, centrifuged and snap frozen prior to use. A purity of 90% was confirmed by immunostaining for known granulosa cell markers forkhead box L2 (FOXL2) and GATA Binding Protein 4 (GATA4) [35, 36].

In vitro maturation and C-peptide treatment

Isolation of mature GV oocytes was conducted as previously described [37]. Briefly, 48 h following eCG injection, ovaries were harvested and pre-ovulatory follicles were repeatedly punctured by a 27 -gauge needle to release mature cumulus-oocyte-complexes

149 (COCs) into MEM α media supplemented with 2.5 μM milrinone to maintain GV arrest.
150 Oocytes were maintained under mineral oil at all times. Only oocytes with an intact layer of
151 cumulus cells were recovered and the mechanical removal of all cumulus cells was achieved
152 via repeated aspiration with a narrow pipette at 37 °C.
153
154 For in vitro maturation (IVM), oocytes were washed free of milrinone by aspiration
155 through four 50 μl droplets of MEM α medium (Thermo Fisher Scientific) supplemented with
156 20% (v/v) fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin pre equilibrated at
157 37˚C under an atmosphere of 5% O_2, 6% CO_2 in N_2, before a 16 h incubation in 100 μl of MEM
158 α with or without 10 nM C-peptide; thus generating a pool of MII stage oocytes that were
159 matured with and without the addition of exogenous C-peptide. Following IVM, oocyte
160 maturation was scored. GV oocytes were identified by the presence of a nuclear envelope and
161 nucleolus, MI oocytes by the absence of the nuclear envelope and nucleolus, MII oocytes
162 identified via the presence of the first polar body, and degenerative oocytes identified via
163 cytoplasmic fragmentation [38, 39].
164
165 To investigate the role of the G-protein coupled receptor, GPR146, in the uptake of C-
166 peptide during oocyte maturation, GV stage oocytes were collected into milrinone
167 supplemented MEM α media. Prior to IVM, oocytes were further supplemented with 10 μg of
168 anti-GPR146 antibody (1 h at 37 ˚C), washed free of milrinone and then matured for 16 h in
169 100 μl of MEM α either in the presence of absence of 10 nM C-peptide. Finally, oocyte
170 maturation was scored and the uptake of C-peptide assessed by immunocytochemistry.
171
172 **In vitro fertilization and artificial activation of oocytes**

In vitro fertilization was conducted as described previously [40, 41]. Briefly, COCs were liberated from the oviductal ampullae and washed in human tubal fluid (HTF) medium 3 ×. COCs were then allocated into a droplet of HTF supplemented with 1 mM reduced glutathione (GSH). Spermatozoa were recovered simultaneously from the cauda epididymides of mature (≥ 8 weeks) Swiss male mice by retrograde perfusion via the vas deferens. Spermatozoa were capacitated by incubation in modified Biggers, Whitten and Whittingham (BWW) medium containing 1 mg/ml polyvinyl alcohol and 1 mg/ml methyl-beta cyclodextrin for 1 h at 37˚C under an atmosphere of 5% O_2, 6% CO_2 in N_2 prior to co-incubated with equilibrated COCs for 4 h at 37˚C.

Alternatively, to promote parthenogenetic activation, oocytes were denuded by incubation in 300 μg/ml hyaluronidase at 37 °C, then held in calcium free KSOM medium containing 10 mM strontium chloride ($SrCl_2$) for 4 h at 37 °C under an atmosphere of 5% O_2, 6% CO_2 in N_2 [41]. Successful fertilization/oocyte activation was determined by identifying pronuclei formation and extrusion of the second polar body. Presumptive zygotes were assessed for signs of successful fertilization (or activation in the case of parthenotes) by recording the extrusion of the second polar body and/or pronucleus formation.

Quantitative Real Time Polymerase chain reaction (qRT-PCR)

Total RNA was extracted from purified populations of granulosa cells and oocytes as well as tissue samples taken from the pancreas and ovary using the TaqMan Small RNA Assay Cells-to-Cell Kit (Thermo Fisher Scientific). RNA extraction was conducted according to the manufacturer's instructions before being incubated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) to remove genomic contamination. Quantitative RT-PCR was performed using a Light Cycler 96 SW 1.1 (Roche Diagnostics) and GoTaq qPCR Master Mix (Promega),

as per the manufacturer's instructions. Reactions were performed in triplicate, and each data set contained 3 individual cell isolations. The primers employed, listed in the 5' to 3' direction, were: Insulin: forward: ACTCGGCCAGTGAGTGCTGC and reverse: GGTGGCTGTCACATTCCCCACC. GPR146: forward: TTGGCCTCTGGACACCTTAC and reverse: TTGAGAAGGCCAGGAACTTG. In addition, cyclophilin: forward CGTCTCCTTCGAGCTGTTT and reverse ACCCTGGCACATGAATCCT primers were used as an internal standard. Importantly, cyclophilin primers shared efficiencies similar to those of our genes of interest. All data were normalized by using the ΔCt method and displayed as relative expression (2e-DT).

Immunohistochemistry

For immunohistochemistry, slides were deparaffinized using serial xylene washes, and subsequently rehydrated using ethanol. Heat-induced antigen retrieval was achieved over 10 minutes using a Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20, pH 9.0) for the anti-insulin antibody or a 10 mM Tris buffer for anti-C-peptide (ab14181). Ovarian sections were blocked using 3% bovine serum albumin (BSA) in Tris buffered saline (TBS) for 1 h at room temperature (RT). Primary antibodies were diluted 1/50 with 1% BSA/TBS and co-incubated with ovary sections overnight at 4˚C (Supplementary Table S1). A negative control in which the primary antibody was omitted and substituted for 1% BSA/TBS alone was also incubated overnight at 4˚C. Following three washes in TBS, slides were incubated in Alexa Fluor 488 (Thermo Fisher Scientific) at a dilution of 1/200 in 1% BSA/TBS for 1 h at RT, washed 3 × in TBS and counterstained with 4′-6-diamidino-2-phenylindole (DAPI). Sections were preserved in Mowiol containing 1,4-diazabicyclo[2.2.2]octane (DABCO) under a glass coverslip. Slides were analyzed using an AXIO Imager.A1 fluorescence microscope (Carl Zeiss Micro Imaging GmbH, Jena, Thuringia, Germany).

223

224 **Immunocytochemistry**

225 Immunocytochemistry was conducted on oocytes as described previously [40]. The
226 following primary antibodies were utilized; anti-insulin, anti-C-peptide, anti-GPR146, anti-
227 phosphorylated AKT1, anti-phosphorylated PI3K (Y467 + Y199) (ab63566) and anti-BRCA2.
228 Each antibody was used at a dilution of 1/100 in 1% BSA in phosphate buffered saline (PBS)
229 and was incubated with oocytes overnight at 4˚C. This step was followed by a 1 h incubation
230 in Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 594 goat anti-rabbit secondary antibodies
231 (Molecular Probes, Carlsbad, CA, USA) or Alexa Fluor 488 donkey anti-goat (for anti-
232 GPR146) at a dilution of 1/1000 in 1% BSA/PBS at 37˚C. All cell preparations were
233 counterstained with the nuclear marker, DAPI and mounted onto Menzel Gläser microscope
234 slides (Thermo Fisher Scientific) in antifade reagent (Prolong Gold Antifade, Thermo Fisher
235 Scientific). The intensity of fluorescent labeling was assessed against a secondary antibody
236 only control using microscopy images captured on an AXIO Imager A1 fluorescence
237 microscope (Carl Zeiss Micro Imaging GmbH), with all imaging parameters kept consistent
238 between treatment groups. Finally, as a complementary approach, confocal laser scanning
239 microscopy was utilized to determine the spatial profile of immunofluorescence signals and to
240 capture all representative images. Confocal microscopy was conducted using an Olympus
241 FV1000 and a 60×/1.2 NA UPLSAPO oil immersion objective lens (Olympus, Notting Hill,
242 VIC, Australia).

243

244 **Cellular Vitality**

245 The cytotoxicity of exogenous C-peptide administration was evaluated using a standard
246 trypan blue dye exclusion assay (T8154). Prior to fixation, oocytes were incubated with the
247 vitality reagent (0.08% v/v) for 10 - 15 minutes at 37 °C. Subsequently, oocytes were washed

3 × in 3 mg/ml polyvinylpyrrolidone in PBS (PVP/PBS) and scored for vitality. An oocyte with a clear cytoplasm was considered viable and those with a blue cytoplasm were considered nonviable.

Detection of extracellular insulin and C-peptide

To detect endogenous insulin and C-peptide levels in follicular fluid, a rat insulin enzyme immunoassay kit (SPI Bio, Montigny Le Bretonneux, France) that accurately detects insulin levels within an approximate range of 0.05 – 10 ng/mL, and a human/mouse/rat C-peptide enzyme immunoassay kit (RayBiotech, Norcross, GA, USA) that detects C-peptide within the range of 0.1 – 1000 ng/mL, were utilized as per the manufacturer's instructions. Recommended controls were performed alongside each replicate of the assays. For collection of follicular fluid, equine ovaries were obtained from a local abattoir and transported on ice. Mature follicles (> 10 mm in size) were punctured with a 24-gauge needle, and the enclosed follicular fluid aspirated, filtered (0.22 µm pore size) and stored at -20˚C until required.

Detection of apoptosis

A terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (Roche Diagnostics) was employed to detect apoptosis in oocytes following extended in vitro culture, with and without the addition of exogenous C-peptide. After fixation and permeabilization, a subset of the cells were treated with 3 µl DNase solution (Promega) as a positive control, while additional oocyte populations served as 'minus label' and 'minus enzyme' negative controls. All cells were incubated with the TUNEL stain master mix as per manufacturer's instructions, washed, and mounted immediately for assessment by fluorescence microscopy. Cells were scored as either TUNEL positive or negative, based on the presence/absence of immunofluorescence.

Immunoprecipitation, immunoblotting and mass spectrometry

Immunoprecipitation was used to identify intracellular binding partners of C-peptide. These protocols were conducted as described previously in Bromfield et al. (2015). Briefly, oocyte protein was extracted using a modified CHAPS lysis buffer (10% glycerin, 10 mM CHAPS, 10 mM HEPES in distilled water) via a 2 h incubation at 4 °C with constant rotation. Simultaneously, 10 µg of anti-C-peptide antibody was cross-linked to Protein G Dynabeads (Thermo Fisher Scientific) using 3,3-dithiobis-sulfosuccinimidyl propionate (DTSSP- 2 mM), as per manufacturer's instructions. Following this, dual incubation of anti-C-peptide-bound Dynabeads with 2 µg of protein lysate (extracted from approximately 200 oocytes previously precleared of nonspecific binding through incubation with unbound Protein G Dynabeads at 4 °C for 1 h) was conducted overnight, as above. Finally, antibody-antigen bound beads were washed in PBS before protein elution was achieved via incubation in SDS loading buffer (10% w/v SDS, 10% w/v sucrose in 0.375 M Tris, pH 6.8, containing a protease inhibitor cocktail (Roche Diagnostics) at 100 °C for 5 minutes. Eluted protein and the following controls were loaded onto NuSep 4–20% Tris–glycine gel (NuSep, Bogart, GA, USA): 0.5 µg C-peptide antibody in H_2O (antibody only), protein lysate prior to preclearing (lysate control), precleared elution and Dynabeads-only, elution from preclearing Dynabeads (not crosslinked with antibody) (preclear), and a 'wash control' containing lysis only buffer.

Following resolution, immunoprecipitated proteins were visualized via a 30 minute incubation with Coomassie blue (0.1% brilliant blue, 45% methanol, 5% glacial acetic acid in dH_20) followed by an overnight incubation in Coomassie de-stain solution (20% ethanol, 5% glacial acetic acid in dH_2O). Dominant elution products were excised from the gel and subjected to further de-staining, dehydration, and trypsin digestion in preparation for mass

298 spectrometry analysis; as described previously [42]. Briefly, Gel bands of interest were excised
299 and subjected to tryptic digestion prior to being separated by reversed-phase nano-liquid
300 chromatography (Dionex Ultimate 3000 RSLCnano, Idstein, Germany) and sequenced by
301 tandem mass spectrometry on an electrospray ionisation 3D Ion Trap Mass Spectrometer
302 (AmaZon ETD, Bruker Daltonik, Bremen, Germany). Raw MS/MS files were converted into
303 MASCOT generic format and imported into Bruker's Proteinscape platform for database
304 searching. Searches were performed using in-house licensed MASCOT server (version 2.3.02,
305 Matrix Science, London, UK), against the UniprotKB database (Mammalia, January 2015).
306 Trypsin was selected as the digestion enzyme with up to two missed cleavages permitted.
307 Peptide mass tolerances were set at 1.4 and 0.7 Da for parent and daughter fragment ions,
308 respectively. Peptide thresholds were set requiring a false-positive rate <0.05% and a
309 MASCOT score >35. Those spectra meeting these criteria were validated by manual inspection
310 to ensure accurate y- and b-ion detection with overlapping sequence coverage.
311
312 Validation of mass spectrometry-identified proteins present in the immunoprecipitation
313 eluates was conducted using standard immunoblotting techniques [43]. Illustrative of these
314 procedures, BRCA2 was confirm as co-eluting with C-peptide via probing of nitrocellulose
315 membranes overnight incubation at 4˚C with a 1/1000 dilution of primary antibody in TBS
316 containing 0.1% Tween 20 (TBST) and 1% BSA. After thorough washing of membranes in
317 TBST (3 × 10 min), they were incubated for 1 h in anti-goat HRP (diluted 1/4000 in 1%
318 BSA/TBST). Membranes were again washed in TBST (3 × 10 min) before being developed
319 using an enhanced chemiluminescence kit (GE Healthcare, Buckinghamshire, England UK)
320 according to the manufacturer’s instructions.
321
322 **Duolink proximity ligation assay (PLA)**

In-situ proximity ligation assays were conducted according to manufacturer's instructions and as described previously [44], using anti-C-peptide and anti-BRCA2 antibodies. Appropriate synthetic oligonucleotide-conjugated secondary antibodies (anti-rabbit for C-peptide and anti-goat for BRCA2) were purchased from Sigma-Aldrich. Oocytes were mounted as previously described for immunocytochemistry and visualized using confocal microscopy. In this assay, discrete red fluorescent foci form whenever the target proteins reside within a maximum distance of 40 nm; thus confirming likely protein-protein interactions.

Statistical analysis

Densitometric analysis and quantification of fluorescence levels within oocytes and pronuclear stage zygotes was achieved using the public sector image processing program, Image J (Version 1.8.0, National Institute of Health, Maryland, USA). Statistical significance was determined using ANOVA, Tukey–Kramer HSD and students t-tests employing JMP (version 13.0.0, SAS Institute, NC, USA). Differences with a value of $P < 0.05$ were considered to be statistically significant. Each experiment was conducted on a minimum of three biological replicates and all graphical data are expressed as mean ± S.E.M.

RESULTS

Insulin and C-peptide are expressed in the mouse ovary

To begin our investigation of the role of insulin and C-peptide in the female reproductive system, quantitative RT-PCR (qRT-PCR) and immunolocalization approaches were utilized to assess gene and protein expression levels within the oocyte and ovarian tissue of the mouse (Figure 1). Contrary to what we were anticipating, the application of qRT-PCR confirmed the expression of appreciable quantities of the *proinsulin* gene (i.e. the gene responsible for producing the precursor from which both of insulin and C-peptide are

generated) in isolated populations of MII oocytes, whole ovary and granulosa cells (Figure 1A). The specificity of this assay was confirmed through the simultaneous amplification of *proinsulin* in whole mouse pancreatic tissue; with this tissue being employed as a positive control for the qRT-PCR assay. Notably, the highest relative expression of *proinsulin* was detected in pancreatic tissue, followed by that of the MII oocytes, whole ovary and finally the granulosa cells. These data raise the intriguing prospect that the female germline may be responsible for the production of an extra-pancreatic source of the insulin and C-peptide hormones, and prompted a detailed investigation of these gene products within female reproductive tissues.

Prior to use, the specificity of the anti-insulin and anti-C-peptide antibodies employed in this study was first verified via immunohistochemistry analyses on mouse pancreatic sections (Supplementary Figure S1A). As expected, intense immunofluorescence was specifically localized to the β-islet cells of the pancreas (see red arrows), with minimal background staining evident in either pancreatic or whole ovarian tissue exposed to secondary antibody only controls (Supplementary Figure S1B and C). Subsequent immunohistochemistry performed on whole mouse ovarian sections revealed the presence of insulin primarily within the oocyte, with additional signals, albeit somewhat more modest, associated with the granulosa cells (white arrow), theca cells (red arrow) and the corpus luteum (CL) (Figure 1B- each follicle is denoted by an asterisk). Specifically, insulin staining was uniformly distributed throughout the ooplasm of the immature oocytes at all follicular stages of development with the exception of the earliest primordial stage, in which insulin appeared restricted to the somatic cells of the ovary. Similarly, C-peptide was again most intensely stained within the oocyte pool distributed throughout the mouse ovary, but was also strongly labeled within the somatic granulosa and theca cells (Figure 1C). Also consistent with insulin, the only developmental time point that differed from this pattern of labeling was that of primordial

follicles, in which C-peptide was restricted to the flattened granulosa cells (white arrow) as opposed to the immature oocyte itself.

To extend these analyses, the insulin and C-peptide proteins were also localized in isolated oocytes during the later stages of their development. This analysis confirmed the presence of insulin in germinal vesicle (GV), metaphase I (MI) and ovulatory stage (MII) oocytes (Figure 1D). Notably however, both the pattern of insulin staining, and the relative abundance of the protein, vary markedly during these phases of maturation. Thus, insulin was initially characterized by intense punctate labeling distributed throughout the cytoplasm and nucleolus of the GV stage oocyte. Thereafter, germinal vesicle breakdown (GVBD) was associated with a significant ($P = 0.0028$) reduction in insulin staining and a concomitant redistribution of the protein to the periphery of MI stage oocytes. Subsequent maturation to form MII oocytes resulted in a significant recovery of insulin ($P = 0.0033$) such that it appeared at levels consistent with those documented in GV oocytes and it was again characterized by a punctate pattern of labeling throughout the oocyte. In marked contrast to insulin, quantification of immunocytochemical data for C-peptide revealed that it was present at relatively constant levels throughout the transition from GV to MII oocytes. Despite this, GV and MI stages of development featured a profile of C-peptide localization that was primarily restricted to the cell periphery, with weaker staining present in the nucleolus and in the chromosomes within the metaphase I plate (Figure 1E). Ovulation however, was accompanied by an apparent redistribution of C-peptide throughout the ooplasm of the oocyte, with intense labeling being maintained in the vicinity of the microtubules of the metaphase II spindle.

Investigating the origins of insulin and C-peptide within the mouse ovary

While our data allude to the prospect that the female germline may be responsible for extra-pancreatic production of proinsulin, this does not discount the possibility that both the

insulin and C-peptide hormones may also be sequestered into the maturing oocyte from exogenous circulation via the surrounding follicular fluid. To explore this possibility, we elected to quantify the relative amount of both hormones within follicular fluid. Unfortunately however, direct assessment of follicular fluid from the mouse ovary proved extremely challenging owing to the difficulty of obtaining a sufficient volume of fluid free of any cellular contamination. We therefore instead undertook an analysis of a heterologous source of follicular fluid isolated from antral and preovulatory follicles within the equine ovary. The application of a sensitive ELISA-based analysis of this follicular fluid revealed the presence of both insulin and C-peptide (Figure 2A). Notably however, the concentration of C-peptide we detected in equine follicular fluid far exceeded that of insulin, equating to a difference in abundance of greater than two orders of magnitude (i.e. 0.03 vs 7.9 ng/mL). Taken together with the relatively high levels of C-peptide recorded in oocytes (Figure 1), these data suggest that this by-product of insulin production may be being actively sequestered into the oocyte to fulfill a hitherto unknown functional role in the development and/or homeostasis of female germ cells.

A putative role for the G coupled protein receptor GPR146 in C-peptide uptake into mouse oocytes

The uptake of C-peptide across cellular membranes necessitates the involvement of membrane associated receptor(s), which facilitate peptide recognition and internalization. Indeed, recently published data has identified a selective receptor for C-peptide in the form of the G-protein coupled receptor, GPR146 [45]. Accordingly, the application of qRT-PCR demonstrated that *Gpr146* is expressed in isolated granulosa cells, whole mouse ovarian tissue, as well as in mature MII oocytes (Figure 2B). Of note, comparatively high expression of *Gpr146* was also detected in isolated granulosa cells. At the protein level, GPR146 was found

423 to concentrate within the plasma membrane of isolated GV, MI and MII oocytes (Figure 2C),
424 with an additional pool of diffuse labeling being recorded throughout the ooplasm.

425 To extend these observations, oocytes were incubated with, or without, exogenous C-
426 peptide during the course of a 16 h in vitro maturation (IVM) regimen (Figure 3). Notably, a
427 highly significant ($P = 0.0096$) reduction was recorded in the levels of C-peptide when
428 comparing MII oocytes matured via IVM (in the absence of C-peptide supplementation) versus
429 those matured in vivo and subsequently harvested by superovulation (Figure 3A). On the basis
430 of these data we infer that endogenous C-peptide is utilized, and not capable of being
431 replenished via intrinsic *proinsulin* expression, within the oocyte during this crucial
432 developmental window. Rather, the accumulation of C-peptide in oocytes collected following
433 superovulation supports the hypothesis that C-peptide is also being actively sequestered by the
434 oocyte whilst bathed in follicular fluid within the ovarian microenvironment. Further support
435 for this model was afforded by the application of exogenous C-peptide to oocytes during IVM,
436 a treatment that completely abrogated the significant depletion of this protein witnessed in the
437 MII oocytes cultured without C-peptide. Indeed, the IVM oocytes receiving C-peptide
438 supplementation retained levels of this hormone that were statistically indistinguishable from
439 those of super-ovulated MII oocytes ($P = 0.8745$). By contrast, none of these treatment
440 regimens elicited any alterations in the relative abundance of either insulin (Figure 3B) or
441 GPR146 (Figure 3C) in the MII oocytes. In addition, preincubation of GV stage oocytes with
442 antibodies directed against an external epitope of the GPR146 receptor [46], significantly ($P <$
443 0.001) attenuated C-peptide-uptake during *in vitro* maturation (Figure 3E).
444
445 ***Assessment of the role of insulin and C-peptide during oocyte development***
446 In view of our collective data implicating C-peptide in oocyte physiology, we next sought to
447 explore the role this protein may play during oocyte development. In guiding this analysis, we

448 note the fact that normal rates of polar body extrusion were recorded in the oocytes matured in
449 vitro in the absence of exogenous C-peptide (Figure 3D). While these data preclude the
450 possibility that C-peptide is indispensable for the GV to MII transition, they do not discount
451 the prospect that it may act as a pro-survival factor to fortify the oocyte against the rigors of
452 post-ovulatory aging. To explore this hypothesis, populations of MII oocytes were exposed to
453 both physiological (pM to low nM range) and supra-physiological levels of either insulin or C-
454 peptide [17, 47] during an 8h in vitro incubation period (Figure 4). Subsequently, the in vitro
455 aged oocytes were assessed for the phosphorylation status of the pro-survival factors
456 phosphoinositide 3–kinase (PI3K) and RAC-alpha serine/threonine-protein kinase (AKT1)
457 [48], as well as the expression of apoptosis signatures using TUNEL reagents. If C-peptide
458 were capable of influencing survival of mouse oocytes, we anticipated an up-regulation in the
459 phosphorylation of PI3K/AKT1 and a commensurate decrease in the levels of TUNEL
460 fluorescence when compared to untreated oocytes over a time frame, which has been associated
461 with deterioration of oocyte quality [49].

462 The results of this experiment indicated that physiological levels of exogenous C-
463 peptide did not appear to impact oocyte quality/survivability in vitro, with no change in the
464 phosphorylation status of either AKT1 (Figure 4A) or PI3K (Figure 4B), or overall cell vitality
465 (Figure 4C). Supra-physiological levels of C-peptide (10 000 nM) seemingly drove oocytes
466 toward an apoptotic demise ($P = 0.0063$), as documented by enhanced TUNEL staining (Figure
467 4D and E). However, physiological concentrations of C-peptide (0.01 nM) exerted no such
468 effect on the apoptotic status of these cells.

469 Since C-peptide did not appear to influence the survival of MII stage oocytes, we turned
470 our attention to examining whether the uptake of C-peptide by the developing oocyte may be
471 a preparatory event for fertilization. To explore this paradigm, we utilized
472 immunocytochemistry to track modifications in C-peptide abundance and/or sub-cellular

473 localization in the pronuclear zygote. Interestingly, distinct alterations to the spatial profile of
474 C-peptide labeling, but not that of insulin, were identified following fertilization of mouse
475 oocytes (Figure 5A). In this context, analysis of MII stage oocytes confirmed a diffuse C-
476 peptide signal throughout the ooplasm as well as in association with the plasma membrane.
477 Following fertilization however, the subcellular localization of C-peptide appeared to
478 concentrate within the vicinity of the pronuclei, a change that occurred at the expense of plasma
479 membrane labeling. Since the functionality of C-peptide has been linked with elevated
480 intracellular calcium in other cell systems [50], we anticipated that this peri-nuclear
481 translocation may have been stimulated, at least in part, by the induction of calcium oscillations
482 that accompany fertilization. To explore this association, parthenotes were generated via
483 activation of oocytes with strontium chloride; a compound known to stimulate artificial
484 calcium oscillations conducive of oocyte activation (Zhang et al., 2005). Notably, the
485 artificially activated parthenotes were also characterized by distinct peri-nuclear localization
486 of C-peptide (Figure 5B). However, the application of the intracellular calcium chelator,
487 BAPTA-AM, to suppress post-activation calcium oscillations, led to a significant reduction in
488 the re-localization of C-peptide to the pronuclei ($P = 0.011$) (Fig. 5B, C).
489
490 ***Identification of putative C-peptide binding partners in the mouse zygote***
491 In order to enhance our understanding of the role of internalized C-peptide in the oocyte
492 both prior to, and following fertilization, co-immunoprecipitation was performed on protein
493 lysates from MII stage oocytes and strontium activated zygotes to capture C-peptide interacting
494 proteins. This strategy proved successful in co-eluting a number of putative interacting partners
495 of C-peptide in both MII oocytes and parthenotes (Figure 6A), the profile of which was
496 remarkably similar. Accordingly, dominant bands appearing in the MII and parthenote
497 immunoprecipitation were selected for identification by mass spectrometry; with parthenotes

498 being selected to avoid any potential contribution from the fertilizing spermatozoon. The
499 protein identified to have the highest MASCOT score in both MII and parthenote eluates was
500 BRCA2 (Table 1); a protein involved in DNA repair [51] and meiotic resumption [52].
501 Additional proteins co-eluted with C-peptide included protein tyrosine phosphatase domain-
502 containing protein 1 (detected in both MII stage and activated oocyte eluates), as well as two
503 chloride channel proteins and an ATP synthase, which were each identified uniquely in
504 activated oocyte eluates (Table 1).

505 On the basis of these data, BRCA2, was selected for further investigation to validate its
506 interaction with C-peptide. When eluates from equivalent C-peptide co-immunoprecipitations
507 were immunoblotted with anti-BRCA2 antibodies, a corresponding band of approximately 390
508 kDa was detected near the origin of both the MII and parthenote lanes (Figure 6B, red arrows).
509 The fact that no such band was detected in any of the control lanes confirms the accuracy of
510 the BRCA2 protein as a legitimate C-peptide partner. This result was strengthened through the
511 application of immunocytochemistry, which confirmed the presence of BRCA2 in both MII
512 stage oocytes and strontium activated parthenotes. Importantly, while BRCA2 was found to be
513 uniformly distributed throughout the ooplasm of MII stage oocytes, a congregation of
514 fluorescence surrounding the pronuclei was identified in the parthenotes (Figure 6C), similar
515 to that previously described for C-peptide (Figure 5A). This association was further confirmed
516 via dual staining of oocytes using alternate fluorophores targeted to BRCA2 and C-peptide
517 primary antibodies. The overlay image clearly revealed a co-localization of BRCA2 and C-
518 peptide throughout the ooplasm but, in particular, highlighted very intense co-labeling of the
519 pronuclei of parthenotes (Figure 6D). Finally, an interaction between BRCA2 and C-peptide
520 was sought using Duolink proximity ligation technology (Figure 6E), wherein punctate red
521 fluorescence is emitted at sites in which target proteins reside within a maximum of ~40 nm of
522 each other. As anticipated, proximity ligation fluorescence was identified throughout the

ooplasm in both MII stage oocytes and parthenotes (Figure 6E; upper panels). However, when focusing on the peri-nuclear domain (Figure 6E – lower panels) only marginal fluorescence was visible within the metaphase plate prior to oocyte activation. By contrast, a prominent fluorescent signal was detected in association with the pronuclei following oocyte activation**;** thus substantiating the putative interaction between BRCA2 and C-peptide within the pronuclei.

DISCUSSION

While it is well-established that insulin is required for regulation of multiple facets of female reproduction, comparatively little information is currently available regarding the role of the C-peptide generated as a consequence of insulin processing. In the present study, we have begun to address this paucity of knowledge, providing the first evidence for the presence of abundant C-peptide within the mouse ovary and oocyte. In addition, we report considerable enrichment of C-peptide in follicular fluid, with concentrations of this peptide far exceeding that of insulin. Accordingly, we have confirmed that the oocyte expresses the selective C-peptide receptor, GPR146 [45] within its membrane, and exhibits the capacity to both internalize and utilize exogenous C-peptide upon initiation of the GV to MII transition during in vitro maturation. Strikingly, we observed a pronounced change in the subcellular localization of C-peptide following fertilization; with evidence for protein translocation from the periphery of the cell to the nucleus. Notably, this change mirrored that of the DNA repair enzyme BRCA2, which we identified as a likely binding partner of C-peptide within the mature oocyte and early embryo.

In terms of tracing the origin of C-peptide and insulin within female reproductive tissues, our data illustrate positive amplification of *proinsulin* transcripts within both the supporting somatic cells (i.e. granulosa) of the ovary and the oocyte itself. These findings add

548 to a growing body of evidence that insulin-producing cells may exist beyond the traditional
549 populations of those residing in the pancreas. Indeed, widespread insulin mRNA and protein
550 expression has been reported in the liver, adipose tissue, spleen, bone marrow, and thymus;
551 albeit in diabetic rodent models [53]. Moreover, such gene expression profiles show
552 concordance with the presence of immunoreactive insulin and C-peptide protein within mouse
553 ovarian tissue and isolated oocytes, thus theoretically supporting the possibility that proinsulin
554 synthesis could occur in the ovary of our non-diabetic mouse line. However, our collective
555 evidence also favors a complementary model whereby intrinsic production of C-peptide and
556 presumably insulin, is supplemented by the uptake of exogenous C-peptide and insulin that are
557 derived from the circulation, before becoming enriched within the ovarian microenvironment
558 and sequestered into the oocyte. Although we were not able to directly substantiate the presence
559 of C-peptide in mouse follicular fluid, assaying of equivalent fluids from the horse ovary
560 confirmed the presence of both insulin and C-peptide. Equine follicular fluid was chosen on
561 the basis of its abundance, but also due to evidence suggesting that the equine insulin/C-peptide
562 response occurs in a secretory pattern similar to that demonstrated in mice [54, 55].
563 Interestingly, the concentrations of C-peptide detected via this strategy were >200-fold higher
564 than those detected for insulin; likely attributed to the extended half-life of circulating C-
565 peptide relative to insulin [56-59]. In confirming the importance of exogenously sourced C-
566 peptide, we demonstrated that mouse oocytes experience a significant depletion of this peptide
567 during IVM. Conversely, when IVM was performed in the presence of C-peptide
568 supplementation, oocytes were able to completely maintain their basal levels of this protein.
569 Of note, no such change was witnessed in intracellular insulin concentration under identical
570 oocyte culture conditions.

571 To address potential mechanisms by which mouse oocytes could internalize C-peptide
572 from their surrounding microenvironment, we investigated the recently characterized C-

peptide receptor, GPR146 [45]. The importance of a GPCR-dependent mechanism of C-peptide uptake was first reported in Swiss 3T3 and HEK-293 cell lines [60]. In this report proinsulin C-peptide was able to internalize to the cytosol and translocate to the nucleus, as demonstrated by confocal microscopy and surface plasmon resonance measurements. Importantly these actions were shown to be sensitive to pertussis toxin, and thus the mechanism of C-peptide uptake was compatible with the actions of a membrane bound GPCR [60].

Encouragingly, GPR146 has since been identified as a putative C-peptide receptor, with corroborating evidence from a human gastric carcinoma cell line (KATOIII), the human embryonic kidney cell line (HEK293), and the human erythroleukemia cell line (TF-1) collected using a Deductive Ligand-Receptor Matching Strategy developed by Yosten et al (2013); which identifies GPCRs candidates for orphan ligands by virtue of structural homology, correlative signaling and expression analyses. This strategy was used to assemble a shortlist of C-peptide receptor candidates, among which the targeted knockdown of GPR146 proved successful in completely abrogating the C-peptide signalosome. In addition, using an antibody directed against GPR146 in KATOIII cells, the authors illustrated the co-localization of C-peptide and GPR146 within the plasma and nuclear membranes with punctate expression in the cytoplasm [45]. Accordingly, we provide novel data supporting both the gene expression and presence of immunoreactive GPR146 protein in mouse oocytes. Whilst the protein was detected throughout the ooplasm, we too recorded the strongest fluorescent foci around the periphery of the cell, presumably corresponding to the plasma membrane. Further support for this model was afforded by the application of anti-GPR146 antibodies directed against an external epitope of the receptor [46], which significantly reduced exogenous C-peptide-uptake during IVM. Importantly, preincubation of oocytes with these antibodies had no effect on their vitality or ability to complete IVM as assessed via rates of polar body extrusion (data not shown). Aside from the oocyte, we also noted particularly abundant expression of the *Gpr146*

598 transcript within isolated populations of granulosa cells. Although we did not have the
599 opportunity to pursue the functional significance of this finding, it nevertheless adds to a
600 growing body of evidence that the maintenance and growth of the oocyte is coordinated by
601 bidirectional intrafollicular signalling between the germline and its somatic counterparts [61]

602 Aside from a putative interaction with GPR146 leading to internalization of C-peptide
603 within the oocyte, we cannot discount the possibility that this peptide may interact with
604 alternative receptors within the oolemma, eliciting signal transduction pathways independent
605 of those orchestrated by the intracellular pool of C-peptide. Support for this mechanism rests
606 with the strong accumulation of C-peptide within the plasma membrane of MII stage oocytes.
607 Accordingly, C-peptide has been shown to bind with high stereospecific affinity to the cell
608 membranes of endothelial cells, renal tubular cells, and fibroblasts with no evidence of
609 accompanying cross-reactivity with insulin, proinsulin or insulin growth factors I and II [62].
610 In this context, binding of C-peptide to cell membranes is believed to stimulate Ca^{2+}-dependent
611 signaling pathways, leading to downstream modulation of Na^{+}-K^{+}-ATPase [63-65] and nitric
612 oxide synthase activities [66, 67]. Of these known targets of C-peptide, the involvement of
613 Ca^{2+}-dependent signaling pathways in developmentally crucial reproductive events such as
614 ovulation, meiotic maturation, cortical granule exocytosis, de-condensation of the sperm
615 nucleus, recruitment of maternal mRNAs, and pronuclear development is very well known
616 [Reviewed in [68]]. Collectively the identification of C-peptide cellular internalization,
617 intracellular binding proteins, absence of rapid C-peptide degradation and apparent nuclear
618 internalization support a maintained activity similar to that of an intracrine peptide hormone.

619 In investigating the biological significance of the abundant C-peptide documented in
620 both follicular fluid and within the oocyte itself, a role in the stimulation of pro-survival
621 pathways was an attractive explanation as seen in KATOIII, HEK293 and TF-1 cell lines;
622 however, our preliminary evidence failed to support this scenario. As a cautionary note

623 however, we cannot completely exclude this possibility considering oocytes utilized in these
624 supplementation studies had received prior exposure to C-peptide during earlier developmental
625 stages in the ovarian microenvironment. Future investigations using a diabetic mouse model
626 would provide critical insight into this response in the complete absence of circulating C-
627 peptide. Nevertheless exposure of oocytes to both physiological and supra-physiological
628 concentrations of C-peptide in our culture system did not influence the PI3K/AKT signaling
629 pathway; contrary to research in other cell types [69-73], nor did these concentrations of C-
630 peptide prolong the viability of oocytes during extended culture periods designed to instigate
631 post-ovulatory ageing [49]. Conversely, these dose-dependent studies actually revealed
632 elevated markers of apoptotic demise in oocyte populations in response to prolonged exposure
633 to high levels of C-peptide; a finding that may be of biological significance when considering
634 the propensity for beta islet cells to over-produce both C-peptide and insulin in patients with
635 Type 2 diabetes, prior to their burnout [74].

636 The most striking finding of this investigation into the functional role of C-peptide
637 within the oocyte was the identification of a dramatic change in its subcellular localization in
638 response to fertilization. Thus, C-peptide immunoreactivity was characterized by a
639 redistribution from relatively uniform labeling of the ooplasm of unfertilized MII stage
640 oocytes, to being concentrated within the pronuclei of zygotes examined at 4 h post-
641 insemination. This was in contrast to intracellular insulin, which failed to undergo any
642 detectable changes in either localization or concentration throughout oocyte maturation and/or
643 fertilization. Consistent with intracellular calcium acting as a key regulator of C-peptide
644 function [50], this translocation of C-peptide occurred coincident with the stimulation of
645 pronounced calcium oscillations that accompany fertilization [75, 76]. Moreover, the extent of
646 C-peptide translocation was significantly compromised in the presence of the calcium chelator
647 BAPTA-AM, which effectively dampens the magnitude of post-fertilization calcium

oscillations [77]. Alternatively, C-peptide translocation was artificially driven using the calcium mimetic, strontium chloride [78]. These collective data mirror previous reports of intracellular C-peptide transport to cell nuclei in model cell lines (e.g. Swiss 3T3 and HEK-293 cells), where it is thought to regulate expression of ribosomal RNA [79]; however, a role for calcium in this process has not yet been confirmed [60]. Nevertheless, the activation of transcription factors resulting from C-peptide / GPCR adhesion on the plasma membrane of human renal tubular cells has been shown to be reliant on intracellular calcium concentration. Notably, this pathway also engages phospholipase C and protein kinase C activities [50], two pivotal elements of the fertilization cascade initiated in mammalian oocytes [80].

In our final experiments, we sought to assess potential C-peptide binding partners and thus account for the potential function of this peptide in the oocyte. Among the proteins identified as co-eluting with C-peptide from activated oocytes were the transcription factor LIM/homeobox protein (LHX8), a homeobox protein involved in oocyte survival [81]; two chloride channel proteins, CFTR (cystic fibrosis transmembrane conductance regulator) [82] and CLCN6 (chloride channel protein 6) [83]; and an ATP synthase, ATP6 [84]. These identifications resonate with previous findings in which C-peptide has been implicated in the activation of both transcription factors [50] and ATPases [50, 85] in response to elevated intracellular calcium. A subset of common proteins were identified in both MII and parthenote eluates, including BRCA2, and the phosphatase enzyme PTPC1 (protein tyrosine phosphatase domain-containing protein 1) [86].

The putative association of C-peptide and BRCA2 is of particular interest owing to the conservation of the reproductive phenotypes observed in *Brca2* mutant mice and those documented in diabetic patients and mouse models. In this context, studies of *Brca2* mutant mouse lines have revealed an essential role for this protein in maintenance of the ovarian reserve [52], DNA repair [87], telomere integrity [88-92], cellular proliferation, differentiation

673 and meiotic resumption [52]. Similarly, the oocytes of diabetic mouse models commonly
674 exhibit abnormalities in meiotic regulation [30, 93], accelerated telomere shortening [94], a
675 predisposition toward DNA damage and a concomitant decreased efficacy for DNA repair [95].
676 Likewise, the spatial and temporal pattern of *Brca2* mRNA expression reported in the ovary of
677 wild-type mice resembles that of C-peptide; with localization of both being detected in
678 granulosa cells, thecal cells, luteal cells, surface epithelium and the oocytes of developing
679 follicles [96, 97]. Despite this evidence, little is currently known of the specific role of either
680 of these proteins in the oocyte or preimplantation embryo beyond the traditional role ascribed
681 to BRCA2 in DNA repair and cellular proliferation.

682 In this regard, mice carrying a homozygous mutation in exons 10 and 11 of the *Brca2*
683 gene are fertile but suffer from embryonic lethality prior to day 9.5 of embryogenesis.
684 Phenotyping of mutant blastocysts has revealed that the *Brca2* mutation leads to significant
685 proliferative abnormalities (including gastrulation) during early embryonic development (i.e.
686 post-embryonic day 7), such that only the extraembryonic regions of the embryo continue to
687 develop *in vitro* and even these regions are severely reduced *in vivo* [98]. Likewise, the
688 embryonic regions of the blastocyst (i.e. the inner cell mass) are also adversely affected in mice
689 and rats with induced diabetes; perturbations, which lead to increased rates of malformation
690 and embryonic absorption [99]. Our own data provides a tentative explanation for these
691 overlapping pathologies by revealing a putative interaction of C-peptide and BRCA2 within
692 the ooplasm of MII oocytes and a subsequent translocation to the pronuclei coincident with
693 fertilization-/oocyte activation. On the basis of these data, we infer that, in addition to its
694 chaperone activity associated with proinsulin folding [100], C-peptide may also influence the
695 function of alternative protein targets such as BRCA2. Indeed, in the context of the embryo,
696 C-peptide could conceivably direct the localization of BRCA2 to the pronuclei in response to
697 fertilization-induced calcium oscillations, thus allowing for meiotic resumption and DNA

repair. In support of this model, fertilization has previously been associated with an upregulation of DNA repair and cellular proliferation mechanisms, which are also driven by nuclear localization of key repair enzymes [101].

In conclusion, this study has characterized a previously unappreciated association between C-peptide and the mouse oocyte. We have demonstrated strong expression of both C-peptide and its purported receptor; GPR146, in mouse oocytes and our data suggest that C-peptide is internalized by oocytes in a GPCR-dependent manner. Internalized C-peptide is responsive to the elevated calcium concentrations experienced in the oocyte at the moment of fertilization, resulting in the subcellular re-localization from the ooplasm to the pronuclei. Moreover, we identified the meiotic resumption/DNA repair protein BRCA2 as a putative binding partner for C-peptide within the oocyte; an interaction that is particularly interesting when considering the propensity for oocytes from diabetic women to experience fertility complications. This study therefore provides the impetus to explore the relationship between dysregulated C-peptide production and the impaired fertility documented in diabetic women.

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AUTHORS CONTRIBUTIONS

J.H.M., T.L., B.N., E.G.B and R.J.A., conceived of the study and designed the experimental approach. Data analysis, experimentation and manuscript preparation was performed by J.H.M

and T.L. Manuscript editing was conducted by E.G.B., B.N., and R.J.A. In addition, S.L.C, J.M.S and E.R.F, S.J.S participated in immunohistochemical and qRT-PCR analysis.

COMPETING INTERESTS STATEMENT

The authors have no competing financial interests to disclose.

FIGURES AND LEGENDS

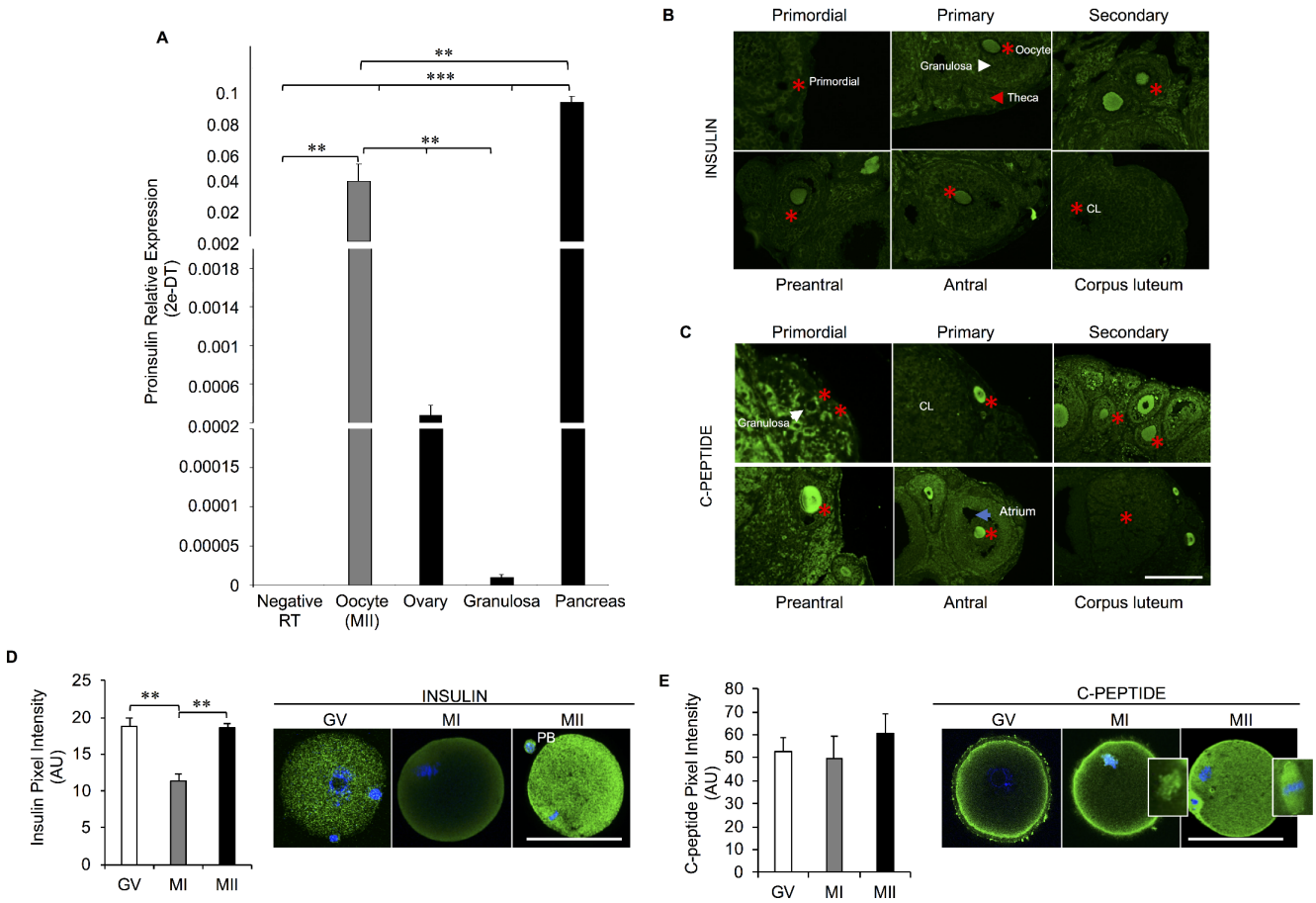


Figure 1: ***Detection of insulin and C-peptide in the mouse ovary and oocyte.***

(A) Quantitative RT-PCR analysis of the Proinsulin gene in the oocyte, ovary and isolated granulosa cells. **(B-C)** Immunohistochemistry and **(D-E)** immunocytochemistry depict the detection of insulin and C-peptide in the mouse ovary, GV, MI and ovulatory stage (MII) oocyte. Histograms display relative pixel intensity analysis. Granulosa cells are denoted with white arrows, theca cells with a red arrowhead, the follicles and the corpus luteum (CL) by red asterisks and the atrium space by a blue arrow. Representative fluorescence images depict the antibody of interest Insulin or C-peptide in green and nuclear counterstain in blue (DAPI). Mean ±SEM values are plotted in histograms. **P < 0.01 and ***P < 0.001. Scale bar= 80 µm.

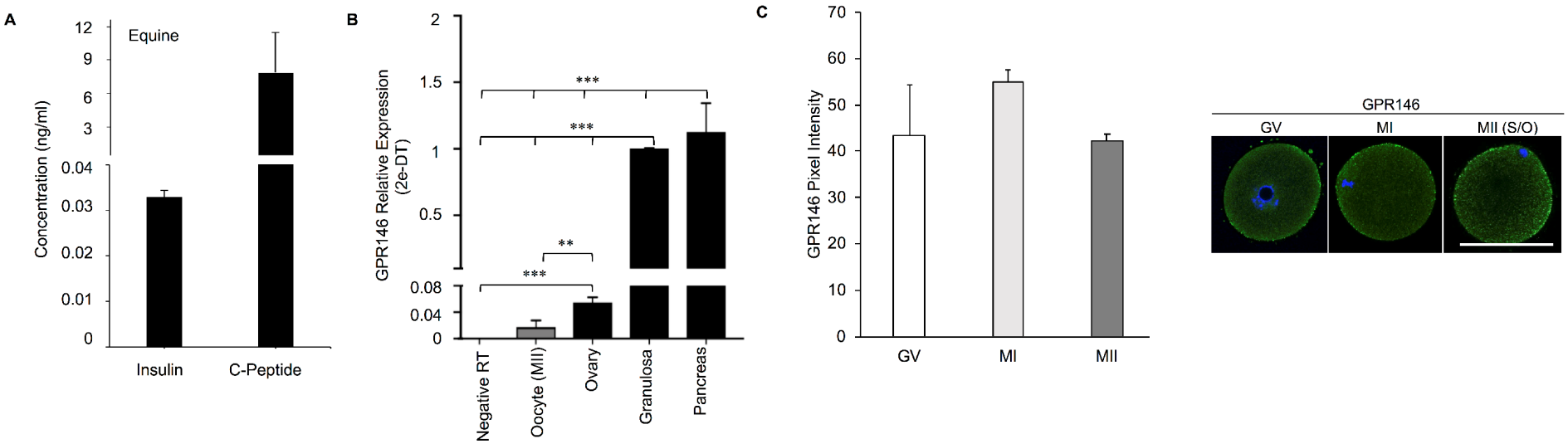


Figure 2: ***Investigating the origins of insulin and C-peptide within the mouse ovary.*** **(A)** ELISA analysis assessing C-peptide and insulin concentrations in equine follicular fluid**. (B)** Quantitative RT-PCR for the GPR146 gene in MII oocyte, ovary, granulosa cell and pancreas. **(C)** Immunocytochemical evaluation of the G-protein coupled receptor; GPR146 (green) in the GV, MI and MII oocyte (counterstained with DAPI). Accompanying histogram displaces relative pixel intensity. Mean ±SEM values are plotted in histograms. **P < 0.01 and ***P < 0.001. Scale bar= 80 µm.

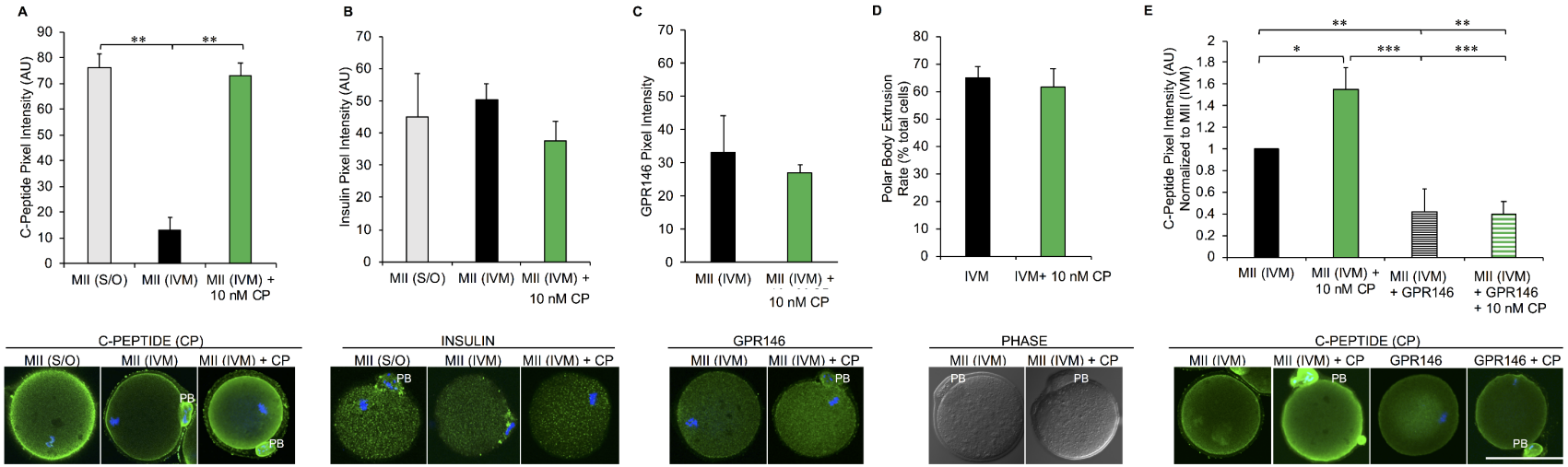


Figure 3: ***Evidence for C-peptide uptake during resumption of meiosis.*** **(A)** Immunocytochemical analyses of MII oocyte populations were performed following in vitro maturation with and without exogenous C-peptide (10 nM) and were compared to super-ovulated control oocytes. **(B-D)** An evaluation of insulin, GPR146 relative pixel intensity and polar body extrusion rate following C-peptide treatment. Mean ±SEM values are plotted in histograms as determined by pixel intensity analysis. **P < 0.01. Scale bar= 80 µm. SO refers to 'superovulated' oocytes and IVM to '*in vitro* matured' oocytes.

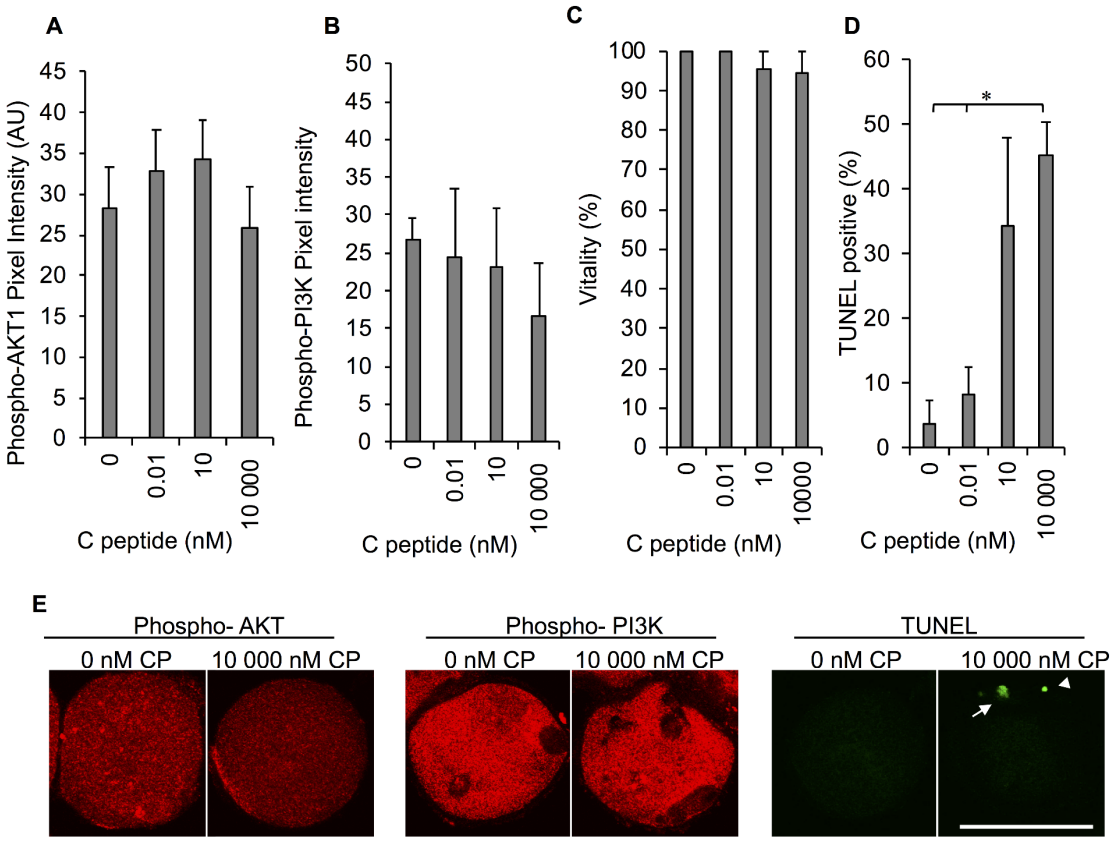
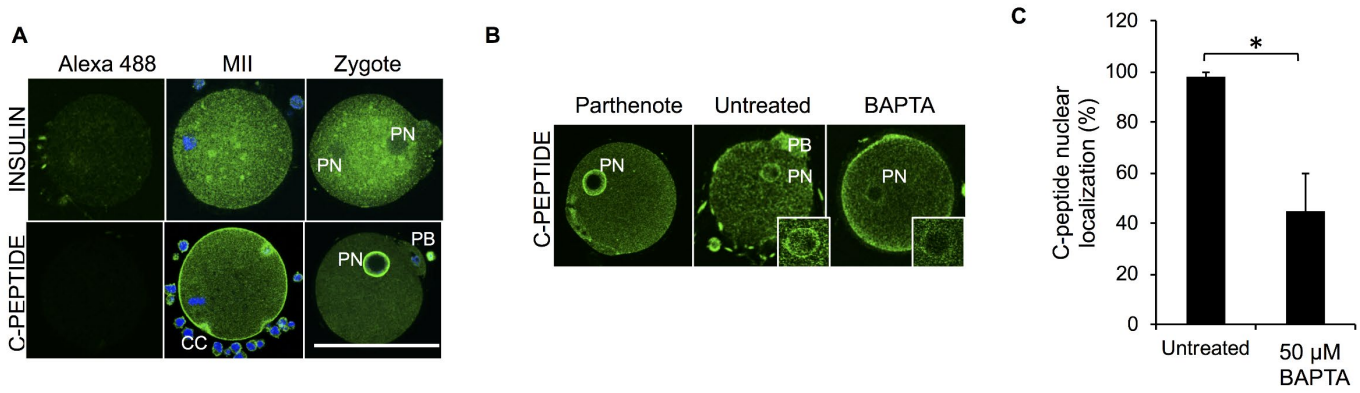


Figure 4: ***Investigating a role for C-peptide and insulin as pro-survival factors in the mouse MII oocyte.*** Immunocytochemical analyses of pro-survival factors; phosphorylated RAC-alpha serine/threonine-protein kinase (AKT1) **(A)** and phosphorylated phosphatidylinositol-3-kinase (PI3K) **(B)** in mouse MII oocytes exposed to physiological and supra-physiological concentrations of C-peptide in culture medium over prolonged culture period (8 h). **(C-D)** Evaluation of vitality (Trypan blue staining) and apoptosis (TUNEL). **(E)** Representative images of P-PI3K, P-AKT and TUNEL staining are included (arrow depicts metaphase plate, arrowhead is polar body). Mean ±SEM values are plotted in histograms. *$P < 0.05$.



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800 **Figure 5:** ***Exploration of a possible link between post-fertilization calcium oscillations and C-peptide activity***

801 ***in the mouse zygote.*** **(A)** Immunocytochemical comparison of C-peptide and insulin (green) in the mouse MII

802 oocyte and presumptive zygote. **(B)** An assessment of C-peptide localization in response to intracellular calcium

803 (Ca^{2+}) was carried out following supplementation of media with a calcium mimetic, strontium chloride, and Ca^{2+}

804 chelator BAPTA-AM (4 h). **(C)** Evaluation of C-peptide nuclear localization after incubation BAPTA-AM. Mean

805 ±SEM values are plotted in histograms. *P < 0.05. Scale bar= 80 µm.

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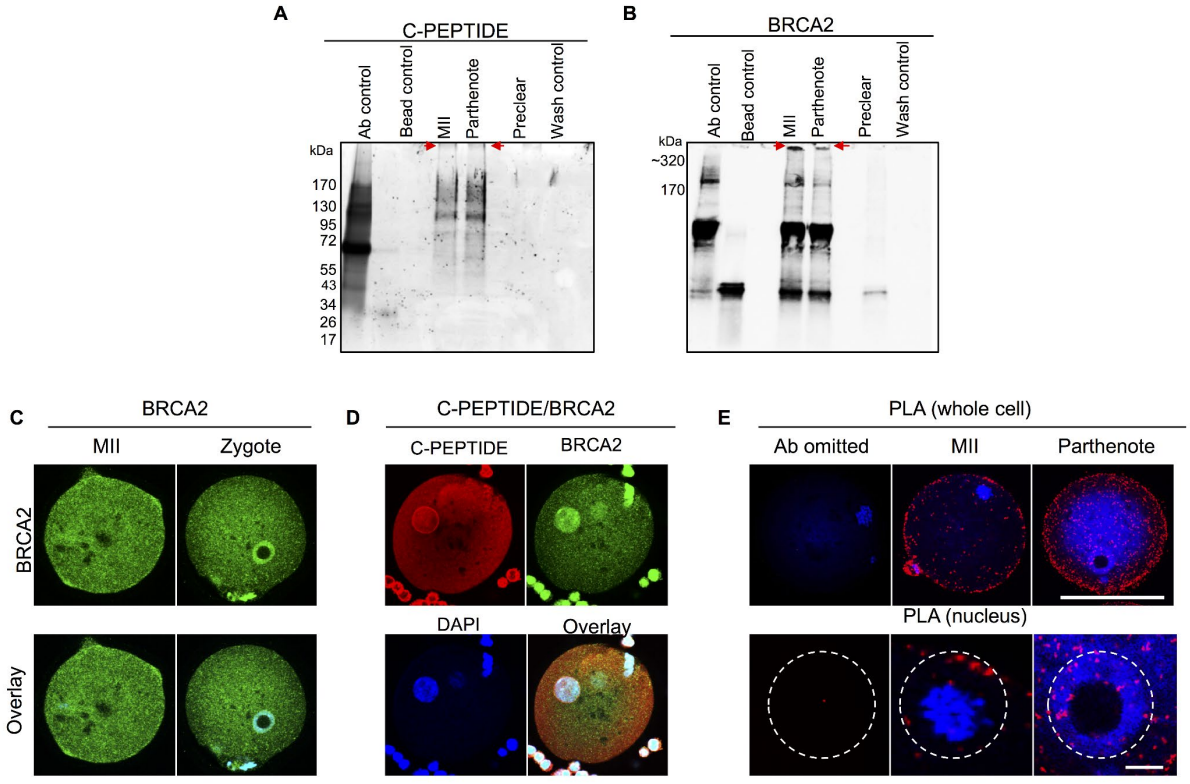

809

810 **Figure 6:** ***Identification of putative C-peptide binding partners in the mouse zygote.*** **(A)** Immunoprecipitation
811 using protein G Dynabeads cross-linked with the C-peptide antibody was used to isolate C-peptide binding
812 partners from MII oocytes and parthenotes. Dominant elution products were identified through Coomassie blue
813 staining of accompanying gels, and gel bands were excised for identification by mass spectrometry. **(B)**
814 Complementary immunoblotting against BRCA2 the highest scoring primary binding partner of C-peptide. **(C)**
815 Immunocytochemistry analyses were performed using the BRCA2 antibody (green, counter stained with DAPI
816 (blue). **(D)** Co-localization of C-peptide and BRCA2 [C-peptide; Alexa 594 (red), BRCA2; Alexa 488 (green)]
817 and a Duolink proximity ligation assay **(E)** were carried out in oocytes, parthenotes and zygotes. Scale bar 1= 80
818 µm. Scale bar 2= 7 µm.

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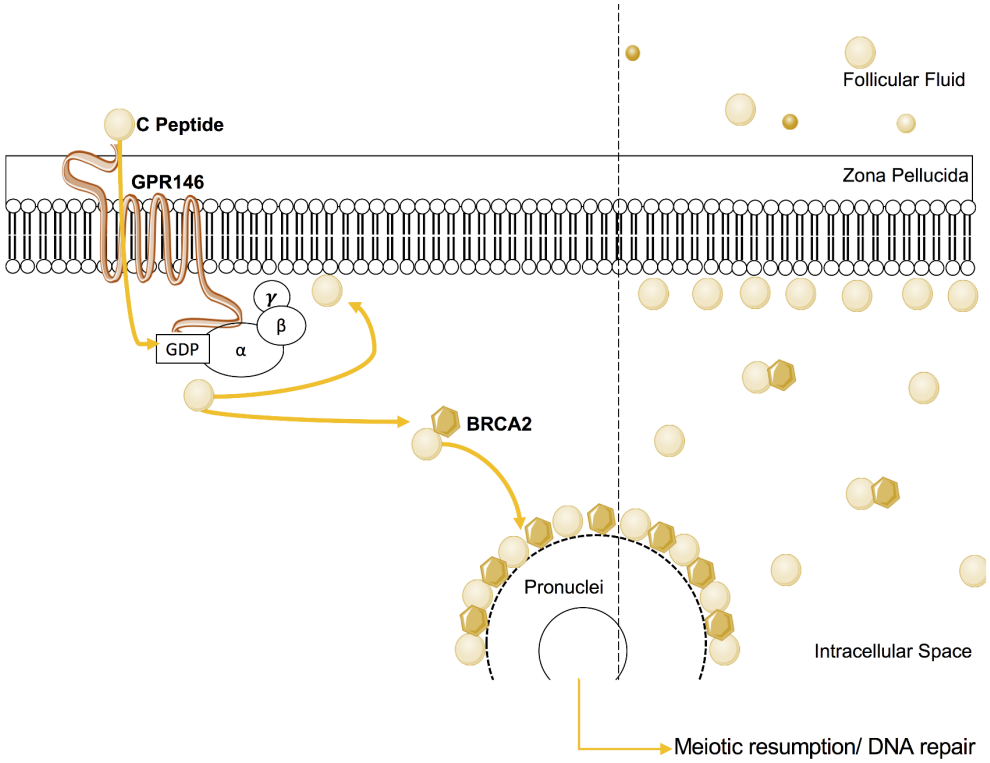

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825 **Figure 7:** ***Proposed function of intracellular C-peptide in the mammalian zygote;*** C-peptide present in follicular
826 fluid is internalized by the G-protein coupled receptor, GPR146. Intracellular C-peptide then binds to breast cancer
827 type 2 susceptibility protein (BRCA2) and directs the translocation of BRCA2 to the pronuclei in response to
828 fertilization stimulating meiotic resumption and DNA repair.

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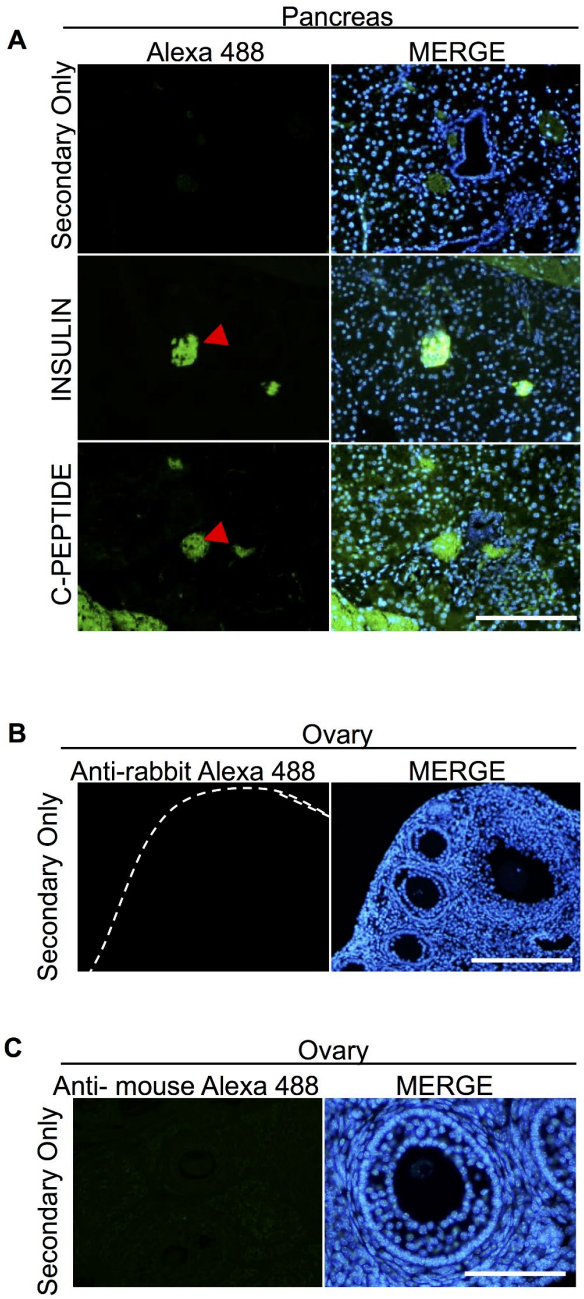
Table 1: ***Identification of C-peptide binding proteins via mass spectrometry.*** Immunoprecipitation was performed using the C-peptide antibody and dominant immunoprecipitated products were excised for trypsin digestion and identification using mass spectrometry. The primary binding partners (highest MASCOT scores) are displayed for both the MII stage oocytes and parthenotes.

		Protein	Gene	Score	MW	Function
MII:	**1.**	Breast cancer type 2 susceptibility protein homolog	BRCA2	74.6	372.0	DNA repair, meiotic resumption
	2.	Peroxisomal membrane protein 2	PXMP2	72.9	22.6	Channel forming protein in peroxisomes
	3.	Telomere-associated protein RIF1	RIF1	46.6	266.1	Involved in DNA damage response
	4.	Protein tyrosine phosphatase domain-containing protein 1	PTPC1	39.6	83.9	Phosphatase enzyme
Parthenote	**1.**	Breast cancer type 2 susceptibility protein homolog	BRCA2	78.2	372.0	DNA repair, meiotic resumption
	2.	Kynurenine/alpha-aminoadipate aminotransferase mitochondrial precursor	AADAT	52.7	47.3	Lysine biosynthesis and degradation
	3.	LIM/homeobox protein	LHX8	49.2	40.7	Transcription factor involved in oogenesis
	4.	Protein tyrosine phosphatase domain-containing protein 1	PTPC1	42.1	83.9	Phosphatase enzyme
	5.	Cystic fibrosis transmembrane conductance regulator	CFTR	38.4	168.4	cAMP-dependent chloride channel
	6.	Chloride channel protein 6	CLCN6	37.6	96.9	Chloride channel/antiporter
	7.	ATPase protein 6	ATP6	35.2	24.8	ATP synthase

Supplementary Table S1: ***Antibody summary table.*** Captured in this table is information relevant to the antibodies used for immunodetection, proximity ligation assays and immunoprecipitation experiments.

Antibody	**Final Concentration**[1]				**Company**	**Catalogue N°**	**Batch N°**	**Concentration**	**RRID**
Primary antibodies	IF	IHC	PLA	IP					
C-peptide	1/100	1/50	1/50	1/100	Abcam	ab14181	GR198034-1	N/A	AB_300968
Insulin (H-86)	0.02 mg/ml	0.04 mg/ml	-	-	Santa Cruz Biotechnology	SC-9168	C1212	0.2 mg/ml	AB_2126540
GPR146	0.02 mg/ml	-	-	-	Santa Cruz Biotechnology	SC-104284	C1609	0.2 mg/ml	AB_2232647
BRCA2 (N-19)	0.02 mg/ml	-	0.04 mg/ml	0.002 mg/ml	Santa Cruz Biotechnology	SC-1819	L2208	0.2 mg/ml	AB_630950
phosphorylated AKT1 (pThr308)	1/100	-	-	-	Calbiochem/Millipore	124001	N/A	N/A	AB_211424
phosphorylated PI3K (Y467 + Y199)	1/100	-	-	-	Abcam	ab63566	N/A	N/A	AB_1142257
Secondary Antibodies									
Anti-rabbit 488 Alexa Fluor	0.02 mg/ml	0.01 mg/ml	-	-	Thermo Fisher Scientific	A11008	1853312	2 mg/ml	AB_143165
Anti-goat 488 Alexa Fluor	0.02 mg/ml	0.01 mg/ml	-	-	Thermo Fisher Scientific	A11005	1396978	2 mg/ml	AB_141372
Anti-rabbit 594 Alexa Fluor	0.02 mg/ml	-	-	-	Thermo Fisher Scientific	A11012	1844440	2 mg/ml	AB_141359

[1] IF, immunofluorescence; IHC, immunohistochemistry, PLA, proximity ligation assay, IP, immunoprecipitation, NA, not available -, not applicable



Supplementary Figure S1: Positive and negative controls for immunodetection using anti-insulin and anti-C-peptide were performed on mouse pancreas sections. The β islet cells of the pancreas are marked by red arrowheads.

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CHAPTER 5: LITERATURE REVIEW

DNA damage and repair in the female germline; contributions to assisted reproductive technologies

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Authors: Martin, J.H., Aitken, R.J., Bromfield, E.G. and Nixon, B. (2018).

Affiliations: [1]Priority Research Centre for Reproductive Biology, School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia.

Correspondence: Jacinta.martin@newcastle.edu.au

CHAPTER OVERVIEW

The final chapter of this thesis provides an overview of our novel findings presented in the context of previously published literature. This review focuses on the innate susceptibility of the female germline to DNA damage and highlights the spectrum of endogenous quality control mechanisms employed by the oocyte and early embryo to prevent and/or remediate DNA damage during the course of development.

Herein, the advances afforded by our studies of maternal DNA damage response mechanisms are summarized and several new avenues for research directed towards increasing the stringency of an oocyte's endogenous defence systems are discussed. With this goal in mind, we contend that future research should look towards improved methods of augmenting the activity of endogenous mechanisms for surveillance of DNA damage, cell cycle arrest, regulated cell death and enhancing the efficacy of DNA repair in the oocyte. Such strategies represent prime targets to fortify the genomic integrity of the oocyte during *in vitro* manipulation, with important implications for novel therapeutics to alleviate the burden of female factor infertility.

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Human Reproduction Update

DNA damage and repair in the female germline: contributions to ART

Jacinta H. Martin [1,2,*], R. John Aitken[1,2], Elizabeth G. Bromfield[1,2,†], and Brett Nixon [1,2,†]

[1]Priority Research Centre for Reproductive Science, Discipline of Biological Sciences, University of Newcastle, Callaghan, NSW 2308, Australia [2]Hunter Medical Research Institute, Kookaburra Circuit, New Lambton Heights, NSW 2305, Australia

*Correspondence address. The University of Newcastle, Callaghan, NSW 2308, Australia. Tel: +61-2-4921 6977; Fax: +61-2-49216923; E-mail: Jacinta.martin@newcastle.edu.au orcid.org/0000-0002-0876-5150

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BACKGROUND: DNA integrity and stability are critical determinants of cell viability. This is especially true in the female germline, wherein DNA integrity underpins successful conception, embryonic development, pregnancy and the production of healthy offspring. However, DNA is not inert; rather, it is subject to assault from various environment factors resulting in chemical modification and/or strand breakage. If structural alterations result and are left unrepaired, they have the potential to cause mutations and propagate disease. In this regard, reduced genetic integrity of the female germline ranks among the leading causes of subfertility in humans. With an estimated 10% of couples in developed countries taking recourse to ART to achieve pregnancy, the need for ongoing research into the capacity of the oocyte to detect DNA damage and thereafter initiate cell cycle arrest, apoptosis or DNA repair is increasingly more pressing.

OBJECTIVE AND RATIONALE: This review documents our current knowledge of the quality control mechanisms utilised by the female germline to prevent and remediate DNA damage during their development from primordial follicles through to the formation of preimplantation embryos.

SEARCH METHODS: The PubMed database was searched using the keywords: primordial follicle, primary follicle, secondary follicle, tertiary follicle, germinal vesical, MI, MII oocyte, zygote, preimplantation embryo, DNA repair, double-strand break and DNA damage. These keywords were combined with other phrases relevant to the topic. Literature was restricted to peer-reviewed original articles in the English language (published 1979–2018) and references within these articles were also searched.

[†]These authors contributed equally to this manuscript.

OUTCOMES: In this review, we explore the quality control mechanisms utilised by the female germline to prevent, detect and remediate DNA damage. We follow the trajectory of development from the primordial follicle stage through to the preimplantation embryo, highlighting findings likely to have important implications for fertility management, age-related subfertility and premature ovarian failure. In addition, we survey the latest discoveries regarding DNA repair within the metaphase II (MII) oocyte and implicate maternal stores of endogenous DNA repair proteins and mRNA transcripts as a primary means by which they defend their genomic integrity. The collective evidence reviewed herein demonstrates that the MII oocyte can engage in the activation of major DNA damage repair pathway(s), therefore encouraging a reappraisal of the long-held paradigm that oocytes are largely refractory to DNA repair upon reaching this late stage of their development. It is also demonstrated that the zygote can exploit a number of protective strategies to mitigate the risk and/or effect the repair, of DNA damage sustained to either parental germline; affirming that DNA protection is largely a maternally driven trait but that some aspects of repair may rely on a collaborative effort between the male and female germlines.

WIDER IMPLICATIONS: The present review highlights the vulnerability of the oocyte to DNA damage and presents a number of opportunities for research to bolster the stringency of the oocyte's endogenous defences, with implications extending to improved diagnostics and novel therapeutic applications to alleviate the burden of infertility.

Key words: ART / cell viability / DNA damage / DNA repair / genetic integrity / infertility / metaphase I/II oocytes / preimplantation embryo / premature ovarian failure / subfertility

Introduction

A remarkable sequence of molecular events govern the successful initiation of embryonic development. Indeed, syngamy between the male and female gametes brings into being an entirely new and untested combination of chromosomes. Given the complexity of these processes, it is perhaps not surprising that two-thirds of all human pregnancies end in miscarriage or spontaneous abortion; with as many as 15–20% being lost before the woman was even aware of the pregnancy (Wilcox *et al.*, 1988; Zinaman *et al.*, 1996; Wang *et al.*, 2003). Following conception, the success or failure of a pregnancy is influenced by two main factors: the peri-conceptual maternal environment and/or the integrity of the embryo itself. The peri-conceptual maternal environment encompasses uterine/endometrial factors as well as abnormalities and/or metabolic conditions that may lead to hostile modifications of these settings (e.g. polycystic ovary syndrome and endometriosis; comprehensively reviewed elsewhere; Ehrmann, 2005; Solopova *et al.*, 2017; Cho *et al.*, 2018). Embryonic abnormalities may be attributed to reduced genetic integrity (Van Blerkom and Davis, 1998) but may also extend to a myriad of other anomalies including aneuploidy, oxidative stress and ooplasmic immaturity (Lacham-Kaplan and Trounson, 2008; Pereira *et al.*, 2016). In this context, defective gamete quality ranks among the leading causes of subfertility and infertility in humans (Testart *et al.*, 1983; Hull *et al.*, 1996; Agarwal *et al.*, 2005). Owing to the central role of DNA as the repository of genetic information, its integrity and stability are critical determinants of cell viability. However, DNA is not inert; rather, it is a chemical entity that is subject to assault from its immediate environment resulting in chemical modification and/or strand breakages. If structural alterations result and are left unrepaired, they have the potential to cause mutations and propagate disease (reviewed in Liu *et al.*, 2016). To ensure fertility, gamete quality must extend to both the nucleic acids (DNA and various RNA transcripts) and a complex array of alterative macromolecules that constitute the membrane and proteome of these cells. As such, gametes must be safeguarded by a sophisticated suite of protective and/or reparative responses to counteract, ameliorate and/or correct damage that may be inherited by the embryo. While this topic has been thoroughly reviewed in somatic cell literature, we are only now beginning to understand the complex array of quality assurance mechanisms contained within the oocyte. In this review, we explore the vulnerability of the female germline to DNA damage during the development window of the primordial follicle through to the preimplantation embryo. This information is considered alongside new findings on the capacity of oocytes to protect their genomic material as they transition through these key developmental phases. Understanding how the protection of the female genome is integrated into the meiotic events that underpin their development has the potential to inform novel strategies to preserve oocyte/embryo quality *in vitro* and thus improve embryo diagnostics and selection procedures in the context of ART.

DNA damage responses within the ovary

Primordial follicles

Oogenesis represents the culmination of both mitotic and meiotic divisions, leading to the production of a mature oocyte (Hartshorne *et al.*, 2009). In species such as our own, oogenesis is initiated and then arrested within the ovary during prenatal development. Between the second and the seventh month of gestation, rapid cell division produces ~7 million oogonia, which either commit to a developmental fate and are segregated into germ cell 'cysts' or alternatively, are lost through atresia (De Pol *et al.*, 1997). Breakdown of the germ cell cysts is accompanied by the transition of precursor oocytes, enveloped by a single layer of flattened follicular epithelial cells, into primordial follicles; a stage of development where they will remain arrested until entry into the first phase of meiosis immediately after birth (Pepling and Spradling, 2001; Pepling, 2006). This extreme longevity, in combination with the unique architecture of the primordial nucleus, marks the primordial follicle as especially vulnerable to DNA damage (Hanoux *et al.*, 2007; Kerr *et al.*, 2012c). Thus, in

order to protect against the induction of germline mutations at the precipice of development, it is critical that the genomic integrity of oocytes is subject to stringent surveillance, thereby facilitating the detection, repair or elimination of those oocytes with compromised genomic fidelity (Ashwood-Smith and Edwards, 1996; Tilly, 2001).

Much of our current understanding of the DNA damage response mounted in primordial follicles is attributed to studies examining the effects of anti-cancer regimens on fertility (reviewed in Winship *et al.*, 2018). In humans and animals, chemotherapeutic agents and radiation therapy inadvertently threaten fertility via the induction of widespread DNA damage and apoptosis in the resting primordial follicle population (Meirow and Nugent, 2001; Suh *et al.*, 2006; Green *et al.*, 2009; Rodriguez-Wallberg and Oktay, 2014; Anderson *et al.*, 2015; Stefansdottir *et al.*, 2016). The innate susceptibility of these cells is especially troubling when considered in the context of child and adolescent cancer sufferers, who, in addition to a loss of fertility, may experience acute or chronic ovarian insufficiency, as well as early menopause and its associated consequences (Agarwal and Chang, 2007; Cvancarova *et al.*, 2009; Jeruss and Woodruff, 2009; Schover, 2009).

At a molecular level, depletion of the ovarian reserve following exposure to genotoxic stressors is commonly attributed to trans-activating p63 (*TAp63*) mediated apoptosis (Suh *et al.*, 2006; Kerr *et al.*, 2012c) (Fig. 1). Engagement of the apoptotic pathway by *TAp63* proceeds through several successive phases. In the first of these, ataxia telangiectasia (mutated) (ATM) kinase and checkpoint kinase 2 (CHK2) orchestrate the phosphorylation event required to activate TAp63 following exposure to a genotoxic insult (Suh *et al.*, 2006; Livera *et al.*, 2008; Bolcun-Filas *et al.*, 2014; Kim and Suh, 2014). This leads to the transcriptional induction of BH3 (BCL2 homology 3)-only pro-apoptotic factors, PUMA (p53-upregulated modulator of apoptosis) and, to a lesser extent, NOXA (also known as phorbol-12-myristate-13-acetate-induced protein 1 [PMAIP1]). The upregulated expression of these proteins facilitates their interaction with the pro-apoptotic BCL2 family members, BAX and BAK (BCL2-associated X protein and BCL2-antagonist/killer, respectively) (Kerr *et al.*, 2012c). This interaction releases BAX and/or BAK for translocation and induction of mitochondrial dysfunction leading to release of mitochondrial apoptogenic proteins and culminating in the activation of caspase-9, a crucial proteolytic enzyme responsible for intrinsic apoptosis and cell death (Elmore, 2007) (Fig. 1).

In an evolutionary context, removal of oocytes containing damage would preclude the risk of disseminating mutations to future generations, but as a consequence can result in infertility or a shortened reproductive lifespan/capacity of the 'parental' organism (Depmann *et al.*, 2015). In this regard, one might expect that their extended developmental arrest should provide primordial follicles with sufficient time to respond to damage and enact repair; theoretically a superior strategy for dealing with genomic instability whilst still maintaining fertility. It is therefore perhaps surprising that, despite its high evolutionary conservation, the functional significance of this prolonged meiotic arrest has received scant attention (Westendorp and Kirkwood, 1998). Nonetheless, with recent medical advances increasing the probability of patients surviving a cancer diagnosis, the expectation and desire for reproductive options following cancer remission is driving the need to develop innovative fertility preservation techniques (Aziz and Rowland, 2003). In this regard, the DNA damage response in primordial follicles has laid the foundation for novel strategies for fertility preservation. For instance, elegant studies using PUMA or PUMA/NOXA (*Puma−/−, Noxa−/− and Puma−/−Noxa−/−*) knockout mice have revealed protection of the female germline during challenge by ovo-toxicants, which are otherwise capable of depleting the ovarian reserve in wild-type mice (WT) (Suh *et al.*, 2006; Kerr *et al.*, 2012a,b,c; Stefansdottir *et al.*, 2016). In these transgenic models, the ovaries maintained their primordial follicle pool and their fertility was preserved as indicated by the production of multiple litters without gross abnormalities among the offspring. Taken together, these data suggest that oocytes derived from *Puma−/−, Noxa−/−* and *Puma−/−Noxa−/−* mouse models were capable of conducting extremely effective DNA repair (Kerr *et al.*, 2012c) (Fig. 2). Considering that it is the primordial follicle pool that represents a woman's reproductive capacity, it remains unclear why these cells default to such a stringent removal strategy in preference to the alternative repair pathways engaged in the knockout animals. Answering this question will have profound implications for mitigating the infertility experienced by women undergoing chemotherapeutic treatment. In the interim, it could be considered an adaptation compatible with the primordial follicle's uniquely important role in the propagation of a species (Suh *et al.*, 2006; Livera *et al.*, 2008; Kerr *et al.*, 2012c).

Primary and transitory follicles

Release from primordial follicle arrest occurs immediately post-partum, whereupon follicular maturation is reinitiated and proceeds to the primary follicle stage. At this point, the oocyte has entered meiotic prophase I, progressing slowly to the first meiotic arrest coinciding with the late diplotene stage. The primary follicle arrest remains in place, pending receipt of appropriate stimuli that lead to recruitment of the oocytes for ovulation, an extended period that can often span many decades (Kawamura *et al.*, 2004). Similar to the primordial follicle, *TAp63* expression remains high in primary follicles that have recently been recruited into the growing follicle pool but is then lost in advanced follicles following their recruitment for ovulation (Suh *et al.*, 2006). Genetic manipulation of the *TAp63* gene has been performed to generate mouse lines lacking exons 2 and 3, which encode the trans-activating amino terminus of the *p63* protein. These experiments revealed that the oocytes in primary follicles also rely on a *TAp63*-mediated DNA damage response. In this regard, the primary follicle pool in *TAp63*-null mice proved resistant to doses of irradiation (0.45 Gy) that eradicated all their equivalent counterparts in wild-type animals. Notably, the pro-apoptotic BH3-only members, PUMA and NOXA, were also not expressed in the primary follicles of the *TAp63*-null animals, thus contributing to the maintenance of normal numbers of these cells in the face of irradiation challenge (Suh *et al.*, 2006). Taken together, these data indicate that *TAp63* fulfils a conserved role in both primary and primordial follicles in the elimination of damaged oocytes from the germline.

Accordingly, the loss of *TAp63* expression coincides with entry into the follicular growth phase. However, this is just one of a suite of dramatic changes in gene expression profiles that accompany follicular growth (Pan *et al.*, 2005). Although non-growing follicles are themselves transcriptionally active, the scale of the changes in gene expression witnessed during follicular recruitment may reflect the role of the corresponding gene products in ensuring developmental

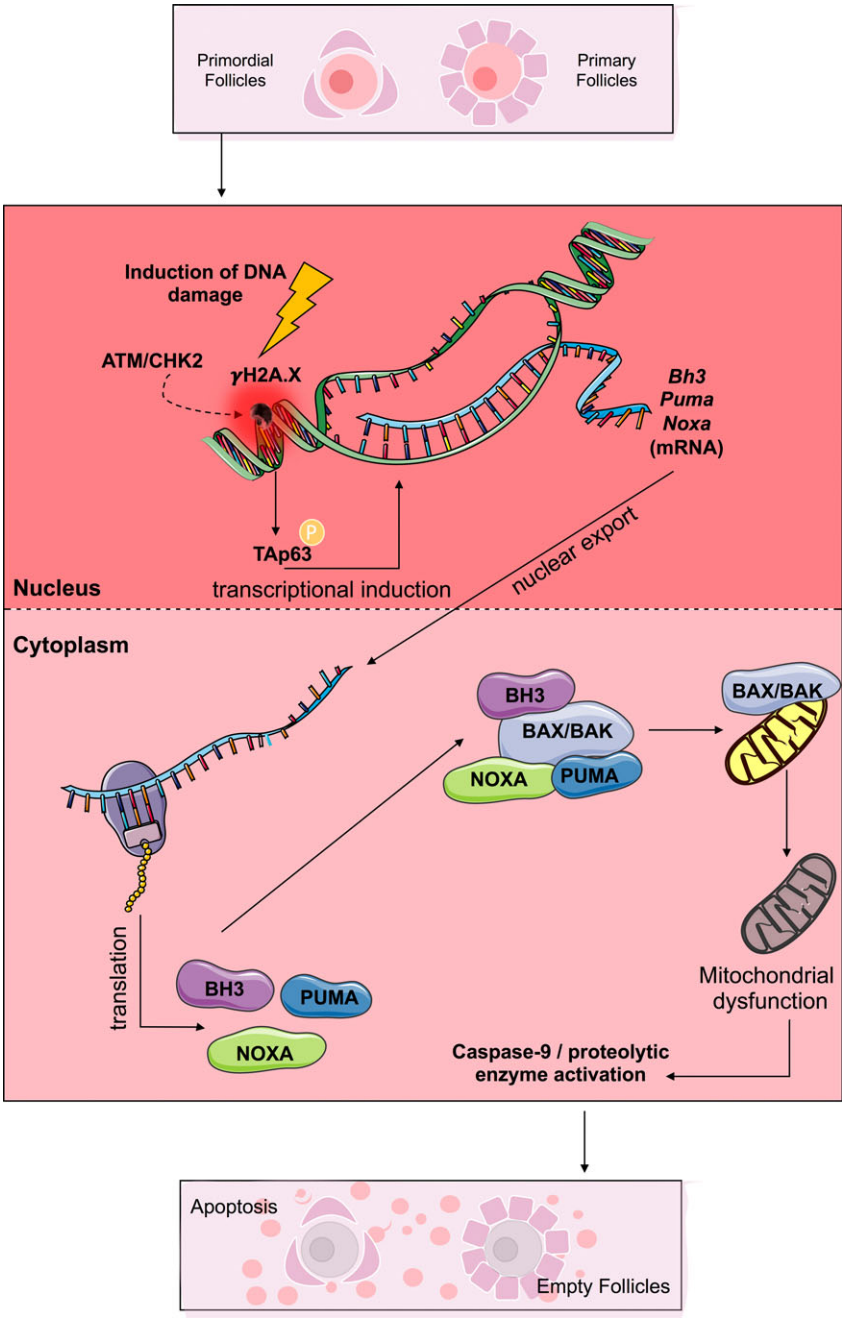

Figure 1 Schematic of the DNA damage response pathway elicited in the primordial follicles. Following the induction of DNA damage, trans-activating p63 (TAp63) mediates widespread apoptotic loss of primordial follicles. The pathway is initiated by a combination of ataxia telangiectasia (mutated) (ATM) kinase and checkpoint kinase 2 (CHK2), which respond to DNA damage through the phosphorylation and hence activation of TAp63. Activation of TAp63 leads to the transcriptional induction of BH3 (BCL2 homology 3)-only pro-apoptotic factors, PUMA (p53-upregulated modulator of apoptosis) and NOXA (phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1)). PUMA and NOXA interact with and release the pro-apoptotic BCL2 family members, BAX and BAK (BCL2-associated X protein and BCL2-antagonist/killer, respectively). The ensuing translocation of BAX and/or BAK to the oocyte's mitochondria leads to their dysfunction and release of mitochondrial apoptogenic proteins and the activation of caspase-9 and proteolytic enzymes, which collectively trigger apoptosis and cell death. The resultant depletion of the primordial follicle pool leads to infertility. Figure adapted from Suh *et al.* (2006) and Kerr *et al.* (2012c).

competence during fertilisation and embryogenesis (Pan *et al.*, 2005). Accordingly, this developmental stage is characterised by an over-representation of genes that encode proteins involved in DNA repair response(s); a strategy that presumably safeguards the genomic integrity of the female germline in the absence of *TAp63* (Zeng *et al.*, 2004; Pan *et al.*, 2005; Zheng *et al.*, 2005). It is therefore likely that

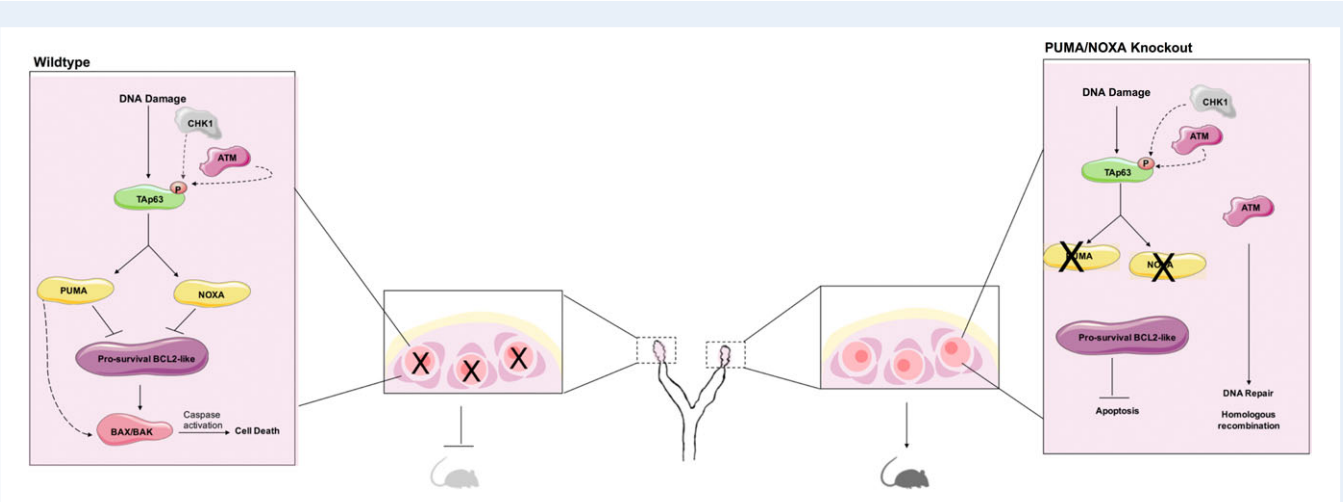

Figure 2 Experimental model for DNA repair in the primordial follicle. Transgenic PUMA or PUMA/NOXA (Puma−/−, Noxa −/− and Puma−/−Noxa−/−) knockout mouse models reveal that in the absence of PUMA and/or NOXA mouse primordial follicles are able to conduct highly effective DNA repair leading to preservation of the primordial follicle pool and the fertility of animals challenged with ovo-toxicants.

upon entering this transition phase, the immature oocyte may gain the capacity to respond to DNA damage via the activation of classic DNA repair pathways. Indeed, in experiments where ovaries obtained from postnatal day 4 rats were cultured in the presence of bisphenol A (BPA; an endocrine disrupting chemical that causes follicular loss and DNA damage in germ cells), a suite of DNA repair genes belonging to the classical double-strand break (DSB) DNA repair pathways (i.e. Atm, DNA-dependent protein kinase catalytic subunit [*DNA* Pkcs], X-ray repair cross complementing 6 [Xrcc6], breast cancer 1 [Brca1], Mre11a, Rad50 and structural maintenance of chromosomes 1A [Smc1a]) were significantly upregulated in as little as 24 hr after exposure (Ganesan and Keating, 2016). In the same report, the authors described a threshold of BPA exposure beyond which the DNA repair processes mounted in the oocyte proved ineffectual, leading to the induction of apoptosis and a concomitant loss of primary and secondary follicles (Ganesan and Keating, 2016).

In a similar context, other groups have demonstrated that exposing mice to low levels of genotoxic agents that target topoisomerase II (TOP2) activity (i.e. 20 nM doxorubicin [DOX] and etoposide [ETP]), elicits only modest inhibition of follicle growth. In contrast, elevation of DOX and ETP exposure to levels exceeding 200 nM are tightly correlated with increased follicle apoptosis, reduced ovarian size and anovulation later in development (Ben-Aharon *et al.*, 2010; Stefansdottir *et al.*, 2016; Xiao *et al.*, 2017). Taken together, these data demonstrate that transitory follicles are not recalcitrant to DNA repair at low doses of ovo-toxicants but that this protective response has a physiological threshold, beyond which, the cells default to an apoptotic pathway leading to permanent ovarian insufficiency and infertility. What remains less certain is the fidelity of the DNA repair enacted in oocytes from the 'low-dose' treatment regimens, and thus, whether these cells retain DNA lesions capable of inducing mutations or chromosomal rearrangements; abnormalities that would only become evident much later in oogenesis during meiotic chromosome segregation, or even in a resulting embryo. While the exact molecular mechanism(s) driving the DNA damage response in these late stages of follicular development remain enigmatic, the growth phase follicles do provide the first evidence of an active DNA damage response in the female germline and thus warrant further investigation.

Germinal vesicle stage oocyte (prophase I arrest)

In contrast to the growing follicle, which remains transcriptionally active until the end of the growth period, a change in chromatin distribution within the nucleolus from the non-surround to the surrounded configuration renders the germinal vesicle (GV) stage oocyte transcriptionally inactive (De La Fuente, 2006). During the preceding growth phase, most transcribed mRNA is immediately translated; however, a significant and developmentally crucial ~30% of mRNA transcripts are translationally repressed and stored in preparation for the transcriptionally inactive meiotic maturation phase and initiation of early embryonic development (Pique *et al.*, 2008). Gene products associated with the DNA repair response have been identified, both at the mRNA and protein level, within the mature GV stage oocyte (Zeng *et al.*, 2004; Pan *et al.*, 2005; Zheng *et al.*, 2005) (Tables I and II). Accordingly, the activity of DNA repair mechanisms in the GV stage oocyte is believed to depend on these stores of pre-synthesised proteins and/or mRNA transcripts accumulated during oogenesis in order to effect DNA repair (Kocabas *et al.*, 2006; Jaroudi *et al.*, 2009).

Indeed, mouse oocytes from preovulatory follicles are capable of detecting DNA damage, as illustrated by γH2AX (phosphorylation of histone H2AX at serine 139) foci formation after exogenous DSB induction (Marangos and Carroll, 2012; Yuen *et al.*, 2012; Ma *et al.*, 2013; Lin *et al.*, 2014). Phosphorylation of γH2AX requires the coordinated action of the MRN complex (comprising the MRE11 [meiotic recombination 11 homolog A], RAD50 [RAD50 recombinase] and NBS1 [Nijmegen breakage syndrome 1] proteins) and ATM activation (reviewed in Stracker and Petrini, 2011). MRN acts as an initial sensor, which binds specifically to sites of DSB DNA damage, promoting the activation of ATM. ATM subsequently catalyses the

Table I Proteomic analysis of the DNA repair proteins in the murine germinal vesicle, metaphase II oocyte and preimplantation embryo.

	GV	MII	Zygote
Single-strand break repair			
XRCC1: X-ray repair cross-complementing protein 1	■	■	■
TDP1: Tyrosyl-DNA phosphodiesterase 1		■	
Base excision repair			
PCNA: proliferating cell nuclear antigen	■	■	■
POLD1: DNA polymerase delta 1, catalytic subunit	■	■	
PARP1: poly [ADP-ribose] polymerase 1	■	■	
LIG1: DNA ligase 1		■	
FEN1: Flap endonuclease 1	■	■	
Nucleotide-excision repair			
RAD23b: RAD23 Homolog B	■	■	■
SIK: Serine/threonine-protein kinase SIK1		■	
POLD1: DNA polymerase delta 1, catalytic subunit	■	■	
POLD2: POLD2 DNA polymerase delta 2, accessory subunit	■	■	■
Double-strand breaks			
BRCA2: breast cancer 2	■		■
TRIP13: thyroid hormone receptor interactor 13,	■	■	■
PRKDC: DNA-PK catalytic subunit	■	■	
SOD 1: superoxide dismutase 1	■	■	■
SOD 2: superoxide dismutase 2	■	■	■
XRCC5: X-ray repair cross complementing 5 (KU80)	■	■	
XRCC6: X-ray repair cross complementing 6 (KU70)	■	■	
HR			
LIG1: DNA ligase 1		■	
BRCA1: breast cancer 1		■	
BRCA2: breast cancer 2	■		■
RAD 51: RAD51 recombinase	■	■	
RPA1: replication protein A 70 kDa DNA-binding subunit	■	■	■
H2AFX: (γH2AX) H2AX becomes phosphorylated on serine 139	■	■	■
SMC6: structural maintenance of chromosomes protein 6		■	
Non-homologus end-joining (NHEJ)			
PRKDC: DNA-PK catalytic subunit	■	■	
XRCC5: X-ray repair cross complementing 5 (KU80)	■	■	
XRCC6: X-ray repair cross complementing 6 (KU70)	■	■	
NONO: Non-POU domain-containing octamer-binding protein	■	■	■
General			
MCM2: minichromosome maintenance complex component 2	■	■	■
MCM3: minichromosome maintenance complex component 3	■	■	■
MCM4: minichromosome maintenance complex component 4	■	■	
MCM5: minichromosome maintenance complex component 5	■	■	■
MCM6: minichromosome maintenance complex component 6	■	■	■
MCM7: minichromosome maintenance complex component 7		■	
NASP: nuclear autoantigenic sperm protein	■	■	■
PHB: prohibitin-1	■	■	■
RBBP4: retinoblastoma-binding protein 4	■	■	
RBBP7: retinoblastoma-binding protein 7	■	■	■

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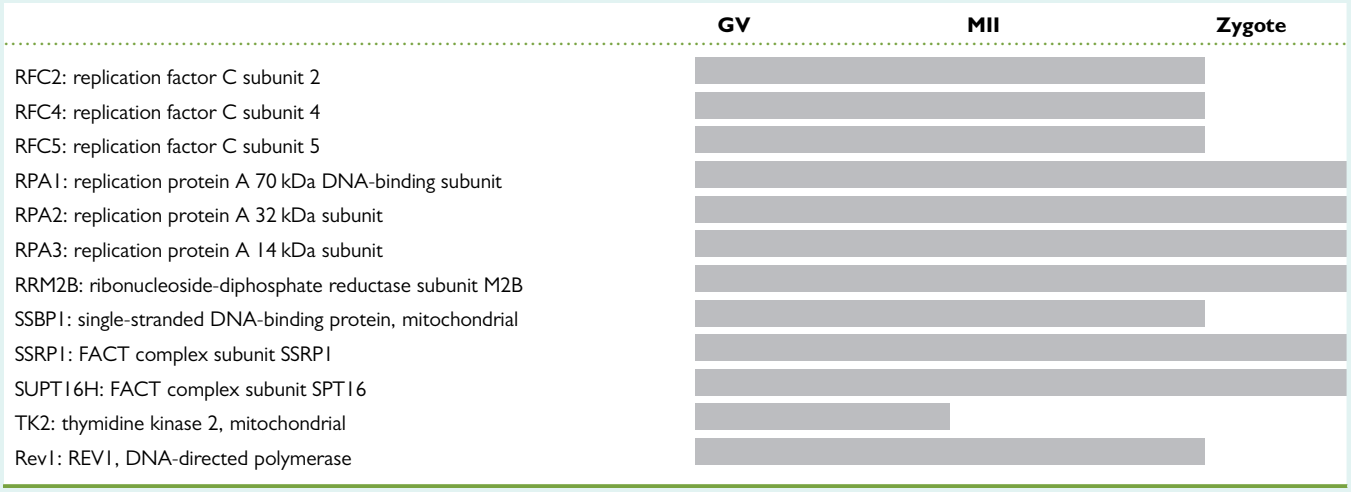
Table I *Continued*



A significant enrichment of mRNA and protein involved in DNA metabolism occurs during the follicular phases of oogenesis, a strategy that safeguards the genomic integrity of the female germline ensuring developmental competence during fertilisation and embryogenesis. The mouse oocyte proteome comprises 53 proteins putatively involved in DNA repair represented during the germinal vesicle (GV), metaphase II (MII) and zygotic stages of development. Among these proteins, 35 are predominantly expressed in the ovulatory stage oocyte, when compared to the GV and zygotic stages of development. Pathways represented include proteins required for single-strand break repair, double-strand break repair, nucleotide-excision repair and base excision repair. The table was adapted from the data collected by Wang *et al.* (2010).

phosphorylation of histone H2AX and this post-translational modification then propagates around the damaged DNA site (Burma *et al.*, 2001; Jungmichel and Stucki, 2010) (Fig. 3). In somatic cells, this cascade succeeds in promoting repair of DSB through homologous recombination (HR) and/or non-homologous end-joining (NHEJ) pathways (Fig. 3). In contrast, the induction of DSB by genotoxic agents such as ETP and DOX, as well as the formation of interstrand crosslinks, does not appear to stimulate a robust DNA damage response within the oocyte beyond the phosphorylation of H2AX. In the absence of such a response, oocytes harbouring DSB DNA are permitted entry into the first meiotic M-phase (MI) (Marangos and Carroll, 2012; Yuen *et al.*, 2012). The reason for this continued developmental passage rests with a failure of the G2/prophase checkpoint, whereby fully-grown GV oocytes are unable to effectively activate the master regulator of the DNA damage response pathway, ATM kinase (Marangos and Carroll, 2012; Marangos *et al.*, 2015). Instead, mature GV oocytes express a relatively low level of ATM compared to either growing oocytes or blastocysts, which likely accounts for the limited activation of the traditional ATM mediated CHK1 (Cell cycle checkpoint 1) arrest pathway after DSB induction (Marangos and Carroll, 2012).

Although the ability of the GV oocyte to detect DNA damage remains high, the establishment of an effective DNA damage response is reserved only for very severe DNA damage inflicted, for instance, by high concentrations of ETP, DOX or neocarzinostatin. Even so, these insults still only serve to delay, rather than prevent entry into M-phase (Yuen *et al.*, 2012). Thus, despite encountering severe DNA damage and prolonged arrest, oocytes retain the ability to eventually enter M-phase, carrying with them the potential for chromosomal aberrations, such as aneuploidy, translocations, chromatid interchanges and breaks (Tease, 1983; Jacquet *et al.*, 2005; Marangos and Carroll, 2012; Yuen *et al.*, 2012; Ma *et al.*, 2013; Lin *et al.*, 2014). Since the integrity of an oocyte's DNA is essential for reproductive success (Ashwood-Smith and Edwards, 1996; Carroll and Marangos, 2013; Titus *et al.*, 2013), it is unclear why the DNA damage threshold for checkpoint activation is elevated in mature GV oocytes. One possible explanation is that there is a selective advantage to repairing the damage later during the cell cycle rather than activating cell cycle checkpoints during the resumption of meiosis. In this sense, it may be a better metabolic 'investment' to delay repair strategies until later in development. This notion accords with the energy demands imposed by DNA repair pathways. Indeed, research on various human syndromes and mouse models has established inherited errors in DNA damage repair pathways tend to appear in concert with pathologies attributed to perturbation of growth and energy metabolism (e.g. ataxia telangiectasia, premature aging, dysregulation of insulin signalling and lipid accumulation) (Hasty *et al.*, 2003; Martin, 2005; Lans and Hoeijmakers, 2006; de Magalhaes and Faragher, 2008). In the female germline, the two meiotic M-phases may therefore pose the only lines of defence against DNA damage mediated developmental abnormalities prior to preimplantation embryonic development.

Metaphase I

Within the G2/M stage of meiosis and, in the absence of a stringent G2/prophase checkpoint, a germline specific cell cycle arrest known as the spindle assembly checkpoint (SAC) is tasked with protecting the integrity of the female germline (Collins *et al.*, 2015; Marangos *et al.*, 2015). The SAC primarily functions by monitoring the status of kinetochore–microtubule attachment during spindle assembly, preventing aneuploidy by inhibiting the activity of the anaphase-promoting complex (APC) before chromosomes are ready to faithfully divide (Musacchio, 2011). The SAC may therefore act as the

Table II Transcriptomic analysis of the DNA repair genes in the human MII oocyte and blastocyst.

Pathway	Transcript	MII oocyte	Blastocyst
Base Excision Repair (BER)	APEX1: Apurinic/Apyrimidinic Endodeoxyribonuclease 1	High	High
	APEX2: Apurinic/Apyrimidinic Endodeoxyribonuclease 2	Low	Low
	LIG3: DNA Ligase 3	Not detected	Not detected
	MBD4: Methyl-CpG Binding Domain 4, DNA Glycosylase	High	Medium
	MPG: N-Methylpurine DNA Glycosylase	Medium	Not detected
	MUTYH: MutY DNA Glycosylase	Low	Low
	NEIL1: Nei-Like DNA Glycosylase 1	Medium	Low
	NEIL2: Nei-Like DNA Glycosylase 2	Not detected	Not detected
	NTHL1: Nth-Like DNA Glycosylase 1	Low	Low
	OGG1:8-Oxoguanine DNA Glycosylase	Medium	Low
	PARP1: Poly(ADP-Ribose) Polymerase 1	Medium	High
	PARP2: Poly(ADP-Ribose) Polymerase 2	Medium	High
	PNKP: Polynucleotide Kinase 3′-Phosphatase	Not detected	Not detected
	SMUG1: Single-Strand-Selective Monofunctional Uracil-DNA Glycosylase 1	Not detected	Medium
	TDG: Thymine DNA Glycosylase	Not detected	High
	UNG: Uracil DNA Glycosylase	High	High
	XRCC1: X-Ray Repair Cross Complementing 1	Low	Medium
Double Strand Break Repair (DSBR): Homologous Recombination (HR)	BRCA1: Breast Cancer 1	Not Detected	Medium
	BRCA2: Breast Cancer 2	Medium	Not detected
	DMC1: DNA Meiotic Recombinase 1	Not detected	Not detected
	EME1: Essential Meiotic Structure-Specific Endonuclease 1	Low	Low
	EME2: Essential Meiotic Structure-Specific Endonuclease Subunit 2	Medium	Medium
	MRE11A: Meiotic Recombination 11 Homolog 1	Medium	Medium
	MUS81: MUS81 Structure-Specific Endonuclease Subunit	Medium	Medium
	NBN (NBS1): Nijmegen Breakage Syndrome 1 (Nibrin)	Not detected	Not detected
	RAD50: RAD50 Recombinase	High	Low
	RAD51: RAD51 Recombinase	High	High
	RAD51C: RAD51 Paralog C	High	High
	RAD51L1: RAD51 Paralog B	Low	Not detected
	RAD51L3: RAD51 Paralog D	Not detected	Medium
	RAD52: RAD52 Homolog	High	High
	RAD54B: RAD54 Homolog B	Medium	Low
	RAD54L: RAD54 Like	Not detected	Not detected
	RBBP8: RB-Binding Protein 8, Endonuclease	High	Medium
	SHFM1: Split Hand/Foot Malformation (Ectrodactyly) Type 1	Low	High
	XRCC2: X-Ray Repair Cross Complementing 2	Low	Low
	XRCC3: X-Ray Repair Cross Complementing 3	Not detected	Not detected
Double Strand Break Repair (DSBR): Non-Homologous End-Joining (NHEJ)	DCLRE1C (Artemis): DNA Cross-Link Repair 1 C	Not Detected	Medium
	LIG4: DNA Ligase 4	Not Detected	Not detected
	PRKDC: Protein Kinase, DNA-Activated, Catalytic Polypeptide	Not detected	Not detected
	XRCC4: X-Ray Repair Cross Complementing 4	Medium	Medium
	XRCC5 (Ku80): X-Ray Repair Cross Complementing 5	Medium	High
	XRCC6 (Ku70): X-Ray Repair Cross Complementing 6	High	High

Continued

Table II *Continued*

Pathway	Transcript	MII oocyte	Blastocyst
Mismatch Repair (MMR)	MLH1: MutL Homolog 1	Medium	High
	MLH3: MutL Homolog 3	Not detected	Not detected
	MSH2: MutS Homolog 2	High	High
	MSH3: MutS Homolog 3	Medium	Low
	MSH4: MutS Homolog 4	Not detected	Not detected
	MSH5: MutS Homolog 5	Low	Not detected
	MSH6: MutS Homolog 6	High	High
	PMS1: PMS1 Homolog 1, Mismatch Repair System Component	Medium	Medium
	PMS2: PMS1 Homolog 2, Mismatch Repair System Component	Medium	Medium
	PMS2L3: PMS1 Homolog 2, Mismatch Repair System Component Pseudogene 3	Not detected	Low
Nucleotide Excision Repair (NER)	CCNH: Cyclin H	High	Medium
	CDK7: Cyclin Dependent Kinase 7	High	High
	CETN2: Centrin 2	Not detected	Not detected
	DDB1: Damage Specific DNA Binding Protein 1	Medium	Medium
	DDB2 (LHX3): Damage Specific DNA Binding Protein 2	Not detected	Not detected
	ERCC1: ERCC Excision Repair 1, Endonuclease Non-Catalytic Subunit	Low	Medium
	ERCC2 (XPD): ERCC Excision Repair 2, TFIIH Core Complex Helicase Subunit	Low	Not detected
	ERCC3 (XPB): ERCC Excision Repair 3, TFIIH Core Complex Helicase Subunit	Not detected	Low
	ERCC4 (XPF): ERCC Excision Repair 4, Endonuclease Catalytic Subunit	Not detected	Not detected
	ERCC5 (XPG): ERCC Excision Repair 5, Endonuclease	Medium	Low
	ERCC6 (CSB): ERCC Excision Repair 6, Chromatin Remodeling Factor	Medium	Not detected
	ERCC8 (CSA/CKN1): ERCC Excision Repair 8, CSA Ubiquitin Ligase Complex Subunit	Low	Low
	GTF2H1: General Transcription Factor IIH Subunit 1	Medium	Medium
	GTF2H2: General Transcription Factor IIH Subunit 2	High	Medium
	GTF2H3: General Transcription Factor IIH Subunit 3	Not detected	Not detected
	GTF2H4 (VARSL): GTF2H4 (VARSL)	Low	Medium
	GTF2H5: General Transcription Factor IIH Subunit 5	High	High
	LIG1: DNA Ligase 1	High	Medium
	MMS19L (MMS19): MMS19 Homolog, Cytosolic Iron-Sulfur Assembly Component	Medium	High
	MNAT1: Menage A Trois one CDK-Activating Kinase Assembly Factor	Medium	Medium
	RAD23A: RAD23 Homolog A	Low	Medium
	RAD23B: RAD23 Homolog B	Not detected	High
	RPA1: Replication Protein A1	Medium	Low
	RPA2: Replication Protein A2	High	High
	RPA3: Replication Protein A3	Not detected	Low
	XAB2: XPA Binding Protein 2	Not detected	Not detected
	XPA: Xeroderma Pigmentosum Group A-Complementing Protein	High	High
	XPC: Xeroderma Pigmentosum, Complementation Group C	Low	Not detected
General	CHAF1A: Chromatin Assembly Factor 1 Subunit A	High	High
	H2AFX: H2A Histone Family Member X	High	High
	DEPC-1: Alkylated DNA Repair Protein Alpha-Ketoglutarate dependent Dioxygenase Homolog 3	Medium	Low

Continued

Table II *Continued*

Pathway	Transcript	MII oocyte	Blastocyst
	ALKBH2 (ABH2): Alpha-Ketoglutarate-Dependent Dioxygenase AlkB Homolog 2	High	High
	MGMT: *O*-6-Methylguanine-DNA Methyltransferase	High	Medium
	RAD18: RAD18	Not detected	Medium
	UBE2A: Ubiquitin Conjugating Enzyme E2 A	High	High
	UBE2B: Ubiquitin Conjugating Enzyme E2 B	Medium	Medium
	UBE2N: Ubiquitin Conjugating Enzyme E2 N	Medium	Medium
	UBE2V2: Ubiquitin Conjugating Enzyme E2 V2	Medium	High
	NUDT1: Nudix Hydrolase 1	Medium	High
	DUT: Deoxyuridine Triphosphatase	High	High
	TDP1: Tyrosyl-DNA Phosphodiesterase 1	High	Low
	RRM2B: Ribonucleotide Reductase Regulatory TP53 Inducible Subunit M2B	Not detected	Low
	MAD2L2: Mitotic Arrest Deficient 2 Like 2	Medium	Medium
	PCNA: Proliferating Cell Nuclear Antigen	High	High
	POLB: DNA Polymerase Beta	High	High
	POLD1: DNA Polymerase Delta 1, Catalytic Subunit	Not detected	Low
	POLE: DNA Polymerase Epsilon, Catalytic Subunit	Low	Low
	POLG: DNA Polymerase Gamma, Catalytic Subunit	Low	Medium
	POLH: DNA Polymerase Eta	Not detected	Not detected
	POLI: DNA Polymerase Iota	Low	Not detected
	POLK: DNA Polymerase Kappa	Low	Not detected
	POLL: DNA Polymerase Lambda	Low	Low
	POLM: DNA Polymerase Mu	Not detected	Low
	POLN: DNA Polymerase Nu	Not detected	Not detected
	POLQ: DNA Polymerase Theta	Medium	Low
	REV1L: Rev1-Like Terminal Deoxycytidyl Transferase	High	Medium
	REV3L: REV3-Like, DNA Directed Polymerase Zeta Catalytic Subunit	Not detected	Not detected
	EXO1: Exonuclease 1	Medium	Medium
	FEN1: Flap Structure-Specific Endonuclease 1	High	High
	FLJ35220: FLJ35220	Not detected	Medium
	SPO11: SPO11, Initiator of Meiotic Double Stranded Breaks	Not detected	Not detected
	TREX1: Three Prime Repair Exonuclease 1	Not detected	Not detected
	TREX2: Three Prime Repair Exonuclease 2	Medium	Medium
	ATM: Ataxia Telangiectasia Mutated	Not detected	Not detected
	BLM: Bloom Syndrome RecQ-Like Helicase	High	High
	FAAP24 (C19orf40): Fanconi Anemia Core Complex-Associated Protein 24	Low	Low
	FANCA: Fanconi Anemia Complementation Group A	Not detected	Not detected
	FANCB: Fanconi Anemia Complementation Group B	Medium	Low
	FANCC: Fanconi Anemia Complementation Group C	Medium	Not detected
	FANCD2: Fanconi Anemia Complementation Group D2	Low	Low
	FANCE: Fanconi Anemia Complementation Group E	Not detected	Low
	FANCF: Fanconi Anemia Complementation Group F	Low	Not detected
	FANCG: Fanconi Anemia Complementation Group G	Not detected	Medium
	FANCL: Fanconi Anemia Complementation Group L	High	Medium
	FANCM: FANCM	Medium	Low
	FANCN (PALB2/FLJ21816): Partner and Localizer of BRCA2	Medium	Not detected

Continued

Table II *Continued*

Pathway	Transcript	MII oocyte	Blastocyst
	KIAA1794: Fanconi Anemia Complementation Group I	High	Medium
	RECQL4: RecQ-Like Helicase 4	Low	Low
	WRN: Werner Syndrome RecQ-Like Helicase	Medium	Low
	ATR: Ataxia Telangiectasia and Rad3-Related Protein	Medium	Not detected
	CHEK1: Checkpoint Kinase 1	Not detected	Medium
	CHEK2: Checkpoint Kinase 2	Medium	Not detected
	CLK2: CDC-Like Kinase 2	Not detected	Not detected
	HUS1: HUS1 Checkpoint Clamp Component	Not detected	Not detected
	MDC1: Mediator of DNA Damage Checkpoint 1	Not detected	Medium
	PER1: Period Circadian Regulator 1	Medium	Medium
	RAD1: RAD1 Checkpoint DNA Exonuclease	Medium	Medium
	RAD17: RAD17 Checkpoint Clamp Loader Component	High	High
	RAD9A: RAD9 Checkpoint Clamp Component A	Medium	Low
	TP53:Tumor Protein P53	Not detected	Not detected
	APTX: Aprataxin	Medium	High
	DCLRE1A: DNA Cross-Link Repair 1A	High	Medium
	DCLRE1B: DNA Cross-Link Repair 1B	Low	Not detected
	HEL308: Helicase, POLQ Like	Low	Low
	NEIL3: Nei-Like DNA Glycosylase 3	Not detected	Not detected
	RDM1: RAD52 Motif Containing 1	Not detected	Not detected
	RECQL: RecQ-Like Helicase	Low	Medium
	RECQL5: RecQ-Like Helicase 5	Not detected	Not detected
	RPA4: Replication Protein A4	Low	Not detected

Transcriptional inactivation of the oocyte during ovulatory recruitment sees a dramatic change in the gene expression profiles that accompany the follicular growth phase. A developmentally crucial pool of mRNA transcripts (~30%) are translationally repressed and stored in preparation for the final stages of meiotic maturation phase and the initiation of early embryonic development. Of these genes, those that encode proteins involved in DNA repair response(s) are significantly enriched. Notably, genes involved in single-strand break repair, double-strand break repair, mismatched base repair, nucleotide-excision repair and base excision repair have all been identified in the oocyte and blastocyst with an unprecedented enrichment for a significant number of these (107) in the ovulatory stage oocyte. The level of expression and/or the absence of expression is indicated by a colour scale where white denotes a transcript which was not detected. The table was adapted from Jaroudi *et al.* (2009).

primary gatekeeper during GV breakdown (passage from GV-metaphase I (MI) oocyte). This may also be true of the transition from MI to MII oocytes, where spindle assembly takes place, co-ordinated by a protein complex that comprises MAD2 (mitotic arrest deficient 2), BUB1 (budding uninhibited by benzimidazoles 1), BUBR1 (BUB1-related protein 1) and MPS1 (Monopolar spindle 1). This complex accumulates on microtubule-free kinetochores during spindle assembly to form the cytoslic APC inhibitory complex to arrest the cell cycle (Musacchio and Salmon, 2007; Elowe, 2011; Homer, 2011; Gui and Homer, 2012; Lara-Gonzalez *et al.*, 2012). If triggered, the SAC only relinquishes cell cycle arrest when all kinetochores are stably bound to microtubules, alleviating the inhibition of anaphase so that the cell cycle can continue (Lara-Gonzalez *et al.*, 2012).

SAC activation can occur in response to genomic instability induced by ovo-toxicants (e.g. bleomycin, phleomycin [PHL], DOX and ETP), ultraviolet B and ionising radiation (Collins *et al.*, 2015; Marangos *et al.*, 2015). SAC activation is correlated with a failure to complete the MI to MII transition by virtue of a long-lived cell cycle arrest (~25 hr) (Collins *et al.*, 2015). This protracted period far exceeds the timeframe required to stimulate and enact DNA repair (2–6 hr) (Kinner *et al.*, 2008), and it is therefore unlikely that the purpose of this arrest is to provide an opportunity for DNA repair. Rather, it has been argued that this arrest may act to substantially desynchronise oocytes from the developmental cascade (Collins *et al.*, 2015). That is, undamaged oocytes will have undergone MI completion (9 hr post-ovulation), have been ovulated (10–11 hr post-ovulation) and fertilised by (15–18 hr post-ovulation), all prior to the release of the damaged cells from their arrested state (Howlett and Bolton, 1985). In agreement with this hypothesis, oocytes evaluated >10 hr post-ovulation show a significantly reduced ability to participate in fertilisation and produce viable embryos (Lord *et al.*, 2013). Thus, even if a damaged oocyte were to escape the SAC, it would likely be precluded from participating in fertilisation.

This interplay between DNA damage and SAC activation remains an entirely novel meiotic defence mechanism, as mitotic DNA damage appears to retain no capacity to activate the SAC (Skoufias *et al.*, 2004). The precise mechanistic link(s) between DNA damage and SAC activation remain elusive but it seems likely that, in the absence of another checkpoint, the protracted length of meiosis (several hours in mouse) compared with mitosis, may allow a DNA-damage signal to propagate for long enough to stimulate SAC mediated arrest. Regardless, the existence of such an effective DNA

checkpoint, which functions to prevent oocytes with genomic instability from progressing to fertilisable mature eggs, provides an important line of defence against mutagenesis in the female germline. Accordingly, emerging data now suggest that age-related compromise of the SAC may be the leading cause of increased chromosomal anomalies in oocytes and embryos from older mothers (Marangos et al., 2015). With up to 50% of oocytes from aged mothers (mice >50 weeks of age) progressing through MI whilst harbouring DNA damage, compared with only 12% of oocytes in young controls (Marangos et al., 2015), this represents a pressing concern; particularly with the current trend for women to frequently delay childbirth (Australian Institute of Health and Welfare, 2015). Adding to this concern are data that oocytes that elude SAC arrest not only experience a significant delay in the timing of polar body extrusion, but also display gross abnormalities including deformed spindle structures, chromosome configuration and cytoplasmic chromatin (Marangos and Carroll, 2012; Collins et al., 2015; Marangos et al., 2015). Improved understanding of the molecular mechanism(s) governing the interplay between DNA damage and SAC activation therefore holds considerable promise for developing novel reproductive interventions to address the age-related decline in female fertility.

The post-ovulatory oocyte (MII arrest)

As reviewed above, mature GV and MI stage oocytes are largely refractory to DNA repair. While it is not yet clear why these cell stages favour cell cycle arrest over the activation of major DNA damage repair pathways, recent research has begun to address a similar question in later stage mature MII oocytes. Like their GV stage counterparts, the mature ovulatory stage oocyte remains transcriptionally silent and must therefore rely on endogenous stores of pre-synthesised proteins and/or mRNA transcripts accumulated during oogenesis to effect DNA repair (Kocabas et al., 2006). Notably, the mouse oocyte proteome comprises some 53 proteins putatively involved in DNA repair, recombination and replication, which are represented during the GV, MII and zygotic stages of their development (Table I). More specifically, these proteins encompass a subset that have been linked to DSB DNA repair, single-strand break (SSB) repair, nucleotide-excision repair (NER) and base excision repair (BER) pathways (Wang et al., 2010). In addition, a detailed transcriptomic analysis of mRNA present in MII oocytes and blastocysts, identified 107 of the 154 transcripts that give rise to proteins implicated in DNA repair. Among differentially expressed DNA repair genes, 40/55 (73%) had lower expression levels in blastocysts compared with MII oocytes (Table II) (Jaroudi et al., 2009).

Despite this evidence, the efficacy of DNA repair activity in MII oocytes has only been reported in two publications to date (Lord and Aitken, 2015; Martin et al., 2018). The paucity of interest in DNA repair at this developmental time point likely reflects the long-held notion that the role of the MII oocyte is to furnish the DNA repair machinery (mRNA transcripts and proteins) required by the zygote to enact DNA repair immediately after fertilisation. Nonetheless, the impetus to study the DNA damage response in MII oocytes rests with our recent findings that these cells are rendered highly vulnerable to DSB DNA damage owing to an inability to engage membrane defences belonging to the multidrug resistance (MDR), or ATP-binding cassette (ABC), transporter superfamily of proteins (Martin et al., 2016a,b). Such proteins reside in the plasma membrane of a wide range of somatic cells, wherein they act as efflux pumps effectively preventing the accumulation of cytotoxic agents before they have the potential to elicit widespread intracellular damage. A key feature of the MDR family is their broad substrate specificity, thus enabling them to fulfil an important first line of cellular defence against a plethora of chemically unrelated compounds (Aller et al., 2009). This has also proven true of the female germline, where the activity of permeability glycoprotein (PGP) confers a MDR phenotype thereby limiting the detrimental impact of genotoxic drugs such as ETP. Notably however, such PGP activity has only been substantiated in fertilised oocytes, with no such activity having been recorded in the MII oocyte prior to fertilisation (Hamdoun et al., 2004; Roepke et al., 2006; Martin et al., 2016a,b). Indeed, the MII oocyte possesses only modest levels of the PGP protein and, what little PGP is expressed in these cells, is distributed throughout the cytosol rather than at its site of action in the plasma membrane. As a consequence, the MII oocytes are not afforded this first line of protection. Instead, these cells harbour a significant and, hitherto unappreciated, enrichment of DNA repair enzymes (Tables I and II), thus inferring that the MII oocyte may be very well equipped to resolve DNA lesions, compared to their zygote counterparts (Zeng et al., 2004; Zheng et al., 2005; Jaroudi et al., 2009; Wang et al., 2010).

Represented amongst this subset of the MII proteome are numerous proteins implicated in each of the recognised DNA repair pathways, namely: SSB, BER, NER and DSB repair via both HR and NHEJ (Tables I and II). Examination of the BER capacity of mouse MII oocytes has revealed that these cells possess basal levels of BER activity and that this pathway is capable of repairing the oxidatively induced DNA lesion, 8-hydroxy-2′-deoxyguanosine (8OHdG) (Lord and Aitken, 2015). Indeed, the MII oocyte appears to contain all of the necessary proteins required to implement 8OHdG lesion repair, a strategy that may help minimise the oxidative DNA damage burden carried by the oocyte and thus decrease the potential for mutagenesis in resultant embryos. The only notable limitation is a relative lack of OGG1, the first enzyme in the BER pathway (Lord and Aitken, 2015). However, upon fertilisation, a suite of post-translational modifications stimulates activation and translocation of key BER repair proteins to the pronucleus. The upregulation of OGG1 during this process permits accelerated excision of oxidatively damaged DNA in the zygote. Together, these data suggest that the oocyte does possess the ability to enact DNA repair prior to fertilisation and, encouragingly, also indicate that this capacity may be further enhanced following the union of the gametes at the moment of fertilisation.

Beyond oxidatively induced DNA lesions, many other forms of DNA damage can precipitate developmentally significant sequelae leading to compromised offspring vitality. Among these, DNA DSB ranks as one of the most pervasive and potentially detrimental. It is known that, prior to completing the second M phase, the immature oocyte is incapable of mitigating the impact of DSB, or repairing this damage once it has been inflicted. However, based on our recent findings, we now appreciate that the post-ovulatory MII oocyte is able to repair DSB DNA damage via NHEJ (Martin et al., 2018). In establishing this paradigm, MII oocytes were cultured in the presence of the genotoxic agents ETP, PHL or DOX, each of which are

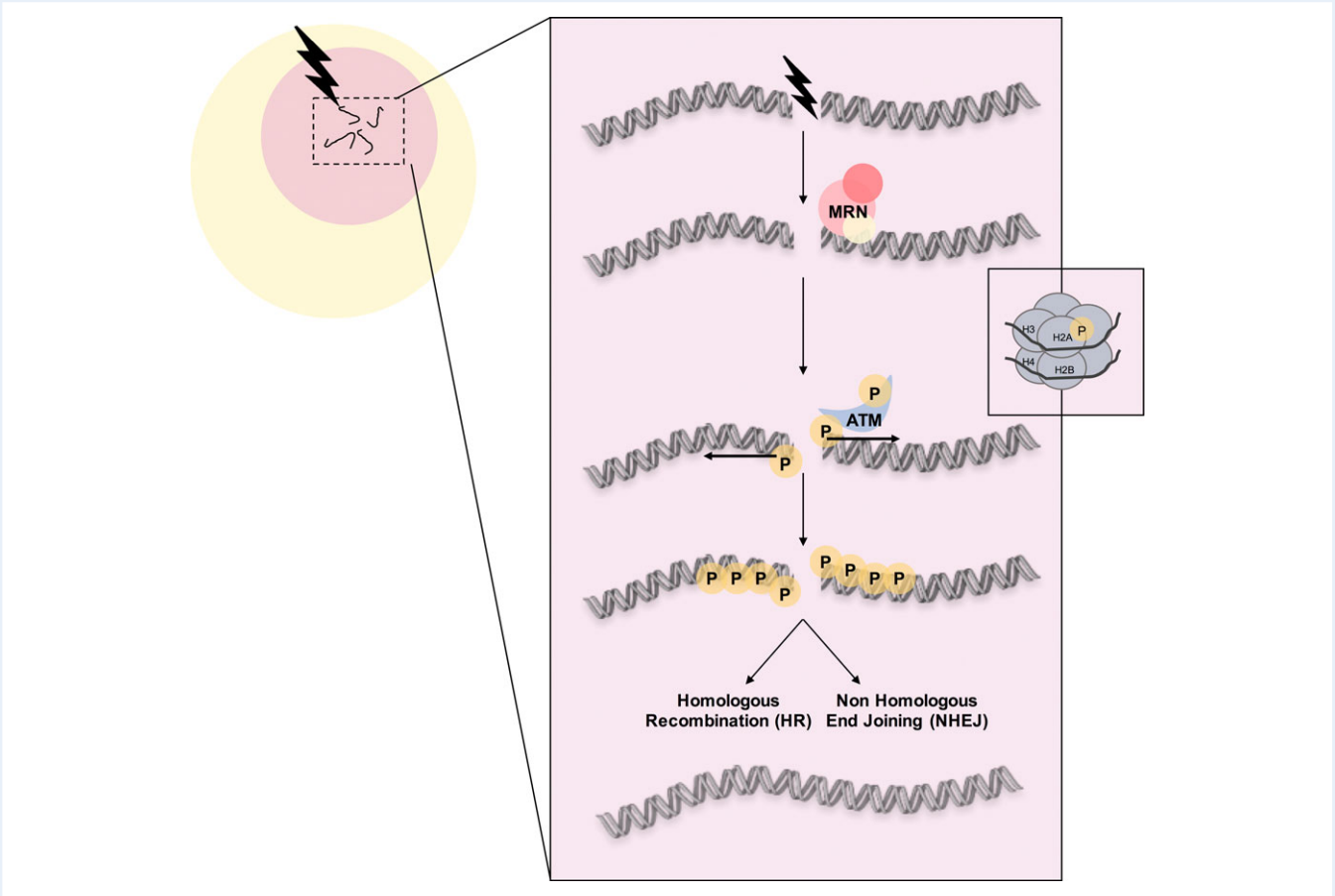


Figure 3 Activation of the double-strand break (DSB) response pathways. The surveillance mechanisms utilised by cells to detect the induction of DSB DNA damage involves the MRN (comprising the protein subunits MRE11, RAD50 and NBS1) complex. MRN acts as an initial sensor, binding specifically to sites of DSB DNA damage and triggering ATM kinase to self- phosphorylate. ATM subsequently catalyses the phosphorylation of histone H2AX at serine 139 to form γH2AX. Finally, this post-translational modification then propagates around the damage site enabling DNA repair proteins to dock and repair damage. DSB repair may occur through two canonical pathways; homologous recombination (HR) and/or non-homologous end-joining (NHEJ). Decisions regarding which repair pathway will conduct repair are driven by the cell cycle.

capable of inducing robust DSB DNA damage (detected via γH2AX labelling). During subsequent kinetic profiling of γH2AX resolution, we witnessed a substantial decline in this biomarker (to basal levels equivalent to the untreated controls) by 4 hr post-treatment; a timeframe consistent with that expected following the recruitment of DSB DNA repair machinery. Accordingly, we were able to confirm the presence of several key enzymes within this pathway, namely: ATM, X-ray repair cross complementing 5 (KU80/XRCC5), DNA PKcs and proliferating cell nuclear antigen (Table I). Moreover, selective pharmacological inhibitors of DNA PKcs (NU7441) and DNA ligase IV (SCR7) were employed to establish the functional significance of NHEJ, both of which effectively prevented γH2A.X resolution. These data represent the first functional characterisation of an active DNA repair pathway in the mature mammalian MII oocyte and encourage a reappraisal of the paradigm that, beyond the early follicular phases of development, oocytes possess relatively inefficient DNA damage response mechanisms and are therefore largely refractory to DNA repair.

Fertilisation and preimplantation embryo protection

Maternal resources for DNA damage repair in early embryogenesis

Of all the oocyte's developmental stages, the best understood protective strategies are those elaborated by the preimplantation embryo. The current, widely accepted paradigm posits that the oocyte assumes responsibility for the repair and remodelling of both the maternal and paternal genomes during the oocyte-embryo transition (Aitken and De Iuliis, 2007; Gonzalez-Marin *et al.*, 2012; Yamauchi *et al.*, 2012; Fernandez-Diez *et al.*, 2015). Damage inherited by the embryo from either gamete must be repaired prior to S phase of the first mitotic division, in order to subvert the risk of

mutagenesis and the attendant dysregulation of cell differentiation and development in the zygote. Despite this, the zygote has several unique features that influence its capacity for effective DNA repair, including: a complete absence of transcription-coupled translation (Schultz, 2002; Hamatani *et al.*, 2004; Bianchi and Sette, 2011); TOP2 mediated remodelling of sperm derived chromatin resulting in the formation of extensive, albeit transient, DSBs (Adenot *et al.*, 1991; Bizzaro *et al.*, 2000; Derijck *et al.*, 2006; Cortes-Gutierrez *et al.*, 2014); and the absence of a traditional G1–S checkpoint following gamete fusion (Shimura *et al.*, 2002; Baart *et al.*, 2004; Adiga *et al.*, 2007; Carbone and Chavez, 2015).

In considering the implications of each of these features, the lack of transcription-coupled translation indicates that all DNA repair in the preimplantation embryo must be of maternal origin. That is, in a situation akin to the mature MII oocyte, the embryo relies on endogenous stores of mRNA transcripts and proteins accumulated during oogenesis in order to correct DNA damage. This remains true until the maternal/zygotic transition, which mostly occurs at the 2-cell stage of embryo development in mice, and the 4- to 8-cell stage in the human embryo (Gurtu *et al.*, 2002; Marchetti *et al.*, 2007; Derijck *et al.*, 2008; Menezo *et al.*, 2010). Interestingly, the bulk of inherited DNA damage appears to come from the paternal germline, as spermatozoa harbouring significant levels of DNA damage commonly retain the ability to negotiate the female reproductive tract, reach the site of fertilisation and fertilise oocytes (both *in vivo* and *in vitro*) (Matsuda *et al.*, 1989; Twigg *et al.*, 1998; Zenzes, 2000; Simon *et al.*, 2014; Sivanarayana *et al.*, 2014; Garcia-Ferreyra *et al.*, 2015; Marchetti *et al.*, 2015).

The contribution of spermatozoa to DNA damage and repair at fertilisation

It is the paucity of DNA repair mechanisms, and an absence of antioxidant capacity, in the mature spermatozoon that dictate that this cell is especially vulnerable to the accumulation of genomic damage, yet is also incapable of repairing this damage (Smith *et al.*, 2013). An active DNA repair programme is therefore mandatory in the zygote to counter the possibility of paternal transmission of mutagenesis in the germline (Derijck *et al.*, 2008). To enable repair of the paternal genome, it must first undergo restoration of a nucleosomal chromatin structure to facilitate the access of signalling and repair factors (Ahmed *et al.*, 2015). This process requires the progressive displacement of protamines by replacement proteins and finally histones (Barral *et al.*, 2017). To facilitate the unique topological rearrangements permissive of chromatin decondensation, a wave of transient DSB are induced by TOP2, but must then be repaired prior to initiation of S phase (Adenot *et al.*, 1991, 1997; Bizzaro *et al.*, 2000; Derijck *et al.*, 2006).

The primary surveillance and signalling mechanisms for DSB produced during decondensation appear to involve phosphorylation of histone variant H2AX (γH2AX). In fact, the mouse zygote contains more of the histone H2A variant, H2AX, than any other cell; suggesting that mouse embryos not only have the capacity to recognise and respond to damaged DSB DNA but also that they are exquisitely sensitive to damage (Rogakou *et al.*, 1998; Nashun *et al.*, 2010; Gawecka *et al.*, 2013). Accordingly, mRNA templates encoding 12/19 proteins involved in HR repair and 3/6 proteins involved in NHEJ, the canonical DSB repair pathways, have been detected in mouse embryos (Jaroudi *et al.*, 2009). Specifically, NHEJ is active during the replacement of sperm protamines by nucleosomes after which both HR and NHEJ are operative in S-phase, correcting damage that may be inherited by both gametes (Generoso *et al.*, 1979; Matsuda *et al.*, 1989; Derijck *et al.*, 2008).

With respect to other common forms of DNA damage, the zygote has been shown to contain the necessary protein machinery to effect repair of the oxidative DNA lesion, 8OHdG (Lord and Aitken, 2015) (see above). As an important caveat however, the ooplasm was found to contain only relatively low levels of OGG1, the enzyme required to excise the oxidatively damaged base (Lord and Aitken, 2015). By contrast, the fertilising spermatozoon carries with it a substantial amount of OGG1 (Smith *et al.*, 2013), which may serve to supplement the endogenous levels of the protein contributed by the oocyte, and thus collude to create abasic sites that can readily be resolved by downstream elements of the BER pathway; apurinic endonuclease 1 (APE1) and X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1), both of which are abundantly present within the oocyte. Intriguingly, mammalian sperm do not possess either APE1 or XRCC1. The presence of two truncated BER pathways in the respective germlines therefore raises the prospect that the gametes must collaborate to effect the repair of oxidative DNA damage within the zygote irrespective of which cell contributed this damage. Cumulatively, these mechanisms are critically important for protecting the genetic integrity of the zygote to allow for unimpeded transition through embryogenesis.

Quality control in the early embryo

Fertilisation-associated post-translational protein modification is a widespread event (reviewed in Martin *et al.*, 2017) and, in the absence of active gene expression and *de novo* protein translation, is likely to serve as the requisite signal that 'reprograms' the oocyte towards embryogenesis and away from default pathways of senescence and apoptosis (Howlett and Bolton, 1985). Critically, this appears to be especially true of DNA repair enzymes, but it may also hold true for elements of the cell's first line of defence, such as those of the transmembrane transporter proteins of the MDR/ABC superfamily. As discussed above (see 'The post-ovulatory oocyte (MII arrest)'), these proteins mediate the transport of many chemically unrelated toxins across cellular membranes, thereby precluding their intracellular accumulation (Aller *et al.*, 2009). In the mouse zygote, one such transporter protein, PGP, experiences a rapid activation immediately after fertilisation owing to post-translational modification. Specifically, PGP undergoes phosphorylation at threonine and, to a lesser extent, serine residues driven by a surge in intracellular calcium (Ca^{2+}) oscillations (Martin *et al.*, 2016a,b). Once activated, likely via the action of either protein kinase A (PKA) and/or PKC serine/threonine kinases (Mechetner *et al.*, 1998; Idriss *et al.*, 2000; Lelong-Rebel *et al.*, 2003; Martin *et al.*, 2016a,b), PGP increases the bi-directional transport capacity of the embryo, directing genotoxic agents out of the intracellular environment and away from the vulnerable genomic material (Roepke *et al.*, 2006; Martin *et al.*, 2016a,b), while shuttling hormones and amino acids from the surrounding environment into the zygote to facilitate growth and development (Elbling *et al.*, 1993; Hamdoun *et al.*, 2004; Roepke *et al.*, 2006; Martin *et al.*, 2016a,b).

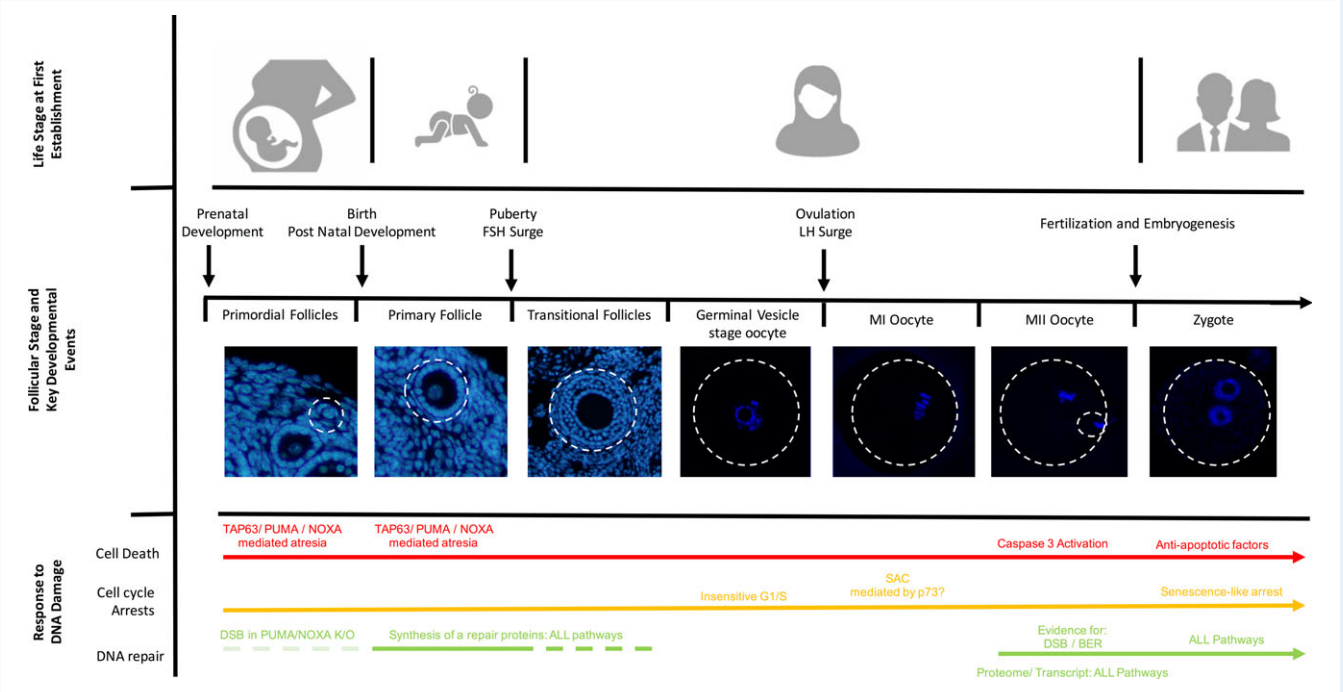


Figure 4 Mammalian ovarian development and the DNA damage response. The capacity of the mammalian oocyte for damage detection, repair or elimination of oocytes with compromised genomic fidelity differs dramatically during oogenesis. Extreme longevity in combination with dramatic changes in chromatin structure, transcriptional activity and chromosome number dictate the diverse responses employed by the oocyte at each developmentally important stage. In the immature primordial and primary oocyte stages, damage detection is primarily mediated by TAp63 expression leading to cell death following the induction of widespread DNA damage, with significant consequences for fertility. Hereafter, inactivation of TAp63 is coincident with the follicular phase of development, which is characterised by an over-representation of genes that encode proteins involved in DNA repair response(s); a strategy that safeguards the genomic integrity in the absence of TAp63. Accordingly, it is likely that at this stage in development, the immature oocyte may gain the capacity to respond to DNA damage via the activation of classic DNA repair pathways. However, little functional evidence has been collected during this developmental timeframe. In addition, during the growth phase a significant and developmentally crucial pool of mRNA transcripts (~30%) are translationally repressed and stored in preparation for the transcriptionally inactive meiotic maturation phase and initiation of early embryonic development. At this point the spindle assembly checkpoint has also been reported to prevent the propagation of DNA damage during the transition from GV-metaphase I (MI). While novel, evidence now suggests that the metaphase II (MII) oocyte, in addition to the pronuclear zygote, can carry out effective DNA repair.

As this example illustrates, the zygote is capable of mounting diverse protective strategies to mitigate the risk imposed by an elevated mutational load being carried by the offspring. Nevertheless, early stage mammalian embryogenesis lacks several traditional elements of the canonical somatic cell DNA damage response pathways, including a G1/S and G2/M checkpoint, as well as an ability to undergo programmed cell death (Shimura *et al.*, 2002; Baart *et al.*, 2004; Adiga *et al.*, 2007). Recent evidence now suggests that auxiliary to DNA repair, the preimplantation embryo has adopted a series of non-conventional cell 'removal' processes. Deficiencies in traditional G1/S and G2/M checkpoints in the zygote mean that embryos carrying substantial DNA damage can still progress through development, prior to the establishment of a functional apoptotic pathway during the latter stages of preimplantation embryonic development (Shoukir *et al.*, 1998; Dumoulin *et al.*, 2000). In this regard, zygotes are capable of recognising and responding to DNA damage but are shielded from cell death by anti-apoptotic protection; a strategy that may provide additional opportunities for DNA repair and the preservation of fertility. Notably, hallmarks of apoptosis, such as cytoplasmic fragmentation, are evident as early as the first cell cycle in mouse embryos at the second cell cycle in their human counterparts (Jurisicova *et al.*, 1996, 1998; Byrne *et al.*, 1999). By contrast, other apoptotic characteristics, including chromatin and cytoplasmic condensation, followed by DNA degradation and cell shrinkage, as well as marginalisation and nuclear fragmentation (Jurisicova *et al.*, 1998), have not been observed until the formation of morulae and blastocysts (Hardy *et al.*, 1989; Hardy, 1997; Byrne *et al.*, 1999; Matwee *et al.*, 2000; Gjorret *et al.*, 2003), after which an active apoptotic programme is achieved. The downregulation of an apoptotic programme may have evolved to enable corrective strategies to ensure the quality of gamete/embryos and preserve fertility. Importantly, the understanding of such diverse mechanisms may also provide the distinctive biological insight that leads to novel interventions to preserve fertility in a clinical setting.

Applications and future perspectives

The evidence summarised in this review highlights that the endogenous quality control mechanisms employed by the female germline vary significantly during the distinct phases of their development. Such information holds promise in terms of informing rational strategies to fortify the genomic integrity, and overall health, of the oocyte during the natural ageing process and/or in the context of *in vitro* manipulation during assisted reproduction. The natural ageing process poses a major challenge to a woman's reproductive health, significantly reducing reproductive potential and simultaneously elevating the prospect of embryonic chromosomal abnormalities (Li *et al.*, 2012). Such pathologies are intimately tied to the attrition of the ovarian follicular reserve (Zhang and Liu, 2015) and a spectrum of lesions in spindle formation, cell cycle control, survival factors, DNA integrity and chromosome separation (Tatone *et al.*, 2008; Tatone, 2008). Among the potential underlying aetiologies responsible for deterioration of the ageing ovarian microenvironment (Tatone *et al.*, 2008), multiple studies have implicated an overproduction of reactive oxygen species (ROS) (Takahashi *et al.*, 2003; Lord *et al.*, 2013; Benkhalifa *et al.*, 2014; Lord and Aitken, 2015), an impaired capacity for spindle maintenance (Marangos *et al.*, 2015) and compromised proficiency for DNA repair (Titus *et al.*, 2013). These causative mechanisms therefore represent prime targets for therapeutic interventions to prolong the fertile window and aid in the diagnosis and prevention of premature ovarian failure.

While conceding that intraovarian ROS may arise from multiple sources (Tatone *et al.*, 2008, 2015; Eichenlaub-Ritter *et al.*, 2011), the elevated levels of oxidative damage repeatedly documented within aged oocytes implicates defective mitochondrial function and electrophilic aldehyde production as a potential contributors (Lord and Aitken, 2015; Mihalas *et al.*, 2017). This raises the prospect of prophylactic administration of traditional antioxidant cocktails to scavenge or otherwise mitigate the bioavailability of damaging ROS. In the context of post-ovulatory aging, antioxidant supplementation has made considerable strides toward ensuring oocyte quality during culture procedures. Melatonin, a potent antioxidant capable of scavenging O_2^- and H_2O_2 (Papadopoulos *et al.*, 2002; Tan *et al.*, 2002), has been found to modulate silent information regulator (sirtuin) proteins (reviewed below) (Yang *et al.*, 2018), successfully preventing the accumulation of ROS within the oocyte: delaying the onset of apoptosis, extending the optimal window for fertilisation and improving the quality of embryos produced from oocytes that had been aged *in vitro* (Lord *et al.*, 2013). In the same manner, penicillamine, a nucleophilic thiol that directly reacts with electrophilic aldehydes (a downstream consequence of ROS) has been shown to attenuate electrophilic aldehyde-induced pathologies associated with the *in vitro* and maternal age-related declines in oocyte quality (Lord *et al.*, 2013; Mihalas *et al.*, 2017). Alternatively, the addition of pharmaceutical compounds that can specifically restore mitochondrial function could also hold considerable promise in *in vitro* culture. A recent study assessing the utility of endoplasmic reticulum stress inhibitors (salubrinal and BGP-15) to combat the effects of obesity-mediated oocyte damage provides an important precedent for this approach (Wu *et al.*, 2015). Briefly, pre-conception administration of these inhibitors to obese female mice effectively restored their oocyte quality, embryo development and the mitochondrial DNA content of foetal tissue to levels equivalent to those in lean mice (Wu *et al.*, 2015). The fact that these benefits were achieved during the final stages of oocyte development and maturation argues that the legacy of damage to oocyte organelles may be reversible. However, whether such protection extends to the female nuclear genome remains to be established.

As an alternative to the normalisation of oocyte organelle function, recent studies also allude to the merit of selectively activating key elements of the oocyte proteome, including those of the sirtuin family. In particular, SIRT2 holds a key role in the regulation of female fertility, with its aged-dependent loss contributing to an increased incidence of infertility, miscarriage and trisomic conceptions in both mice and humans (Nagaoka *et al.*, 2012; Zhang *et al.*, 2014; Qiu *et al.*, 2018). In terms of its mechanism of action, SIRT2 has been shown to mediate the essential deacetylation of lysine 243 on BUBR1 (BUBR1-K243R)(Qiu *et al.*, 2018), a core component of the SAC. Such findings lend support to the possibility of targeting SIRT2 for pharmacological activation as a means by which to counter the age-associated decline in oocyte quality; a notion supported by extensive research in mouse models that experience accelerated ageing phenotypes (North *et al.*, 2014). Notably, the druggability of sirtuins has been established in the context of numerous disease states (de Oliveira *et al.*, 2012), which have provided pre-clinical evidence for a multitude of SIRT activating compounds, including: resveratrol (3,5,4′-hydroxystilbene), SRT1720, SRT1460, SRT2183, imidazoquinoxalines, pyrroloquinoxalines and 1,4-dihydropyridines (Cohen *et al.*, 2004; Nayagam *et al.*, 2006; Boily *et al.*, 2008; Anderson *et al.*, 2009; Wang *et al.*, 2014; Valente *et al.*, 2016).

Aside from SIRT2, alternative members of the sirtuin family have also been implicated as key regulators of numerous DNA repair pathways. Indeed, the use of knockout mouse models has confirmed a causal link between SIRT1, SIRT6 and SIRT7 and the promotion of HR, BER and NHEJ pathways. Moreover, the loss of SIRT1, SIRT6 or SIRT7 exacerbates degenerative 'aging-like' phenotypes (Mostoslavsky *et al.*, 2006; McCord *et al.*, 2009; Uhl *et al.*, 2010; Mao *et al.*, 2012; Vazquez *et al.*, 2017). Considering that the oocyte is equipped with the necessary proteins to enact DNA repair (Jaroudi *et al.*, 2009; Wang *et al.*, 2010), yet seemingly remains recalcitrant in their utilisation, investigation of the ability of sirtuins to stimulate these pathways may form a novel means to encourage DNA repair during *in vitro* culture. Notably, resveratrol (a SIRT1 activator), has already found application as a media supplement for the IVM of oocytes from several mammalian species (murine, porcine and bovine); with favourable reports of significant improvements in oocyte quality and embryonic development (Liu *et al.*, 2013, 2018; Takeo *et al.*, 2014; Wang *et al.*, 2014, 2018; Itami *et al.*, 2015; Li *et al.*, 2016; Khan *et al.*, 2017; Lee *et al.*, 2018; Rocha *et al.*, 2018; Santos *et al.*, 2018). As an important extension of this model, i.p. injection of resveratrol has also been shown to alleviate the burden of DNA damage encountered in the male gamete following chronic exposure of mice to the environmental toxicant, acrylamide (Katen *et al.*, 2016). It is therefore tempting to speculate that the administration of a sirtuin activator (such as resveratrol) in either a reparative and/or a prophylactic context could offer promise in terms of alleviating oocyte DNA damage, meiotic defects and aneuploidy. At present however, it remains to be investigated whether resveratrol exerts any influence over the genomic integrity of the female germline.

More generally, maternal nutrition and lifestyle choices have emerged as important factors in the regulation of oocyte and embryo quality *in vivo* (Szostak and Laurant, 2011; Gilbert *et al.*, 2012; Fleming *et al.*, 2018). In this context, both over- and under- nutrition have been shown to significantly alter postnatal phenotype, affecting growth, physiological and metabolic parameters associated with the onset of adult non-communicable disorders (Fleming *et al.*, 2009). Notably, the lasting effects of obesity have been attributed to oxidative stress (Igosheva *et al.*, 2010; Kazemi *et al.*, 2013), leading to a consequential increase in meiotic spindle fragmentation, chromosome misalignment and clustering, DNA damage and epigenetic reprogramming (Jungheim *et al.*, 2011; Luzzo *et al.*, 2012).

Encouragingly, the implementation of dietary and exercise interventions can counter, at least in part, the adverse effects of obesity on reproduction (Moran *et al.*, 2009; Panidis *et al.*, 2013). Indeed, studies of obese women and experimental animal models have shown that the combination of diet and exercise can significantly improve pregnancy rate (Moran *et al.*, 2011; Sim *et al.*, 2014), cumulative live birth rate (Sim *et al.*, 2014; Mutsaerts, 2016; Espinos *et al.*, 2017) and reduce the incidence of miscarriage (Clark *et al.*, 1998). While it remains unclear how diet specifically modulates the adverse sequelae of obesity on reproduction, physical activity has been shown to significantly reduce NADPH oxidase activity and decrease superoxide anion production, circumventing ROS generation and normalising the metabolic status and endocrine function of oocytes in obese rats (Szostak and Laurant, 2011; Gilbert *et al.*, 2012). While encouraging information regarding the effects of exercise and diet for reproduction remain in their relative infancy, these data suggest that preventative strategies such as lifestyle interventions may hold considerable promise in improving the reproductive health of women.

The genomic integrity of the female germline in also challenged by procedures accompanying the increased uptake of ART, primarily in the form of cryopreservation, designed to extent the reproductive window. Indeed, the combined elements of freezing and thawing represent a dynamic process during which a number of mechanical, thermal and chemical factors can fluctuate over non-physiological ranges [reviewed in (Kopeika *et al.*, 2015)]. Historically, cryopreservation strategies were associated with significant losses in post-thaw vitality (Papadopoulos *et al.*, 2002; Lim *et al.*, 2007, 2008) owing to excursions in osmotic stress, hydrostatic pressure, intracellular ionic content and pH; all of which have the potential to negatively impact genomic integrity (Kultz and Chakravarty, 2001; McCarthy *et al.*, 2010; Kopeika *et al.*, 2015). Indeed, investigation of the impact of oocyte freeze-thawing has uncovered several detrimental consequences including, chromosomal misalignment, oxidative stress and an attendant increase in oxidative DNA damage (Coticchio *et al.*, 2009; Martinez-Burgos *et al.*, 2011), as well as elevations in sister chromatid exchange frequency, strand breakage and reductions in DNA repair capabilities (Galloway *et al.*, 1987; Kalweit *et al.*, 1990). Notably, each of these consequences may impair gene expression and ultimately affect the genetic and functional competence of oocytes and embryos (Aye *et al.*, 2010). Thus, maintaining the developmental potential of oocytes subsequent to cryopreservation is of little value under conditions in which their DNA can be adversely affected.

To alleviate the negative consequences associated with traditional freezing methods, advances in cryopreservation technologies have seen a shift toward the application of vitrification as the preferred freezing modality in clinical embryology laboratories. Vitrification procedures solidify samples into a 'glass-like state' by virtue of the application of extremely rapid cooling rates (15 000–30 000°C/min), thus avoiding intra- and extracellular ice formation (Vajta and Kuwayama, 2006), as well as modulating osmotic tension within tolerable limits (Fabbri *et al.*, 2001; Martinez-Burgos *et al.*, 2011) and thus eliminating key variables that would otherwise challenge the genomic integrity of cells during cryopreservation. Indeed, analysis of vitrified oocytes and embryos has confirmed statistically significant increases in embryo survival, implantation and clinical pregnancy compared to traditional slow-freezing protocols (AbdelHafez *et al.*, 2010). Despite this knowledge, very few studies have specifically addressed the issue of DNA damage/fragmentation during vitrification and even fewer provide a direct comparison between cryopreservation techniques. Further experiments investigating the effect of current cryopreservation regimens on genomic integrity relative to developmental competence are therefore critical for continued advances in fertility preservation.

Conclusion

The maintenance of gamete quality is a prerequisite for successful conception, embryonic development and pregnancy. In this review, we have explored the extant quality control measures employed by the female germline to mitigate the impact of DNA damage during development (Fig. 4). Improved understanding of how the protection of the female genome is integrated into normal developmental programmes has the potential to reveal critical insights to alleviate the burden of infertility. With this goal in mind, we contend that, in addition to tailored strategies devised on the basis of improved mechanistic understanding of oocyte biology, simple interventions such as lifestyle choices and/or technical improvements in ART protocols can have a profound impact on genomic integrity in the female germline and it is thus important to integrate such knowledge in devising the most effective strategies for fertility preservation.

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Authors' roles

J.H.M., B.N. and E.G.B conceived the thematic content of this manuscript. Primary literature review and manuscript preparation was performed by J.H.M. E.G.B., B.N. and R.J.A participated in literature review and manuscript editing.

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Conflict of interest

The authors have no conflicts of interest to disclose.

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CHAPTER 6:
APPLICATIONS AND FUTURE RESEARCH DIRECTIONS

The maintenance of gamete fidelity is a prerequisite for fertilisation, the establishment of a healthy pregnancy and normal embryonic development. In the studies described, we have explored the endogenous protection and repair machinery employed by the female germline to mitigate the impact of DNA damage during development. Improved understanding of how the protection of the female genome is integrated into normal developmental programs has the potential to contribute to novel means of alleviating the burden of infertility. With this goal in mind, we contend that future research should look towards improved methods of augmenting the activity of the oocyte's native mechanisms for the surveillance, detection and repair of DNA damage.

During the course of my candidature, I have uncovered several novel protective strategies, which the oocyte and early preimplantation embryo engage to ensure their genomic integrity. These include the identification of an unconventional defence mechanism involving enhanced cellular efflux activity associated with the expression of permeability glycoprotein (PGP; Chapter 2); a large ATP-dependent efflux pump belonging to the multidrug resistance (MDR) or ATP-binding cassette (ABC) transporter superfamily of proteins, which directs the transport of macromolecules across extra- and intracellular membranes (Aller et al., 2009). Our collective data demonstrate that PGP fulfils an important first line of defence in the zygote, increasing the bi-directional transport capacity precluding the induction of DNA damage following fertilisation (Martin et al., 2016a; Martin et al., 2016b) (Figure 1A). Notably however, PGP activity has only been substantiated in fertilised oocytes, with no such action having been recorded in the ovary or MII oocyte prior to fertilisation (Hamdoun et al., 2004; Martin et al., 2016a; Martin et al., 2016b; Roepke et al., 2006).

Thus, exploiting the potential of the PGP transmembrane transporter protein family for enhancing drug resistance is an intriguing avenue for the improvement of oocyte viability during *in vitro* manipulations and/or in the context of fortifying protection of the intra-ovarian oocyte reserve. As an example, discretional control over the expression of MDR-like transporters has emerged as a promising avenue for protection of oocytes during exposure to chemotherapeutic regimens, which otherwise elicit a devastating legacy on the quality of the female germline and hence a woman's fertility (Brayboy et al., 2017). Encouragingly, the expression of MDR proteins is not restricted to the mouse zygote, having recently been discovery in the ovarian stroma, granulosa and the follicle itself (Brayboy et al., 2017). As an important precedent for this approach, pharmacological interventions (e.g. forskolin, rifampicin, and/or progesterone) have been shown to elevate MDR transporter protein expression and function, and thereby significantly improve the cryotolerance of bovine and porcine embryos (Kfir-Erenfeld et al., 2010; Mori et al., 2013). Moreover, it has long been known that injection of mouse *Pgp* mRNA into *Xenopus* oocytes enables these cells to adopt a MDR-like phenotype. Thus, these oocytes express PGP and consequently exhibit a decreased accumulation of cytotoxic [^{3}H]vinblastine owing to an enhanced capacity to extrude the drug compared to control oocytes not expressing the PGP (Castillo et al., 1990). Although the efficacy of these approaches needs to be established in human gametes, this strategy of artificially promoting PGP expression could find clinical application in the context of fortifying the genomic integrity, and overall health, of the oocyte and early embryo against a plethora of chemically unrelated toxins (Aller et al., 2009). Moreover, the merit of selectively activating key elements of the oocyte proteome, could hold considerable promise in an IVF setting

where chemical stresses (e.g. arising from cell culture medium) may manifest in, or further exacerbate, DNA damage.

Our analysis of DNA repair effected by the non-homologous end joining (NHEJ) pathway, has offered up a number of alternative protein candidates that could also be manipulated for the purpose of improving DNA repair following exposure to gonadotoxic agents (Chapter 3). Indeed, we demonstrate that the preservation of the maternal genome may be enhanced by an endogenous store of DNA repair proteins accumulated during oogenesis (Martin et al., 2018) (Figure 1C). In this regard, targeting DNA repair pathways for pharmacological activation is an approach that has been trialed to preserve cellular function in fields as diverse as ageing and tumorigenesis. In particular, the silent information regulators (sirtuins) have emerged as promising candidates to modulate cellular homeostasis (Dang, 2014). As a corollary, the 'drugability' of sirtuins has led to the synthesis of a multitude of SIRT activating compounds, many of which have already been used to generate pre-clinical evidence in preparation for human application (Anderson et al., 2009; Boily et al., 2008; Cohen et al., 2004; Nayagam et al., 2006; Valente et al., 2016; Wang et al., 2014). In regard to DNA repair, several members of the sirtuin family (SIRT1, SIRT6 and SIRT7) have been implicated as key regulators and/or promotors DNA repair pathways including homologous recombination (HR), base excision repair (BER) and NHEJ pathways (Mao et al., 2012; McCord et al., 2009; Mostoslavsky et al., 2006; Uhl et al., 2010; Vazquez et al., 2017). Encouragingly, resveratrol (a SIRT1 activator) has already found application as a media supplement during the *in vitro* maturation of oocytes from several mammalian species (murine, porcine and bovine); with favourable reports of significant improvements in oocyte quality, *in vitro* maturation rates and embryonic development (Itami et al., 2015; Khan et al., 2017; Lee et al.,

2018; Li et al., 2016; Liu et al., 2013; Liu et al., 2018; Rocha et al., 2018; Santos et al., 2018; Takeo et al., 2014; Wang et al., 2014; Wang et al., 2018).

As an alternative approach, in the age of personalised medicine, gene-therapy has emerged as an alternative strategy to modulate gene expression and/or activity. Genome editing tools, such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR/Cas9 system, are able to be toused correct or modulate the expression of potentially beneficial genes (Reviewed in (Limp-Foster and Kelley, 2000)). In cancer cells, overexpression of selective repair genes (such as those involved in BER) has been shown to confer resistance to ionizing radiation and chemotherapeutic agents such as thiotepa and bleomycin (Xu et al., 2001). Such findings have important implications for *in vitro* fertility management, age-related subfertility and premature ovarian failure; diagnoses in which oocytes typically display a reduced capacity for DNA repair. Conversely, the targeted inactivation of proteins that prevent DNA repair, such as *TAp63* (trans activating p63), or the proapoptotic genes of *Bax* (Bcl-2-associated X protein) (Perez et al., 1999), *Puma* (p53-upregulated modulator of apoptosis) and *Noxa* (also known as *Pmaip1*; phorbol-12-myristate-13-acetate-induced protein 1), has been shown to have positive outcomes in the context of preserving fertility following challenge with ovo-toxicants, which are otherwise capable of depleting the ovarian reserve in wildtype mice (Kerr et al., 2012a; Kerr et al., 2012b; Kerr et al., 2012c; Suh et al., 2006). In this way, removal of gatekeeper genes or pro-apoptotic factors, thus preventing apoptosis can instead drive highly efficient DNA repair pathways in the mouse primordial follicles. Such an approach has been used to preserve fertility and reduce the negative sequelae that accompanies depletion of the ovarian reserve (Kerr et al., 2012c; Suh et al., 2006).

As a more immediate approach, interventions that prevent, rather than repair, DNA damage have also proven effective. For this purpose, we assessed the capacity of co-administration of two compounds, N-acetylcysteine (NAC) and sodium salicylate (SS) alongside a gonadotoxic insult (Etoposide; ETP) (Chapter 3). In this study, we demonstrated that SS, in particular, holds significant promise in mitigating the deleterious consequences of genotoxic exposure (Martin et al., 2018). Such activity is likely attributed to the ability of SS to act as a catalytic inhibitor of topoisomerase IIα, thereby blocking genotoxic agent induced topoisomerase IIα-DNA cleavable complex formation and DNA damage (Bau and Kurz, 2011). Akin to the administration of traditional antioxidant cocktails to curtail the bioavailability of damaging reactive oxygen species, these data raise the prospect of prophylactic administration of genotoxic scavengers to mitigate genomic instability and preserve oocyte quality during *in vitro* manipulation or cancer therapy alike.

Finally, in chapter 4 we focused on the capacity of proinsulin C-peptide, to modulate oocyte and early embryo biology. Our study uncovered a novel interaction between C-peptide and the DNA repair enzyme, breast cancer type 2 susceptibility protein (BRCA2), leading us to posit that, in addition to its hormonal characteristics, C-peptide may possess chaperone-like activity that assists the re-localisation of its protein targets to the pronuclei in preparation for meiotic resumption and DNA repair (Figure 1B). Certainly, fertilisation is known to be associated with an upregulation of DNA repair mechanisms that are also driven by nuclear localisation of key repair enzymes (Lord and Aitken, 2015); a crucial step in the prevention of mutagenesis in the embryo. Collectively, these findings lend support to a novel role for C-peptide in the female germline and one that warrants further investigation.

CONCLUDING REMARKS:

Taken together, the findings reported in this thesis support our original hypothesis that the female germline is endowed with a suite of mechanisms designed to safeguard its genomic integrity. On the basis of these new findings, we have prepared a cohesive model to illustrate the integration of these protective strategies (Figure 1). This model depicts interplay between bi-directional transport machinery, such as PGP, for the exclusion of genotoxic agents, the chaperone and intracrine actions of the proinsulin C-peptide and DNA repair machinery essential for the maintenance of genomic integrity in the postovulatory oocyte. Importantly, this collection of studies offers a molecular understanding of the endogenous capacity of the oocyte and preimplantation embryo to detect and subsequently respond to DNA damage and, in turn, identifies novel clinical targets to enhance oocyte competence *in vitro* and potentially contribute to improvements in assisted reproductive technologies.

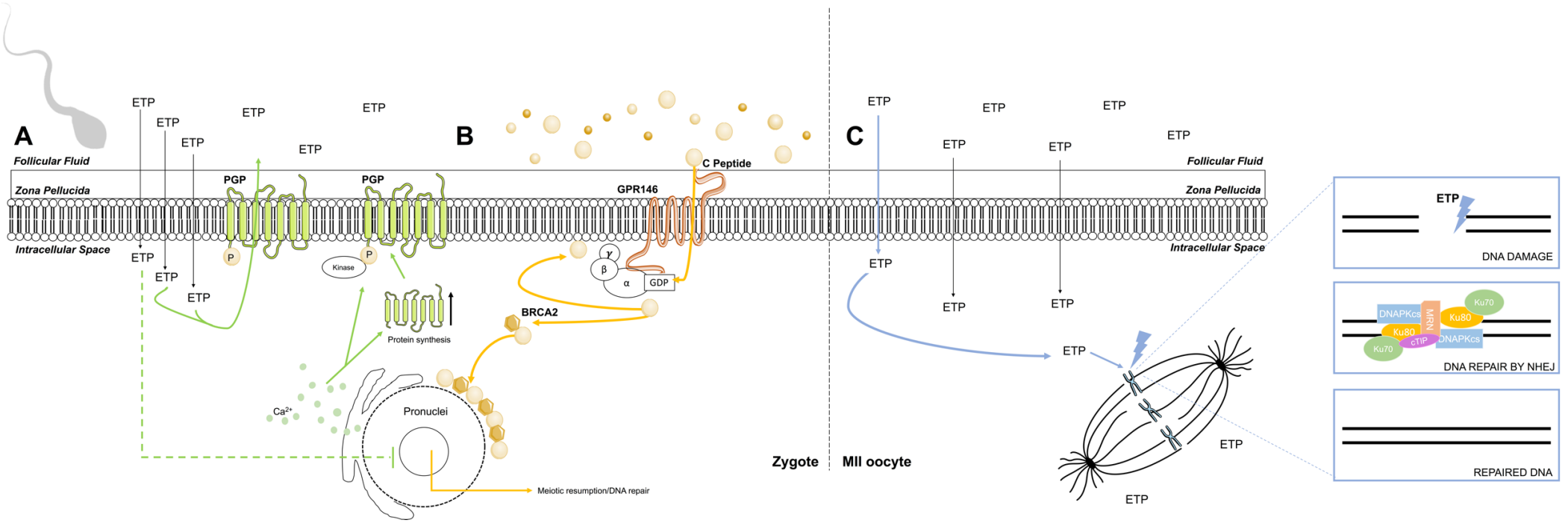

Figure 1: ***Schematic of quality control mechanisms in the post ovulatory MII oocyte and the preimplantation embryo. (A)*** Fertilisation induced Calcium (Ca^{2+}) oscillations trigger significant biochemical and physiological modifications within the oocyte leading to post translational synthesis of transporter proteins and significant reorganization to the plasma membrane. This results in an increase in permeability glycoprotein (PGP) and subsequent increases in the efflux capacity of the zygote, thereby preventing DNA damage to the pronucleus. **(B)** C-peptide within follicular fluid is internalized by oocytes in a G protein coupled receptor (GPCR)-dependent manner, by GPR146. Intracellular C-peptide then binds to breast cancer type 2 susceptibility protein (BRCA2) and directs the translocation of BRCA2 to the pronucleus prior to meiotic resumption and DNA repair. **(C)** DNA repair in the mature MII oocytes requires the co-ordinated by action of pre-synthesised DNA repair proteins that accumulate during oogenesis. Proteins required for non-homologous end joining (NHEJ) converge at the site of DNA damage following genotoxic insult (ETP; etoposide) where they conduct DNA repair. Figure adapted from Martin et al., 2016a; Martin et al., 2016b; Martin et al., 2018a and Martin et al., 2018b (Under review; BOR).

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