Regulation of membrane domains and mitochondrial dynamics during normal oocyte maturation and embryogenesis and in response to physiological stress

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A thesis submitted to the University of Adelaide in fulfillment of the requirements for admission to the degree of Doctor of Philosophy

December 2016

Abstract

Oocytes acquire developmental competence during the final stages of folliculogenesis and during oocyte maturation to enable successful preimplantation embryo development. It is increasingly evident that the peri-conceptual environment surrounding the oocyte has a significant impact on the developmental competence and long-term health of offspring. Thus alterations to oocytes are emerging as significant contributors to the reduced conception and pregnancy rates that are commonly observed in conditions such as maternal diabetes or obesity. This thesis investigated a number of mechanisms by which alterations to oocytes, particularly lipids and oxidative stress, impinge upon the developmental program, namely the mitochondria and differentiation potential of embryonic cells.

Firstly, the role of membrane functional domains in oocyte developmental competence was examined. Oocytes are cholesterol-laden cells and I sought to determine whether oocyte cholesterol stores are important for establishing membrane structure and functional lipid rafts during embryogenesis (via cholesterol depletion). I established that cholesterol depletion resulted in a significant reduction of cholesterol in the oocytes and disrupted oocyte raft distribution as verified by staining. I also showed that cholesterol depletion resulted in a failure to cleave in some embryos and delayed cleavage in others. Even when they developed to morphologically normal blastocysts, comprehensive analysis demonstrated that cholesterol depletion of oocytes led to embryos with reduced cell number, lower neutral lipid content and a disruption in raft distribution. These experiments underscored the importance of oocyte cholesterol stores for preimplantation development and membrane lipid raft formation and function during embryogenesis.

To extend these findings, lipid raft and mitochondrial phenotypes were examined in oocytes and embryos of mice with high-fat diet (HFD)-induced obesity. I showed that oocytes and embryos from HFD mice exhibited disorganised lipid raft distribution and that mitochondrial activity was significantly reduced in the oocytes. Interestingly, BGP-15 treatment, an orally active compound that is currently in human clinical trials for type II diabetes, was able to restore oocyte mitochondrial activity, lipid raft distribution in the oocytes and to a lesser extent in the HFD embryos. In particular, there was a massive induction of mitochondrial DNA (mtDNA) copy number in oocytes following BGP-15 treatment to HFD mice.

To understand the mechanism by which BGP-15 improves oocyte developmental competence, I focused on the mitochondria, essential organelles that are critical for embryo development and transgenerationally inherited. The kinetics of mtDNA replication and ability of BGP-15 to counteract stress was examined in multiple species and *in vitro* models of lipid-induced or oxidative stress. There was an accumulation of mtDNA levels during oocyte maturation from germinal vesicle (GV) to metaphase II (MII) oocytes in the mice and a significant mtDNA replication during embryo development in the mice, bovine and macaque. Supplementation of *in vitro* maturation (IVM) culture medium with high lipids suppressed mtDNA levels in the oocytes and embryos and this was partially prevented by BGP-15; an observation consistent in both mice and bovine embryos. Thus, supplementation of maturation medium with BGP-15 provides a means to increase mtDNA in both oocytes and embryos, thereby improving oocyte viability and developmental competence, particularly in the context of elevated lipids.

Building on these results, BGP-15 was tested as a supplement to *in vitro* fertilisation (IVF) medium for its ability to counteract oxidative stress in cumulus-oocyte complexes (COCs) exposed to hydrogen peroxide (H_2O_2). Exposure of COCs to H_2O_2 significantly impaired cleavage rate following IVF. Embryo cell numbers, reactive oxygen species, mtDNA levels were altered but restored to control levels by BGP-15 treatment. Thus, oxidative stress in COCs recapitulates many of the defects seen with obesity and these are similarly normalised by BGP-15.

Taken together, this study demonstrates that changes or alterations, as a result of environmental insult, before conception alter the phenotype of oocytes and that this is retained through to the course of later preimplantation development. I also demonstrated that BGP-15 is able to alleviate the effects of cellular stress via effects on mitochondria. These findings provide important benchmarks for future pre-clinical and clinical evaluations of preconception pharmaceuticals. This research is significant because increasing numbers of women seeking pregnancy have an altered follicular environment due to obesity, diabetes or poor diet; and my research has generated critically needed knowledge towards understanding how these lifestyle factors affect fertility and identified possible avenues for new therapies.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, 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 in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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December 2016

Acknowledgements

I would first like to sincerely thank my supervisors Associate Professor Rebecca Robker and Dr. Linda Wu for giving me the precious opportunity to undertake this PhD. Becky, thank you for providing continuous support, guidance and feedback whenever I needed it, and for so much encouragement and patience throughout the challenging past 3 and a half years. And to Linda, thank you for your supervision, being supportive and caring, guidance with experimental design, techniques, data analysis and keeping me positive and being a great friend.

I would like to acknowledge the support of the National Health and Medical Research Council for grant funding and my postgraduate scholarship. I would also like to acknowledge the Faculty of Health Sciences, University of Adelaide, the Discipline of Obstetrics and Gynaecology, the Robinson Research Institute, Oregon Health and Science University and the Oregon National Primate Research Centre for international and domestic travel opportunities. Thank you to all the staff in the Discipline of Obstetrics and Gynaecology and the Robinson Research Institute for excellent resources throughout my studies.

Thank you to Darryl, Macarena, Laura, Victor, Jamie, Kylie, and Sonja for all your help in the lab and with troubleshooting. To Rob and Jeremy, thanks for your critical comments and discussion during my talks. Bihong, Sally, Lih Yin, Noor, Nicole, Marie, Mel White and Mel McDowall, thank you for your friendship and encouragement throughout the past few years. Thanks to Adrian, Lesley and Annie for experimental and technical assistances. Many thanks to Jon, Dick, Cathy, Melinda, Alison, Jill, Nathan, Fernanda and Carol from the Oregon National Primate Research Centre, for helping me when I was in the United States. I am honoured to have worked with you and really enjoyed the time in Portland. Previous and present honours and PhD students for their assistance throughout my studies: Izza, Dexter, Thao, Yasmyn, Rachel, Javi, Monica, Jasmine and Qianhui. Thank you all.

Thank you to my best friend, Ching, for your friendship and support in good and hard times. To Karen, Michelle, Anna and Rebecca, thank you for listening, offering me advice, and supporting me through this entire process. Last, but not least, I would like to thank my family, especially my loving parents, Ngeh Thung and Ngie Em and my siblings and family members, Siew Kieong, Siew Horng, Leh Kee, Bustami, Ai Lan and my nephew, Zi Chong for their endless love, support and encouragement. I've been blessed to have all of you in my life. I don't know how I would have gone through this without you all. I appreciate it all!

Publications Arising During PhD Candidature

1. Wu, L.L., Russell, D.L., **Wong, S.L**., Chen, M., Tsai, T., St John, J.C., Norman, R.J., Febbraio, M.A., Carroll, J. and Robker, R.L. (2015) *Mitochondrial dysfunction in oocytes of obese mothers: transmission to offspring and reversal by pharmacological endoplasmic reticulum stress inhibitors*. Development, 142: 681-691.

2. **Wong, S.L**., Wu, L.L., Robker, R.L., Thompson, J.G. and McDowall, M.L. (2015) *Hyperglycaemia and lipid differentially impair mouse oocyte developmental competence*. Reproduction, Fertility and Development, 27(4):583-92.

Publications Contributing to This Thesis

1. Wu, L.L., Russell, D.L., **Wong, S.L**., Chen, M., Tsai, T., St John, J.C., Norman, R.J., Febbraio, M.A., Carroll, J. and Robker, R.L. (2015) *Mitochondrial dysfunction in oocytes of obese mothers: transmission to offspring and reversal by pharmacological endoplasmic reticulum stress inhibitors*. Development, 142: 681-691.

Abstracts Arising from This Thesis

2016

WONG, S.L., WU, L.L., RUSSELL, D.L. & ROBKER, R.L. Cholesterol in mouse oocytes is required for embryogenesis and formation of membrane functional domains in blastocysts, EMBL Australia Postgraduate Symposium 2016, Adelaide, Australia. **(poster)**

WONG, S.L., WU, L.L. & ROBKER, R.L. Cholesterol in oocytes is required for embryogenesis and membrane functional domains in blastocysts, 6th Congress of the Asia Pacific Initiative on Reproduction (ASPIRE 2016), Jakarta, Indonesia. **(oral)**

2015

WONG, S.L., WU, L.L. & ROBKER, R.L. Cholesterol in oocytes is required for embryogenesis and membrane functional domains in blastocysts, Robinson Research Institute (RRI) Symposium 2015, Adelaide, Australia. **(poster)**

People's Choice Poster award & Highly Commended Student Poster Award

WONG, S.L., GONZALEZ, M.B., WU, L.L. & ROBKER, R.L. Cholesterol depletion or high-fat diet disturbs oocyte and embryo membrane domains that are restored by insulin sensitising drug, BGP-15, 9th Annual Florey Postgraduate Research Conference, Adelaide, Australia. **(poster)**

Finalist for the Adelaide Research & Innovation Pty Ltd (ARI) Prize

WONG, S.L., WU, L.L., FEBBRAIO, M.A. & ROBKER, R.L. Impaired embryo development due to oxidative stress can be normalised by BGP-15 treatment *in vitro*, The Joint Annual Scientific Meetings of the Endocrine Society of Australia and the Society for Reproductive Biology (ESA-SRB) 2015, Adelaide, Australia. **(oral)**

Finalist for the David Healy New Investigator Award;

SRB-Reproduction Fertility and Development publication of the year award

WONG, S.L., GONZALEZ, M.B., WU, L.L. & ROBKER, R.L. Cholesterol depletion or high-fat diet disturbs oocyte and embryo membrane domains that are restored by insulin sensitising drug, BGP-15, 9th Annual Florey Postgraduate Research Conference, Adelaide, Australia. **(oral)**

2014

WONG, S.L., WU, L.L. & ROBKER, R.L. Cholesterol in oocytes is required for embryogenesis and membrane functional domains in blastocysts, The Joint Annual Scientific Meetings of the Endocrine Society of Australia and the Society for Reproductive Biology (ESA-SRB) 2015, Melbourne, Australia. **(oral)**

WONG, S.L., WU, L.L. & ROBKER, R.L. Cholesterol in oocytes is required for embryogenesis and membrane functional domains in blastocysts, The Australian Society of Medical Research 2014, Adelaide, Australia. **(oral)**

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Abbreviations

µg	Microgram
µL	Microlitre
µm	Micrometre
2C	2-cell
3C	3-cell
4C	4-cell
Adamts1	ADAM Metallopeptidase with Thrombospondin Type 1 Motif 1
ADP	Adenosie diphosphate
AF	Alexa Fluor
AICAR	AICA ribonucleotide
ANOVA	Analysis of variance
ART	Assisted Reproductive Technology
Atf4	Activating transcription factor 4
ATP	Adenosine triphosphate
Bax	BCL2-Associated X Protein
BL	Blastocyst
BMI	Body mass index
BODIPY	Boron-dipyrromethene
BSA	Bovine serum albumin
cAMP	Cyclic adenosine 3′,5′-cyclic monophosphate
CD	Control diet
Cdx2	Caudal-type homeobox transcription factor-2
CEI	Cumulus expansion index
CM-H_2DCFDA	5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate
CO_2	Carbon dioxide
COC	Cumulus-oocyte complex

COCs	Cumulus-oocyte complexes
CTB	Cholera toxin subunit B
DMSO	Dimethyl sulfoxide
DNP	Dinitrophenyl
eCG	Equine chorionic gonadotrophin
EGF	Epidermal growth factor
Eomes	Eomesodermin
ER	Endoplasmic reticulum
FAF	fatty acid-free
FCS	Fetal calf serum
FFAs	Follicular fatty acids
FSH	Follicle-stimulating hormone
g	Gram
Gata3	GATA Binding Protein 3
GM1	Ganglioside monosialotetrahexosylganglioside
GPI	Glycosylphosphatidylinositol
Grp78	Glucose-regulated protein 78
GTP	Guanosine 5'-Triphosphate
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
h	Hour
H_2O_2	Hydrogen peroxide
hCG	Human chorionic gonadotrophin
HDL	High density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	High-fat diet
HREC	Human Research and Ethics Committee
Hsp	Heat-shock protein

i.p.	Intraperitoneal
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IU	International unit
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide
kcal	Kilocalorie
kg	Kilogram
Klf5	Kruppel-Like Factor 5
KO	Knockout
LH	Luteinising hormone
M	Molar
m^2	Square metre
MAM	Mitochondria-associated ER membrane
MEM	Minimum essential medium
mg	Milligram
min	Minutes
MI	Metaphase I
MII	Metaphase II
mL	Millilitre
ML	Morula
mM	Millimolar
MMP	Mitochondrial membrane potential
MOPS	3-(N-morpholino)propanesulfonic acid
mtDNA	Mitochondrial Deoxyribonucleic acid
MTG	MitoTracker Green
MTO	MitoTracker Orange

MβCD	Methyl-beta-cyclodextrin
NADH	Nicotinamide Adenine Dinucleotide Hydride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
Nanog	Homeobox Transcription Factor Nanog
NaOH	Sodium hydroxide
NEFA	Non-esterified fatty acids
ng	Nanogram
NGS	Normal goat serum
nM	Nanomolar
nm	nanometre
Oct4	Octamer binding transcription factor 3/4
OS	Oxidative stress
PA	Palmitic acid
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCOS	Polycystic Ovary Syndrome
PCR	Polymerase chain reaction
PGS	Preimplantation genetic screening
PI	Propidium iodide
PI	Post-insemination
PMSG	Pregnant mare's serum gonadotrophin
PN	Pronuclei
Pou5f1	POU Class 5 Homeobox 1
PUFA	Polyunsaturated fatty acid
PVP	Polyvinylpyrrolidone
Rac1	Tas-related C3 botulinum toxin substrate 1
RNA	ribonucleic acid
ROS	Reactive oxygen species

SEM	Standard error of mean
Sox2	SRY-related HMG-box 2
SRBI	Scavenger receptor, type B class I
SSEA4	Stage-specific embryonic antigen 4
TAG	Triacylglycerols
TALP	Tyrode's Albumen Lactate Pyruvate
TE	Trophectoderm
Tead4	TEA Domain Transcription Factor 4
Tfam	Transcription factor 1, mitochondrial
TMRM	Tetramethyl Rhodamine Methyl Ester
TNBS	2,4,6-Trinitrobenzenesulfonic acid
UPR	Unfolded protein response
Yap1	Yes-associated protein 1
ZP	Zona pellucida

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

The establishment and maintenance of embryo and fetal development, pregnancy, and even adult diseases are determined by the oocyte quality (Krisher 2004). Oocyte quality, i.e. its developmental competence (the ability of the oocyte to support fertilisation and subsequent embryo development) are acquired during folliculogenesis as the oocyte grows, and during the period of oocyte maturation (Krisher 2004). Oocytes are highly responsive to environmental signals (Leroy *et al.* 2011). Thus alterations to the oocytes are emerging as significant contributors to the reduced conception and pregnancy rates that are commonly observed in conditions such as maternal diabetes or obesity (Turner and Robker 2015).

Maternal nutrition/metabolism and metabolic rate of the oocyte and its surrounding cumulus cells are very important for early embryo development (Gu *et al.* 2015). Perturbations in any of these can skew embryo metabolism and development. For example, oocytes recovered from mice fed with high levels of omega-3 fatty acids exhibited altered mitochondrial distribution and calcium levels and increased production of reactive oxygen species (Wakefield *et al.* 2008). Furthermore, the embryos resulting from fertilisation of these oocytes had poorer morphology and decreased developmental ability to the blastocyst stage (Wakefield *et al.* 2008).

Previous studies on the effects of maternal nutrition have mainly focused on the later stages of pregnancy or the early neonatal period because demand for maternal nutrients for growth is highest during these periods (Morrison and Regnault 2016). However, the preimplantation embryo passes through distinct metabolic phases, undergoing changes in protein synthesis, energy requirements and amino acid uptake. Therefore the mother's diet prior to pregnancy and during early pregnancy, the period of preimplantation embryo development, creates a nutritional environment that is critical in determining embryo health. For instance, embryo quality in obese women was significantly reduced when compared to non-obese women with a body mass index (BMI) between 20 and 30 (Carrell *et al.* 2001). Furthermore, oocyte quality is also altered with reduced oocyte number (Fedorcsak *et al.* 2000). This scenario is more likely to result in pregnancy loss in obese women, suggesting alterations in oocyte quality with obesity. In mice, high-fat diet (HFD) females exhibited impaired oocyte developmental competence, characterised by altered mitochondrial metabolism, more

apoptotic ovarian follicles, smaller and fewer mature oocytes, and decreased rates of oocyte nuclear maturation and fertilisation when compared to the controls (Jungheim *et al.* 2010; Wu *et al.* 2010; Wu *et al.* 2012). Alterations such as these have been shown to result in smaller offspring with compromised health (Luzzo *et al.* 2012). Thus, obese women exhibit altered oocyte quality, which is likely to influence embryo development and mitochondrial metabolism as early as during the periconception period.

It remains to be determined however, the precise molecular mechanisms by which obesity alters oocytes; and these are likely to be multi-factorial. Recent reports have revealed the involvement of membrane raft in the determination of fertilisation rate and index in mice (Buschiazzo *et al.* 2013). Lipid rafts are scaffolds for many molecular entities, including communicating extracellular stimuli to the intracellular organelles (Head *et al.* 2014). Mitochondria also play an important role in oocyte biology (Chappel 2013), raising the intriguing possibility that their function may represent one of the key molecular defects responsible for sub-fertility. Identifying how these defects can be reversed in poor quality oocytes also requires further research. This review will describe conditions associated with maternal overnutrition, particularly obesity, the clinical and biological consequences on early embryogenesis, the involvement of membrane rafts in fertilisation and their association with intracellular organelles, the role of mitochondria and oxidative stress on reproduction.

1.2 Mammalian ovarian folliculogenesis and preimplantation development: from oocyte to embryo

1.2.1 Folliculogenesis and ovulation

The follicle is the basic unit of the female ovary which contains the oocyte and the surrounding somatic cells; and folliculogenesis is the growth maturation of the ovarian follicle from a primordial stage through ovulation (Figure 1). Females are born with a finite number of primordial follicles which varies between species, with approximately 4270 in the mouse (Gosden and Telfer 1987) and about 400,000 follicles in women at puberty (Krogh 2011). The primordial follicle is an immature oocyte encircled by a simple squamous layer of follicular cells, and these are scattered in the outer portions of the ovarian cortex, just beneath the tunica albuginea (Chang *et al.* 2009). At this stage, each oocyte has pale

cytoplasm and a large, round nucleus with a prominent nucleolus (Yadav 2004). The first step of folliculogenesis is the development of primordial follicles into primary follicles. Development of follicles to this stage appears to be relatively independent of pituitary control but it probably influences by intraovarian non-steroidal processes that remain to be understood (Hsueh *et al.* 2015). In a primary follicle, the follicular cells enlarge and undergo repeated cell divisions. This division creates several layers of follicular cells around the oocyte. As the wall of the follicle thickens further and the diameter of the developing oocyte increases, a thick zona pellucida (ZP) layer is formed around the oocyte. Although many primordial follicles develop into primary follicles, usually only a few will take the next step. The transformation begins as ZP formation and the thickened follicular cells begin secreting small amounts of fluid. Fluid also accumulates due to transudation from the plasma and surrounding tissue. The accumulation of follicular fluid gradually expands to separate the inner and outer layers of the follicle. At this stage, the complex is known as a secondary follicle. The oocyte continues to grow. The follicle as a whole enlarges rapidly due to this accumulation of fluid. The formation of a fluid-filled cavity surrounding the oocyte characterises the stage of follicle development, as an antral follicle. Granulosa and theca cells continue to undergo mitosis concomitant with an increase in antrum volume. As follicle enlarge, the granulosa cells and theca cells progressively stop dividing to express differentiated functions. Granulosa cells become dependent on FSH and theca cells express receptors for luteinising hormone (LH) for further growth and follicle maturation. Theca cells express LH receptors and pituitary LH induces the production of androgens by the theca cells, which are aromatised by granulosa cells to produce estrogens, primarily estradiol. Consequently, estrogen levels begin to rise. As more estrogen is secreted, more LH receptors are made by the theca cells. At this stage the follicle spans the entire width of the ovarian cortex and stretches the ovarian capsule, creating a prominent bulge in the surface of the ovary. The oocyte, surrounded by the specialised cumulus cells, projects into the expanded central chamber. The LH surge triggers ovulation of mature cumulus-oocyte complex (COC). Bidirectional communication between oocyte and its surrounding granulosa cells is vital, both for oocyte development and for granulosa cells differentiation (Thomas and Vanderhyden 2006). Oocytes depend on differentiated cumulus cells, which provide them with nutrients and regulatory signals needed to promote oocyte nuclear and cytoplasmic maturation and consequently the acquisition of developmental competence.

The LH surge also triggers the terminal differentiation of the granulosa and theca cells to form the corpus luteum. Initially, the high levels of estrogen from the follicles cause the release of LH from the pituitary gland. This LH is temporarily associated with the transcriptional regulation of numerous genes and synthesis and/or activation of specific proteases such as Adamts1 (ADAM Metallopeptidase with Thrombospondin Type 1 Motif 1) that stimulates the rupturing of the follicle. Rupturing occurs at the apex of the preovulatory follicle, and involves degradation of the follicle wall and expulsion of the expanded COC from the ovary, which is then picked up by the oviduct (or Fallopian tube) where fertilisation may occur (Russell and Robker 2007). The granulosa and theca cells of the follicle undergo luteinisation and transform into a corpus luteum that makes the progesterone (Figure 1). The progesterone functions to prepare the uterus for pregnancy, by modifying the endometrium to make it suitable for implantation and nourishment of the early embryo.

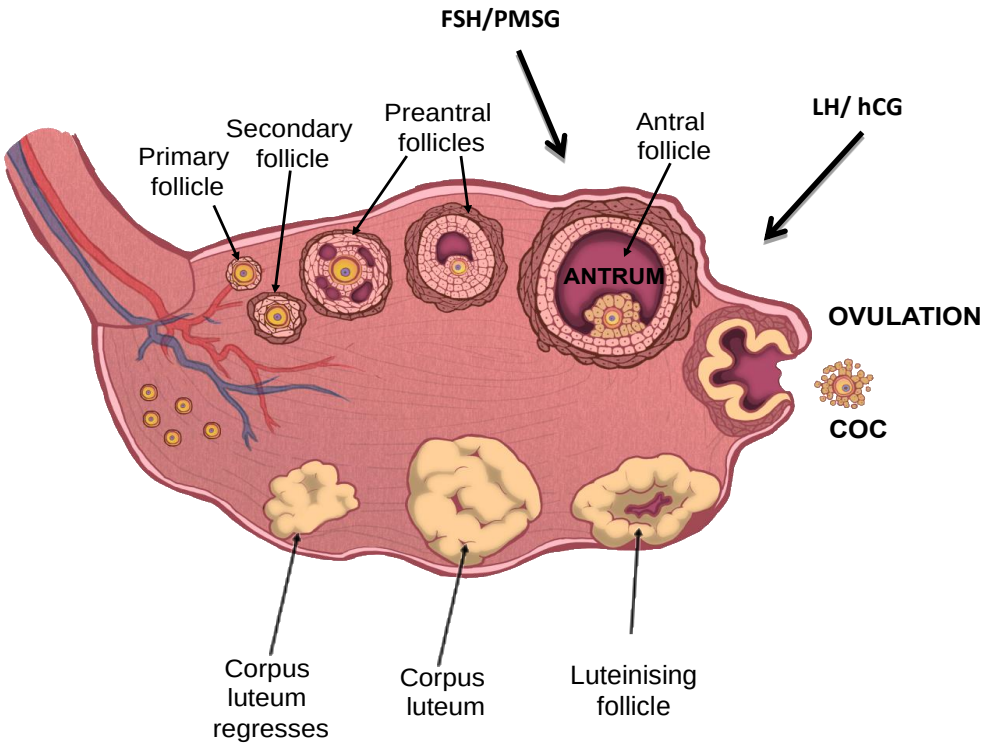

Figure 1 Schematic of folliculogenesis, oocyte maturation and ovulation.

Primordial follicles consist of an immature oocyte surrounded by a layer of flattened somatic granulosa cells. The transition to a primary follicle is marked by the transformation of the granulosa cells into a cuboidal shape. Pre-antral follicles develop layers of a second somatic cell type, theca cells, and antral follicles contain a fluid-filled antrum, which increases in size under the influence of follicle-stimulating hormone (FSH) or its analogue pregnant mare's serum gonadotropin (PMSG). The oocyte directs the differentiation of a sub-population of granulosa cells into cumulus cells, forming the cumulus-oocyte complex (COC). Pre-ovulatory follicles which do not undergo atresia respond to the luteinising hormone (LH) or hCG surge to induce final maturation of the oocyte, ovulation and corpus luteum formation. Moreover, bi-directional communication between cumulus cells and the oocyte regulates numerous aspects of oocyte maturation, including control of meiotic induction, regulation of membrane potential, and suppression of transcriptional activity in the oocyte. Concurrently, the oocyte also regulates aspects of the development and activity of granulosa cells including proliferation and differentiation (Kidder and Vanderhyden 2010).

1.2.2 Oocyte maturation

Oocytes remain arrested in an immature state in the follicle at the prophase I stage of meiosis. The acquisition of developmental competence by an oocyte, encompasses a number of critical events (Takeuchi *et al.* 2005). The final development of oocyte competence usually occurs during the process of meiosis, and is referred to as oocyte maturation. Oocyte maturation includes nuclear maturation and cytoplasmic maturation accompanied by expansion of surrounding cumulus cells, and both are essential for fertilisation and embryo development (Eppig 1991; Albertini *et al.* 2001; Terasaki *et al.* 2001; Krisher 2004) . Nuclear maturation refers to the meiotic process of chromosomal reduction to a haploid content, so as to produce a diploid organism upon fusion with sperm (Cooper 2000). Cytoplasmic maturation involves the cytoplasmic changes in organelles such as the endoplasmic reticulum (ER) and mitochondria required to prepare the cell for fertilisation, activation, and embryo development. Aberrations in these processes result in arrested development and embryo demise.

The process of oocyte nuclear or meiotic maturation traditionally has been described by changes in chromosome morphology during meiosis. Before maturation starts, the oocyte contains a large germinal vesicle (GV) with a large nucleolus. The chromosomes in the GV are mostly decondensed, dispersed, and transcriptionally active (Coticchio *et al.* 2015). Once growth is achieved and the oocyte develops the ability to resume meiosis and progress to the mature stage in the later stages of folliculogenesis, the chromatin is subject to an extensive process of condensation. In several species, chromatin condensation is accompanied by intense transcription down-regulation followed by the germinal vesicle breakdown (GVBD), and nucleoli disperse (Takeuchi *et al.* 2005). As maturation progresses, the paired homologous chromosomes align in the middle of the forming meiotic spindle during metaphase I (MI), followed by the first polar body formation. Then the chromosomes remaining in the oocyte are again arranged on a meiotic spindle at MII and following ovulation are capable of fertilisation.

Lipid metabolism in oocytes is critical for oocyte growth, fertilisation, and the development of early embryos (Dunning *et al.* 2014). Failure of proper lipid delivery leads to abnormal oocytes, a low egg production rate, and low viability of embryos (Grant and Hirsh 1999;

Brock *et al.* 2007). Notably, the lipid content of oocytes varies considerably between species. Triglycerides are the most abundant lipid in oocytes, constituting over 50% of all lipid material (Homa *et al.* 1986; Ferguson and Leese 1999; Kim *et al.* 2001) and provide a large potential energy reserve. They are typically stored as neutral lipid droplets in the cell cytoplasm. The lipid droplets are small and diffuse in oocytes of pre-ovulatory COCs, accumulate during oocyte growth and undergo temporal and spatial changes during maturation. They become larger and more centrally located in response to ovulatory hCG *in vivo*, or FSH + epidermal growth factor (EGF) *in vitro* (Yang *et al.* 2010). In species such as porcine, ovine and bovine, these endogenous lipids are so high that the oocytes appear dark when viewed under the light microscope (Leroy *et al.* 2005; Prates *et al.* 2013). However, mouse oocytes and embryos are pale, indicating its low content of endogenous lipid. There has been very little study examining the specific lipid content of the human oocyte; however, their lipid profiles in general are thought to be relatively similar to those in other species (Dunning *et al.* 2014).

The process of oocyte maturation is also accompanied by fundamental changes in cellular organelles in the cytoplasm. Oocytes possess a variety of organelles typical of most cells (such as Golgi apparatus, mitochondria, ER) as well as oocyte-specific organelles such as cortical granules, annulate lamellae, acidic granules, and pigment granules. (Luciano *et al.* 2012), Cytoplasmic maturation involves modifications in oocyte mitochondrial distribution, clustering and activity in response to changing energy demands of the cell machinery (Dalton and Carroll 2013), and ER reorganisation and maturation finalised to intracellular Ca^{2+} signaling (Mann *et al.* 2010). Van Blerkom and Runner (1984) reported the first redistribution of mitochondria in mouse oocytes. They described a mitochondrial translocation to the perinuclear region during formation of the first meiotic spindle, followed by a redistribution of mitochondria at the time of polar body extrusion (Van Blerkom and Runner 1984). These changes were only observed in maturing oocytes and therefore proposed to be a necessary feature of oocyte maturation. Furthermore, in MII oocytes, the mitochondria are also dispersed throughout the cytoplasm (Dalton and Carroll 2013; Wakai *et al.* 2014). The translocations of mitochondria during this period appears to be controlled by oocyte microtubule networks and is likely to involve cytoplasmic rather the spindle associated microtubules (Van Blerkom 1991; Sun *et al.* 2001; Brevini *et al.* 2005; Liu *et al.*

2010). The redistributions of mitochondria have also been observed in the human oocyte and bovine oocyte where they were correlated with increased development to blastocyst and higher adenosine triphosphate (ATP) levels (Stojkovic *et al.* 2001).

On the other hand, *in vitro* maturation (IVM) of oocytes is a reproductive technology by which immature oocytes are retrieved from unstimulated or minimally stimulated ovaries and subsequently matured *in vitro* in appropriate medium until they reach MII (Figure 2). However, there is a discrepancy in the success rate of embryos derived from *in vitro* matured oocytes compared to *in vivo* oocytes, with the pregnancy rate post-IVM less than half of the pregnancy of post-IVF (*in vitro* fertilisation) in women with Polycystic Ovary Syndrome (PCOS) (Gremeau *et al.* 2012). In mice and bovine, when compared to *in vivo* matured oocytes, embryos derived from IVM oocytes have decreased cleavage and blastocyst development rates, and poorer blastocyst quality (Rizos *et al.* 2002; Sanfins *et al.* 2015). Factors affecting oocyte developmental competence *in vitro* include culture media additives such as serum, hormones and growth factors, as well as the companion somatic cells which surround the oocyte. To date, many ingredients have been added to IVM medium to nurture the immature oocytes in the Petri dish (Demyda and Genero 2011). However, further development is needed to achieve an acceptable pregnancy success rate for IVM-derived oocytes because pregnancy rates with IVM are lower than those achieved with conventional IVF. Importantly, IVM can also be used to experimentally test the effects of physiological factors added exogenously to the culture on oocyte maturation and competence, as well as to test the beneficial effects of new supplements on early embryo development.

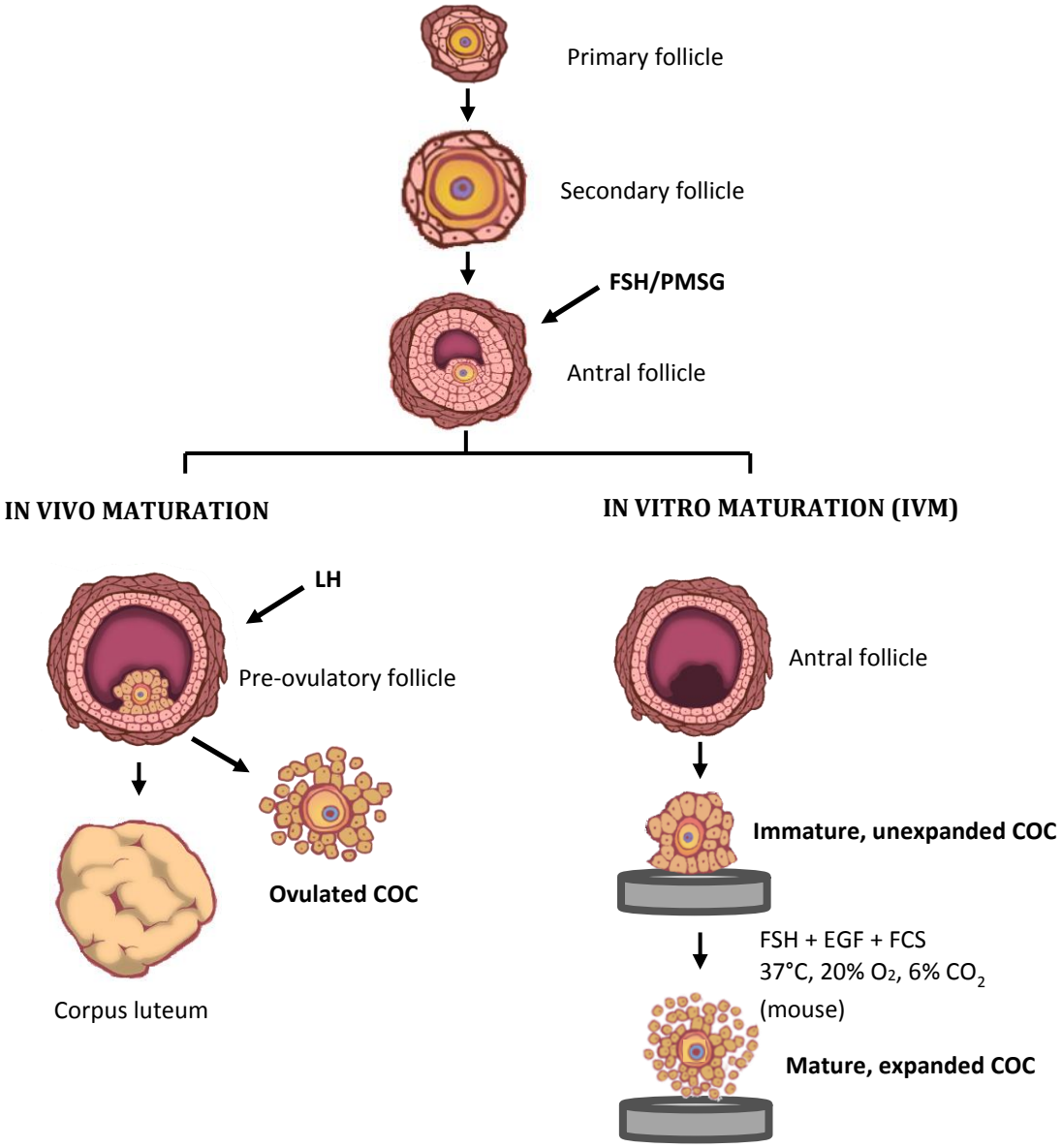

Figure 2 Schematic of oocyte maturation *in vivo* and *in vitro* (mouse).

In vivo maturation involves folliculogenesis until the LH surge or administration of hCG. Meiosis is then resumed concurrent with cumulus expansion. The COC is then ovulated, extrudes its first polar body to reach MII and is capable of being fertilised. In *in vitro* maturation (IVM), following FSH or PMSG administration, immature oocytes in the large antral follicles are extracted and matured in laboratory conditions. These oocytes are not exposed to LH but rather FSH and epidermal growth factor (EGF) which trigger cumulus expansion and oocyte maturation.

1.2.3 Fertilisation and embryo development

Once the mature oocyte is released into the fallopian tube at ovulation, it can be fertilised by a sperm cell, forming a single nucleus in the cytoplasm of the fertilised oocyte which contains both the female and male chromosomes. This unit is called a zygote. Mitochondrial redistribution also occurs during early development in embryos from several species. Following fertilisation, they became progressively aggregated and localised in the perinuclear region (Bavister and Squirrell 2000; Van Blerkom *et al.* 2000; Wilding *et al.* 2001). Abnormal distribution of mitochondria at the pronucleus stage results in some blastomeres with reduced mitochondrial content and diminished ATP generating capacity (Van Blerkom *et al.* 2000). Thus, mitochondrial redistributions appear to be a feature of competent oocytes and successful early development. However, the exact way in which mitochondrial translocations contribute to developmental competence has not yet been established.

Following fertilisation, the zygote further divides into a 2-cell embryo and is transported through the oviduct by cilia beating and oviductal muscle contractions. The 2-cell embryo further divides into a 4-cell embryo then becomes eight, and so forth. Successive cell divisions result in a morula which is an embryonic stage consisting of a solid, compact mass of 16 or more cells. From this stage onwards, morphological appearance becomes distinct, with the outer cell layer differentiating into trophectoderm (TE) and the cells within the embryo forming a compact inner cell mass (ICM). A blastocoel, a fluid-filled cavity, develops and grows and the embryo is now called a blastocyst. In human and mouse, the embryo reaches the uterus around the blastocyst stage where it expands and breaks out of the zona pellucida, a process known as "hatching". The TE cells then adhere to uterine epithelial cells and continue to develop, a process called implantation. The ICM contributes to the formation of fetal tissue, whilst the TE burrows into the wall of uterus and develops into the placental tissues. The distribution of TE and ICM lineage specific proteins in preimplantation embryos have been extensively studied and Caudal-type homeobox transcription factor-2 (Cdx2) and Octamer binding transcription factor 3/4 (Oct4) are the best-known markers for distinguishing TE and ICM respectively. The distribution of other lineage-specific protein in preimplantation embryos found in prior reports is shown in the Appendix, Table 3. Successful implantation involves intimate molecular and cellular

interactions between the competent blastocyst and the uterus, however, there are substantial differences among species in the process of implantation, particularly with regard to "invasiveness", or how much the embryo erodes into maternal tissues.

1.3 Impact of obesity on embryo development and female fertility

1.3.1 Obesity and metabolic syndrome

A significant global health problem is that an increasing number of reproductive age women are overweight, obese or diabetic. In 2015, 63.4% of Australians over 18 of age were overweight or obese (Australian Bureau Statistics 2016). Obesity is most commonly defined as a BMI; weight (kg)/ height (m^2) of 30 or more whereas a BMI of 25 to 29.9 is defined as overweight (AIHW 2012). Obesity, particularly abdominal obesity, is intimately associated with the development of metabolic syndromes (Eckel *et al.* 2005).

Two major systemic perturbations are associated with being overweight or obese: elevations in plasma insulin, that leads to increased circulating glucose, and elevations in free fatty acids (Eckel *et al.* 2005). As obesity progresses, tissues become resistant to the effects of insulin and this leads to type II diabetes mellitus, which is becoming a leading cause of health problems around the world. Obese individuals tend to have high plasma follicular fatty acids (FFAs) concentrations, especially saturated fatty acids (Boden and Shulman 2002). Elevation of plasma FFAs leads to insulin resistance and hepatic inflammation, thus contributing to type II diabetes, hypertension and dyslipidemia.

1.3.2 Clinical consequences of obesity on female reproduction

It is well-established that maternal obesity is associated with reduced conception rates and with complications in the maintenance of pregnancy and delivery of a healthy baby (Purcell and Moley 2011). Obesity is associated with PCOS, which affects about 50% of obese women (Norman *et al.* 2004; Azziz *et al.* 2009). PCOS can be characterised by elevated androgens and insulin resistance leading to irregular menstrual cycles, anovulation, subfertility and pregnancy loss (Jain *et al.* 2007; Metwally *et al.* 2008a; Brewer and Balen 2010). Obese women are also more at risk of having spontaneous abortions, preeclampsia,

miscarriage, pre-term birth or a high birth weight baby when compared with normal-weight women (O'Brien *et al.* 2003; Dokras *et al.* 2006; Metwally *et al.* 2008b; McDonald *et al.* 2010). Specifically, obesity in women has been shown to increase time to conception compared with women of healthy weight (Gesink Law *et al.* 2007; Nohr *et al.* 2009). When their pregnancies are successful, obese women are more likely to give birth to large-for-gestational infants, who are then themselves at increased risk of childhood (2-9 years) and adolescent (14 years) obesity (Maftei *et al.* 2015). Babies born from obese mothers are also at an increased risk of developing metabolic syndromes (including insulin resistance, hypertension, and diabetes) later in life, suggesting the effects of maternal obesity are transgenerational and life-long (Rooney and Ozanne 2011).

1.3.3 Impacts of obesity on oocytes

One of the ways that obesity exerts detrimental effects on female fertility, particularly conception and pregnancy rates, is by causing numerous perturbations in oocyte structure and function prior to fertilisation.

Many studies using mouse models of obesity and *in vitro* studies which culture oocytes in lipid-rich media have shown delays in meiotic maturation accompanied by spindle defects and chromosome misalignment during oocyte maturation in response to these conditions (Jungheim *et al.* 2010; Luzzo *et al.* 2012; Yang *et al.* 2012; Sohrabi *et al.* 2015). Studies from human assisted reproductive technology (ART) clinics have shown an impairment in oocyte maturation associated with increasing BMI (Wittemer *et al.* 2000; Carrell *et al.* 2001; Dokras *et al.* 2006), however, others found no difference in oocyte maturation between different BMI scores (Metwally *et al.* 2007).

In women, increasing BMI is associated with increased insulin, glucose, lactate, triglycerides, non-esterified fatty acids (NEFA) and C-reactive protein, an inflammatory marker in follicular fluid (Robker *et al.* 2009; Jungheim *et al.* 2011b; Valckx *et al.* 2012; Yang *et al.* 2012; Valckx *et al.* 2014). The most abundant free fatty acids detected in the follicular fluid of women were oleic, palmitic, linoleic, and stearic acids (Jungheim *et al.* 2011b; Valckx *et al.* 2014). Elevated follicular free fatty acids in women are associated with poor COC morphology (Jungheim *et al.* 2011b). Abnormal lipid accumulation and distribution within

oocytes have been demonstrated in mice on an obesogenic diet for as little as four weeks (Wu *et al.* 2010; Reynolds *et al.* 2015). This result was also observed in a genetic obese mouse model (Wu *et al.* 2015). These consistent observations of excess lipid in the follicular fluid as well as in the oocytes suggest that lipotoxicity mechanisms maybe activated in the ovarian follicular environment in response to obesity. Lipotoxicity is a cellular response to a high lipid environment that leads to endoplasmic reticulum (ER) stress, mitochondrial damage and reactive oxygen species (ROS) release and can ultimately lead to apoptosis.

Evidence of lipotoxicity mechanisms within the ovarian preovulatory follicles of obese female mice is that a high-fat diet (HFD) induces apoptosis in both granulosa cells and COCs (Wu *et al.* 2010; Jungheim *et al.* 2011b). This would likely contribute to poor gamete quality due to the tight bi-directional communication between these cells and the oocyte. Moreover, data from clinical studies of IVF patients has shown that cumulus cells apoptosis correlate with poor pregnancy rates (Wittmaack *et al.* 1994; Arnot *et al.* 1995; Bergh *et al.* 1998). Consistent with the data from mice with HFD-induced obesity, genetic mutation-induced obese mice also exhibit oocytes with high levels of apoptosis and autophagy when compared to oocytes from control non-obese mice (Wu *et al.* 2015; Hou *et al.* 2016).

A number of studies have reported altered mitochondrial function in mouse oocytes in response to a HFD or genetic obesity. The mitochondria in oocytes from HFD mice have altered structure and distribution, increased mitochondrial membrane potential (MMP) and increased mitochondrial DNA (mtDNA) copy number, the latter usually associated with increased oocyte competence (Igosheva *et al.* 2010). In contrast, Wu *et al*. (2010) found that MMP of oocytes from HFD mice was lower than that of oocyte mitochondria from mice fed a control diet. Differences between the two studies include the use of different diets, strains of mice and different dyes used. In genetic mutation induced obese mouse models, impairments in mitochondria have also been reported. Oocytes from obese mice had abnormal mitochondrial distributions and structures (Hou *et al.* 2016) and reduced MMP (Wu *et al.* 2015).

The redox states of the Nicotinamide Adenine Dinucleotide Hydride (NADH) and Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH) play critical roles in

defining the activity of energy production pathways, in driving oxidative stress and in maintaining antioxidant defences (Blacker and Duchen 2016). Redox state of oocytes from obese mice was also previously measured by assessing NADPH levels. Oocytes from HFD fed mice contained more oxidised NADPH than those from the control diet mice, suggesting that they were in a state of oxidative stress (Igosheva *et al.* 2010). Additionally, the intracellular ROS levels in the oocytes of both diet-induced and genetic mutation induced obese mice were also significantly higher than those of the lean mice (Igosheva *et al.* 2010).

COCs from mice fed a HFD as well as Blobby mice, were shown to have increased expression of ER stress marker genes, activating transcription factor 4 (Atf4) and glucose-regulated protein 78 (Grp78) (Wu *et al.* 2010; Wu *et al.* 2015), similar to those matured in lipid-rich follicular fluid (Yang *et al.* 2012). Similar events occur in human ovarian cells, with increased *ATF4* expression observed in granulosa cells of obese women (Wu *et al.* 2010). Moreover, oocyte size is also affected by obesity, with oocytes from diet-induced obese mice smaller than control oocytes (Jungheim and Moley 2010). The link between maternal obesity and small oocytes is also observed in humans, with smaller oocytes collected from obese women undergoing IVF or intracytoplasmic sperm injection (ICSI) than from non-obese women (Marquard *et al.* 2011). Previous studies in humans have shown that small oocyte size correlates with poor pregnancy outcomes (Wittmaack *et al.* 1994; Arnot *et al.* 1995; Bergh *et al.* 1998).

1.3.4 Consequences of obesity on embryos

Obesity also has detrimental effects on preimplantation embryogenesis which are likely due to ER stress and mitochondrial alterations in the COC. Obese women have been reported to have significantly poorer quality embryos than lean women (Carrell *et al.* 2001), and another group observed a similar effect, but only in women aged below 35 years old (Metwally *et al.* 2007). However, others have been unable to demonstrate a significant correlation between embryo quality and BMI (Fedorcsak *et al.* 2001; Spandorfer *et al.* 2004; Dechaud *et al.* 2006; Esinler *et al.* 2008; Shalom-Paz *et al.* 2011). In an obese mouse model, significantly fewer embryos reached the blastocyst stage of development when compared to control mice and cell type distribution of the embryos was also disrupted (Minge *et al.* 2008). The blastocysts from these resultant embryos displayed a lower ICM to TE ratio. This altered differentiation

was reversed by the insulin sensitiser rosiglitazone. Similarly, Wakefield *et al.* also showed decreased developmental ability to the blastocyst stage in mouse embryos after exposure of oocytes to an environment of high n-3 polyunsaturated fatty acid (PUFA) (Wakefield *et al.* 2008). Moreover, following the exposure of *in vivo* flushed morulae to a high concentration of palmitic acid for 30 hours, the resulting mouse blastocyst had reduced ICM cells and the fetuses were growth restricted (Jungheim *et al.* 2011a). This implies that exposure to a high concentration of palmitic acid leads to aberrant metabolism and long-term metabolic perturbation.

Early embryogenesis alone is susceptible to damage, with embryos from HFD mice impaired as early as the 2-cell stage (Luzzo *et al.* 2012; Sohrabi *et al.* 2015). In addition, embryos from HFD mice also exhibited delayed on-time progression to all developmental milestones (4-8-cell stage, morula/early blastocyst stage, and the expanded/hatching blastocyst stage) (Minge *et al.* 2008; Luzzo *et al.* 2012) and a higher frequency of embryo degradation (Luzzo *et al.* 2012). Our group has previously showed that the *in vivo* fertilisation rate was lower in HFD mice than in the controls (Wu *et al.* 2010). By using a severely obese Blobby model, we showed that Day 2 cleavage rate was lower when compared to those of embryos from lean mice and there was a further reduction in blastocyst formation rate following IVF in the Blobby mice. Blastocysts generated from these obese mice have reduced mtDNA copy number.

In women, being overweight or obesity also induces phenotypic and metabolic abnormalities in the embryos. Blastocysts from obese women have reduced glucose consumption, modified amino acid metabolism and increased levels of endogenous triglyceride (Leary *et al.* 2015). In cattle, oocytes that were exposed to elevated NEFA concentrations yielded blastocysts with lower cell number, increased apoptotic cell ratio, reduced oxygen, pyruvate and glucose consumption and higher amino acid metabolism (Van Hoeck *et al.* 2011). In mice, maternal obesity increased the lipid content and apoptosis rate in the embryos. Alternatively, embryos from diet-induced obese female mice also exhibited altered mitochondrial properties and produced significantly higher ROS levels when compared to those from the lean mice (Igosheva *et al.* 2010).

Together, these are compelling evidence showing that maternal overnutrition has detrimental effects as early as the oocyte stage, which further predisposes them to embryo developmental abnormalities and even metabolic diseases in the offspring. In addition, there are also studies showing changes in dietary composition, in particular n-3 PUFA can have detrimental effects without a change in kcal intake or over-nutrition, and independent of females being obese (Wakefield *et al.* 2008).

1.3.5 Strategies to improve oocyte quality and fertility in obese females

Despite the significant problems caused by being overweight or obese on oocytes and embryos, strategies to sustain or improve oocyte quality have remained elusive. Diet or exercise interventions are the most common suggestions for obese patients to improve their egg quality. A recent study showed that an exercise intervention on HFD mice can improve lipid metabolism and reverse lipid accumulation in GV oocytes (Boudoures *et al.* 2016). But delays in meiosis and disorganised metaphase II (MII) spindles remained unresolved. Thus, exercise intervention is able to improve but not reverse the damage imposed on oocytes as a result of a high-fat diet and obesity.

Besides exercise, pharmaceutical treatment could be another option. We have previously shown that *in vivo* administration of rosiglitazone (four day treatment) in the periovulatory period was able to reverse the negative effects of obesity and a high-fat diet on oocyte developmental competence in the mouse (Minge *et al.* 2008). Rosiglitazone concomitantly lowered blood glucose, insulin and triglyceride levels indicating that oocytes are affected by insulin sensitivity, and that treatment of obese/insulin-resistant females with an insulin sensitiser before conception can significantly improve embryonic developmental competence (Robker 2008). However, it is unclear if this effect results from the regulation of systemic insulin or from actions directly on the oocyte and/or ovarian cells. Importantly this insulin sensitiser uniquely lowered circulating triglycerides while others that failed to improve oocyte quality (AICAR, salicylate) did not (Minge *et al.* 2008)); which again indicates that lipotoxicity is likely a problem for oocytes.

An alternative therapy to reduce lipotoxicity/ER stress in the oocytes involves directly targeting molecules that regulate the unfolded protein response (UPR). A previous mouse

study from our laboratory showed that salubrinal, an ER stress inhibitor, was able to restore both oocyte mitochondrial activity and oocyte developmental competence in COCs that were exposed to high lipid (Wu *et al.* 2012). These findings were further demonstrated in bovine COCs *in vitro* (Sutton-McDowall *et al.* 2016) demonstrating that ER stress is a key mechanism mediating fatty acid-induced defects in oocyte developmental potential. Furthermore, treatment of obese female mice with salubrinal before ovulation completely restored oocyte quality, embryo development, blastocyst mtDNA and the mtDNA content of fetal tissue to levels equivalent to those derived from lean mice (Wu *et al.* 2015). Salubrinal is a laboratory reagent that is well understood but not tested for its safety in humans. Alternatively, another pharmaceutical compound, BGP-15, which is known to alleviate ER stress via different pathways is currently being tested for human use in clinical trials. So, one goal of the current project was to assess the therapeutic potential of this compound in improving oocyte quality.

Our group recently focused on a new pharmaceutical compound to reverse the effects of obesity on oocytes and to restore embryo development. BGP-15 is a hydroximic acid derivative which is a chaperone inducer and protects against obesity-induced insulin resistance (Chung *et al.* 2008). BGP-15 was developed by N-Gene Research Laboratory (USA and Hungary) and is a compound that has undergone human clinical trials for type II diabetes (Literati-Nagy *et al.* 2009; Literati-Nagy *et al.* 2010). BGP-15 has been shown to have beneficial effects in different cell types and Table 1 summarises the effects of BGP-15 in different cell types of several species. It has been shown to act as an inhibitor of poly(ADP-ribose) polymerase (PARP) to aid DNA repair (Szabados *et al.* 2000) and affects ER chaperone GRP78 (Sapra *et al.* 2014), as well as activates Heat Shock Protein 72 (HSP72) (Gehrig *et al.* 2012). In particular, we have recently discovered that treatment of obese female mice with BGP-15 before ovulation increased the amount of mitochondrial replication factors and mtDNA content in the oocytes, as well as restored oocyte quality, embryo development and normalised mtDNA content of fetal tissues to levels that were comparable to the controls, revealing its mechanism of action might be via the mitochondria (Wu *et al.* 2015). BGP-15 was also found to influence the level and size distribution pattern of cholesterol-rich membrane rafts in melanoma cells; and likely triggers raft-associated stress sensing and signaling pathways (Gombos *et al.* 2011). In a HFD mouse model of type

II diabetes, BGP-15 increases mitochondrial number and oxidative metabolism in skeletal muscle and improves insulin sensitivity (Henstridge *et al.* 2014). Consistent with these results in muscle, treatment of obese female Blobby mice with BGP-15 restored mitochondrial activity in oocytes, and induced mtDNA levels (Wu *et al.* 2015). However, in general the mechanism of action of BGP-15, including its binding targets, is not known in oocytes.

Table 1 Effects of BGP-15 on different cell types and its proposed mechanism of action.

Species	Tissues	Findings	Mechanism of action
Mice	Oocyte (Wu *et al.* 2015)	Reverse mitochondrial dysfunction caused by obesity	Likely via mitochondrial replication
	Heart (Sapra *et al.* 2014)	Improve cardiac function and reduce arrhythmic episodes in murine models with heart failure and atrial fibrillation	Not via induction of heat shock protein 70 (Hsp70). Likely to be associated with phosphorylated insulin-like growth factor 1 receptor (pIGF1R)
	Muscle (Gehrig *et al.* 2012)	Improve muscle architecture, strength and contractile function in Duchenne muscular dystrophy	Inducer of the chaperone heat shock protein 72 (Hsp72)
	Liver and Muscle (Chung *et al.* 2008)	Protect against obesity-induced insulin resistance	Likely to be via up-regulation of Hsp72
	Hepatoma Xenografts (Kardon *et al.* 2006)	Suppress hepatoma development by affecting angiogenesis	Via hypoxia signaling
Rats	Langendorff heart perfusion (Szabados *et al.* 2000)	Decrease reactive oxygen species levels and cell injury during ischemia-reperfusion in the heart	Via poly(ADP-ribose) polymerase (PARP) activity
	Langendorff heart perfusion (Sarszegi *et al.* 2012)	Prevent Imatinib-induced cardiotoxicity	Likely via mitochondria protective role
	Skeletal muscle (Henstridge *et al.* 2014)	Increase mitochondrial number, oxidative capacity and insulin sensitivity in type II diabetes	Likely to be via up-regulation of Hsp72
	Diaphragm muscle (Salah *et al.* 2016)	Alleviate ventilation-induced diaphragm dysfunction	Likely to be via Hsp72 induction and PARP-1 inhibition
Rabbits, Rats	Systemic (Literati-Nagy *et al.* 2014)	Increase insulin sensitivity in cholesterol-fed rabbits, protect against streptozotocin-induced changes in vasorelaxation	Likely via Hsp induction

Human	Systemic (Literati-Nagy *et al.* 2009)	Increase insulin sensitivity, total body glucose utilisation, muscle tissue glucose utilisation and fat-free body mass glucose utilisation	Likely via Hsp induction
	Systemic (Literati-Nagy *et al.* 2010)	Reduce olanzapine-induced metabolic side effects, such as weight gain, insulin resistance and blood glucose abnormalities	Not exclusively mediated by Hsp. Possibly a direct effect on lipid metabolism
***In vitro* molecular dynamic simulation**	Mouse melanoma cells, human embryonic kidney cell line (Gombos *et al.* 2011)	Prevent transient structural disintegration of rafts induced by fever-type heat stress. Able to remodel cholesterol-enriched lipid platforms	Likely to be via GTPase protein Rac1 and heat shock factor (HSF) signaling

1.4 Mechanisms by which obesity might affect embryos

The fundamental mechanisms by which the preimplantation embryo is changed by obesity or other metabolic syndromes are not fully understood. However, based on the available literature published to date it is likely that the defects are due to changes in the oocyte. This was previously confirmed in a study where the increased pregnancy failure rate in obese women returned to a normal rate if donor oocytes were used rather than autologous oocytes (Luke *et al.* 2011). A large number of studies, primarily in mice, have elucidated some of the cellular mechanisms by which obesity impacts oocyte developmental competence. These include excess lipid in oocytes, induction of ER stress and mitochondrial dysfunction and it is likely that several aspects of embryogenesis are thus subsequently disrupted. In particular I hypothesise that these aspects are: membrane integrity, mitochondrial replication and function, and oxidative stress responses. Thus the aims of this thesis are to explore each of these biochemical parameters in embryogenesis, the impact of obesity and the ability of BGP-15 to normalise any defects (Figure 3).

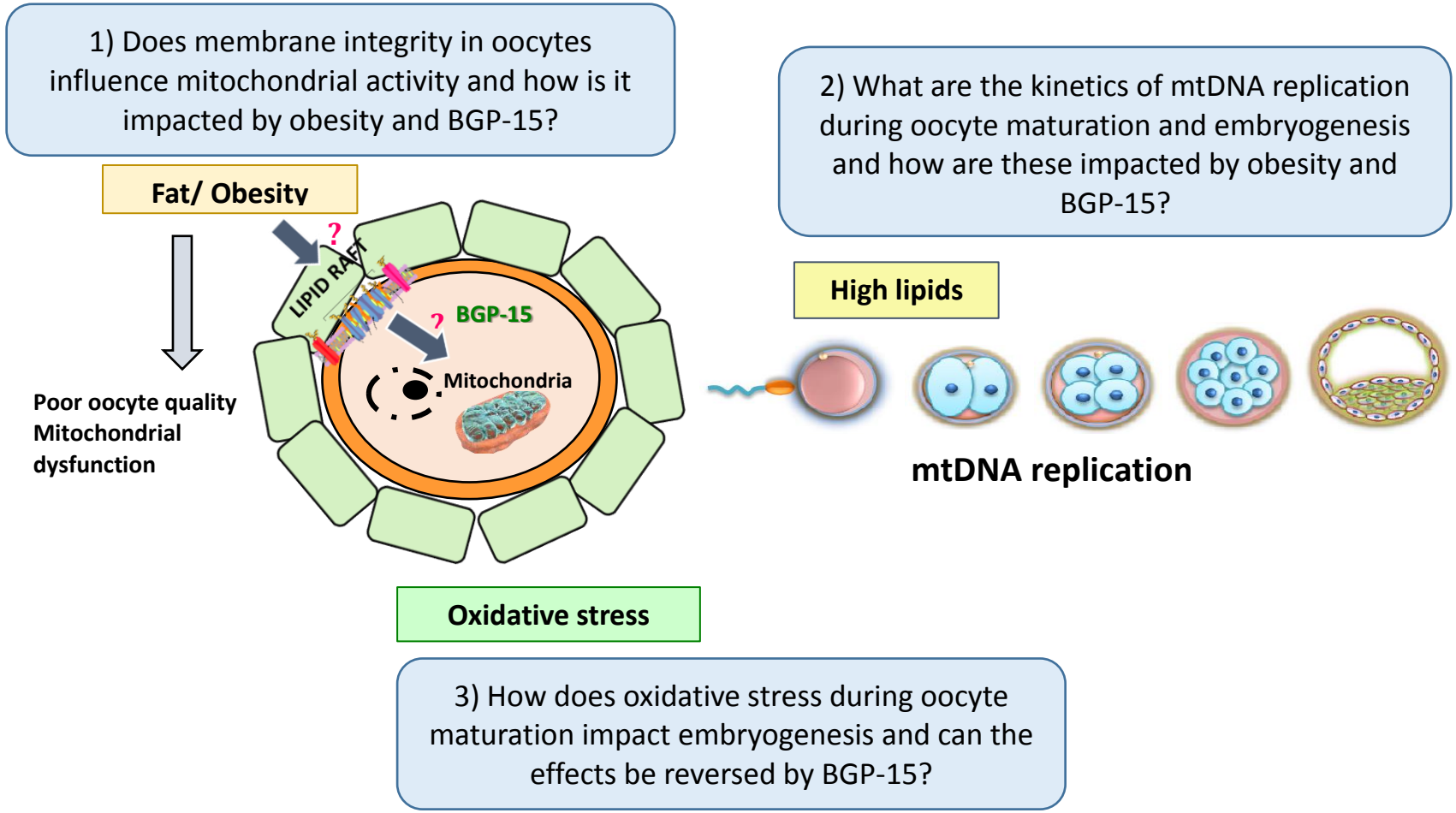

Figure 3 Schematic diagram of the research project and questions.

1.5 Summary and Outline of Aims

The preconception environment is known to influence the developmental competence of the oocyte. In particular, it is well known that obesity/HFD conditions during this time are detrimental to subsequent embryo and fetal health; but the mechanisms for this effect are poorly understood. The importance of membrane structure and mitochondria as the effectors is becoming increasingly evident in a wide variety of fields. Additionally, there is also very little known about the contribution of cholesterol to oocytes or whether it acts to influence membrane rafts, particularly under HFD conditions, and how BGP-15 may act to counteract the stress. Understanding these will generate critically needed knowledge towards how obesity affects fertility, and identify possible therapeutic targets.

Specific hypotheses and aims

Hypothesis 1: Membrane integrity in oocytes influences mitochondrial activity and these are disrupted by obesity but normalised by BGP-15

Chapter 2 will investigate whether oocyte membrane integrity influences oocyte mitochondrial activity and embryogenesis by examining the effect of oocyte membrane disruption (via cholesterol depletion) on embryogenesis and mitochondrial activity and whether BGP-15 can stabilise oocyte membranes.

Chapter 3 will determine whether membrane integrity in embryos is disrupted by maternal obesity and whether this is reversible by BGP-15 treatment.

Hypothesis 2: Mitochondrial replication during oocyte maturation and embryogenesis is disrupted by exposure to excess lipid but induced by treatment with BGP-15

Chapter 4 will examine the kinetics of mtDNA replication during oocyte maturation and embryogenesis in mouse, cow, macaque and humans, the effects of high lipid during oocyte maturation on mtDNA replication and the effects of BGP-15.

Hypothesis 3: Oxidative stress during oocyte maturation impairs embryogenesis similar to obesity but these effects are reversible with BGP-15 treatment.

Chapter 5 will utilise a brief treatment with H_2O_2 to induce oxidative stress in oocytes and then determine whether the changes in ROS, mtDNA and embryo quality are reversible by supplementing the IVF media with BGP-15.

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CHAPTER 2

OOCYTE MEMBRANE INTEGRITY INFLUENCES EMBRYO CLEAVAGE KINETICS AND LIPID RAFTS BUT NOT VIA ALTERED MITOCHONDRIAL ACTIVITY

Overview

Follicular fluid of obese women contains elevated levels of triglyceride and findings from our laboratory showed that exposure of cumulus-oocyte complexes (COCs) to high level of fatty acids increased lipid content and altered mitochondrial activity in the oocytes. After fatty acids are taken up by somatic cells, they are esterified into triacylglycerols (TAG) and cholesterol-esters and stored as neutral lipids in lipid droplets. Lipid droplets are not simple lipid storage depots, but rather complex organelles that have multiple cellular functions. One of its proposed functions is to distribute neutral lipid as well as phospholipids to various membrane-bound organelles, including the oocytes. It has been suggested that neutral lipid in the oocytes fulfill an important function in supplying energy and in the biosynthesis of membranes during early embryonic development (Kruip *et al.* 1983; Ferguson and Leese 1999; McEvoy *et al.* 2000; Kim *et al.* 2001; Sturmey *et al.* 2006). To date, membrane dynamics in the oocyte are not well understood. Recent findings showed that membrane rafts play an important role in oocyte maturation, fertilisation and oocyte quality (Buschiazzo *et al.* 2013). Furthermore, based on the evidence that there was a build-up of lipid droplets in the oocytes of obese mice (Wu *et al.* 2015) and elevated follicular fluid fatty acids have been associated with poor oocyte morphology, I hypothesise that membrane integrity in the oocyte influences mitochondrial activity and these are disrupted by obesity but normalised by BGP-15. Therefore, the first aim of this thesis will investigate whether oocyte membrane integrity influences oocyte mitochondrial activity by examining the effect of membrane disruption (via cholesterol depletion) on embryogenesis and mitochondrial activity and whether BGP-15 can stabilise oocyte membranes. I also speculate that overabundant fatty acids in an obesogenic environment can severely compromise membrane integrity and subsequently lead to mitochondrial dysfunction. Therefore, an additional aim of this thesis is to determine whether membrane integrity on embryos is disrupted by maternal obesity and whether this is reversible by BGP-15 treatment.

2.1 Introduction

Mammalian embryos undergo dramatic membrane reorganisation as they develop from a single-celled zygote to a differentiated multicellular blastocyst capable of implantation in the maternal uterus. In addition to a structural role, cell membrane integrity also serves as an important platform for cell adhesion and attachment, ion conductivity and signaling processes (Miner *et al.* 2004; Tutuncu *et al.* 2004; Yurchenco 2011). Lipid rafts are highly specialised, liquid-ordered subdomains of the cell membrane. The current understanding of lipid rafts under basal conditions sees them as extremely small and/or dynamic assemblies of cholesterol, sphingolipids and proteins that are directly involved in a variety of cell membrane functions including membrane signaling and trafficking (Simons and Toomre 2000).

In mammalian oocytes, lipid raft microdomains of the plasma membrane contain the ganglioside monosialotetrahexosylganglioside (GM1) and tetraspanin CD9, and previous studies have shown that these domains are involved in gamete fusion and adhesion (Kaji *et al.* 2000; Miyado *et al.* 2000; Alfieri *et al.* 2003). In oocytes, cell membrane integrity serves as an important platform for cell adhesion and attachment, ion conductivity and signaling processes (Evans 2012; McGinnis *et al.* 2013). The dynamic changes in membrane bioactivity can be mediated by the nanometer-sized lipid rafts. In respect to the lipid raft component, ganglioside GM1 is multi-functional in the exoplasmic leaflet, with essential roles in cell adhesion, cell recognition, ligand binding and signal transduction (Janes *et al.* 1999; Lopez and Schnaar 2009). The cholera toxin subunit B (CTB) which binds to the extracellular domain of GM1 is an established marker of lipid rafts. Other techniques used to identify rafts are shown in the Appendix, Table 4.

Lipid rafts were reported to play a critical role in spindle assembly during cell cleavage in embryogenesis, as well as cleavage furrow ingression (Albertson *et al.* 2005). For instance, GM1 was shown to contribute to cytokinesis during the cleavage of mouse preimplantation embryos and sea urchin eggs (Ng *et al.* 2005; Comiskey and Warner 2007).

Cholesterol is a key component of cell membranes and is essential for lipid raft formation and function. Thus, cholesterol depletion is often used to elucidate functional roles of

membrane integrity and lipid raft microdomain signaling. To date, relatively little is known about the essential roles of lipid rafts in oocytes and their functions during preimplantation embryo development.

In mouse oocytes, cholesterol synthesis is at least in part mediated by the surrounding cumulus cells and then transferred to the oocytes. In other words, cumulus cells are the source of cholesterol for mouse oocytes (Su *et al.* 2008). Interestingly, mice deficient in high density lipoprotein (HDL) receptor scavenger receptor, type B class I (SRBI) display abnormally large HDL particles, enriched in unesterified cholesterol and are infertile due to severely impaired oocyte developmental competence. The oocytes from these SRBI knockout (KO) mice showed abnormally high levels of cholesterol (Yesilaltay *et al.* 2014). However, fertility in these mice can be rescued by a HDL cholesterol lowering drug probucol (Miettinen *et al.* 2001). The SRBI KO oocytes were also prematurely activated-exiting metaphase II (MII) and completing meiotic progression under *in vitro* maturation (IVM) conditions. Similar events were also observed when these premature eggs were treated with a molecular complex containing cholesterol, again possibly because of an excess of cholesterol in the eggs (Yesilaltay *et al.* 2014).

In contrast, treatment of one-cell mouse zygotes with a cholesterol-depleting drug, methyl-β-cyclodextrin (MβCD) prevented embryonic development beyond 2 to 4 cells stages in culture (Comiskey and Warner 2007) further implicating the importance of cholesterol during preimplantation development. However, these embryos were not extensively characterised and the basis for arrested development was not determined. Thus although these studies cumulatively show that cholesterol is essential for normal oocyte developmental competence, the cellular mechanisms on which it impinges are not clear.

Intracellularly, cholesterol is found unevenly distributed among cellular membranes with the endoplasmic reticulum (ER) containing about 0.5-1% of total cellular cholesterol (Miller and Bose 2011). In contrast, mitochondria are relatively cholesterol-poor organelles, with the majority of cholesterol located in the outer mitochondrial membrane. A combination of *in vivo* and *in vitro* analyses reveal that cholesterol accumulation promotes organelle dysfunction via different levels, one of which is via reducing the fluidity of the mitochondrial

membrane, thereby clearly pinpointing causality of cholesterol levels in organelle malfunction. Additionally, it is well-established that ER is in physical contact with the mitochondria, interacting through a subregion of ER referred to as the mitochondria-associated ER membrane (MAM), a unique lipid composition enriched in cholesterol and phospholipids, with characteristics of lipid rafts (de Meis *et al.* 2010; Area-Gomez *et al.* 2012; Hedskog *et al.* 2013). It was recently suggested that lipid rafts may form contacts between organelles and transfer membrane-derived signals. For instance, they play a role in ER sorting by stabilising the association of glycosylphosphatidylinositol (GPI)-proteins with the ER membrane (Helms and Zurzolo 2004). It is possible that the MAM domains would act as a contact site for facilitating cholesterol transport to the mitochondria (Flis and Daum 2013). So, changes in cellular cholesterol levels might also disturb these contact sites and may ultimately cause a dysfunction in signaling between the ER and mitochondria. In oocytes, mitochondria are consistently found in clusters in close proximity to ER membranes. Given the proximity of the ER and mitochondria, it was reasoned that changes in local cholesterol levels would specifically affect these organelles, predominantly the mitochondria and in turn trigger cell death and impaired embryogenesis. In human oocytes, it was previously shown that mitochondria participate in GM1 lipid raft microdomain organisation (Van Blerkom and Caltrider 2013).

To date, no study has explored the relationship between rafts and mitochondria in oocytes; thus we also sought to examine mitochondrial membrane potential (MMP) (a marker for oocyte or embryo health) in response to oocyte cholesterol depletion and membrane disruption. In addition we have recently identified a compound, BGP-15 which increases oocyte mitochondrial membrane potential when given to mice (Wu *et al.* 2015). It has also been found to increase skeletal muscle mitochondrial number (Henstridge *et al.* 2014). Interestingly, in other cells BGP-15 acts a membrane fluidiser (Sapra *et al.* 2014) and can counteract the effects of cellular cholesterol depletion (Gombos *et al.* 2011). Thus, the aim of this study was to investigate the functional importance and significance of lipid rafts in mouse embryogenesis using MβCD, which extracts cholesterol from the cell membrane. The effect of MβCD-mediated cholesterol depletion on oocyte mitochondrial activity and subsequent embryo health was evaluated. The effect of BGP-15 on stabilising membrane rafts was also examined.

2.2 Materials and Methods

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

2.2.1 Animals

All experimental procedures were approved by the University of Adelaide Animal Ethics Committee and were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. C57BL/6 mice were obtained from the University of Adelaide Laboratory Animal Services and housed in the Animal Facility under a 14:10 hour light:dark cycle with *ad libitum* access to food and water**.**

Female mice (6 weeks of age) were hormonally induced to ovulate by intraperitoneal (i.p.) injection of 5 IU (international units) per 12 g body weight of *pregnant mare's serum gonadotrophin* (PMSG; National Hormone and Peptide Program, Torrance, CA, USA) followed 48 h later by 5 IU per 12 g body weight human chorionic gonadotrophin (hCG; Merck, Sharp and Dohme), each in 0.1 mL 0.9 % saline. Mice were humanely killed by cervical dislocation 13 h to 16 h post-hCG and COCs obtained from oviducts and collected in HEPES-buffered ∝-minimum essential medium (MEM) handling media (Life Technologies, Invitrogen, Carlsbad, CA, USA) supplemented with 1% fetal calf serum (FCS) (Life Technologies, Invitrogen, Carlsbad, CA, USA) and pre-warmed to 37°C prior to use. Male mice (8 weeks of age) of proven fertility were humanely killed by cervical dislocation and vas deferens/epididymis dissected for isolation of sperm for IVF.

2.2.2 Cholesterol Depletion of Oocytes

Methyl-β-cyclodextrin (MβCD) was used to deplete cholesterol from ovulated oocytes. A stock solution (1 M) in HEPES-buffered α-MEM culture media (Life Technologies, Invitrogen, Carlsbad, CA, USA) was stored at 4°C. The stock solution was vortexed at room temperature before preparing working solution (15 mM) in HEPES-buffered α-MEM handling media. Before use, solutions were pre-warmed at 37°C in an atmosphere of 5% CO_2 and 95% air until the reagent was completely dissolved. A control aliquot of HEPES-

buffered α-MEM handling media was also pre-warmed in the same conditions. Ovulated COCs were placed in drops with or without 15 mM MβCD in HEPES-buffered α-MEM handling media and incubated for 30 min at 37°C in an atmosphere of 5% CO_2, 20% O_2 and 95% air.

The dose (15 mM) and timing (30 min) of MβCD treatment were chosen based on preliminary dose-finding experiments (Supplemental Figure 1) which tested the effects of doses from 5 mM MβCD to 40 mM MβCD for either 30 min or 1 h incubation on oocyte viability.

2.2.3 *In vitro* Fertilisation (IVF) and Embryo Culture

Fertilisation, wash and embryo culture media were Research Vitro Fertilisation, Research Vitro Wash and Research Vitro Cleave, respectively, from Cook Medical (William A. Cook Australia Pty. Ltd., Queensland, Australia). Ovulated COCs isolated from oviducts were cultured in the presence or absence of 15 mM MβCD for 30 min at 37°C and then used for *in vitro* fertilisation (IVF). Epididymides and vasa deferentia from male mice were collected into 1 mL of warm (37°C) fertilisation medium. Sperm were extracted into the medium and allowed to capacitate at 37°C in an atmosphere of 5% CO_2, 20% O_2 and 95% air for 1 h. Capacitated sperm (10 μL) was added to each fertilisation drop (90 μL).

Following cholesterol depletion in MβCD (or control) media as above, COCs were washed twice in wash medium, and co-incubated with the sperm for 4 h at 37°C in an atmosphere of 5% CO_2, 20% O_2 and 95% air. Presumptive zygotes were then washed in embryo culture medium and placed into culture drops (15-20 zygotes per 20 μL drop) at 37°C in an atmosphere of 5% CO_2, 20% O_2 and 95% air. On Day 2, fertilisation was assessed and 2-cell embryos were transferred into a fresh 20 μL drop of embryo culture medium (10 embryos per drop). Embryo morphology was classified as appropriately developed ('on-time') using the following criteria; on Day 2, embryos at the 2-cell stage; and on Day 5, blastocysts or hatching blastocysts. Development rate was assessed on Day 2 as the percentage of embryos meeting the on-time development criteria from starting number of oocytes; while development on Day 5 as the percentage of embryos meeting the on-time

development criteria from 2-cell embryos. Twenty-three experimental replicates were performed, with 30 COCs used per treatment group within replicates.

2.2.4 Time-Lapse Embryo Culture

In separate experiments, time-lapse imaging was employed to more closely analyse developmental kinetics and cell size of oocytes. Oocytes were fertilised as previously described and then presumptive zygotes placed in 16-well dishes (Primovision, Vitrolife, Sweden), in culture media and conditions as described above, and monitored by Primo Vision time-lapse embryo monitoring system (Primovision, Thermo Scientific HERAcell VIOS 160i CO_2 incubator) with images of individual embryos generated every 15 min throughout culture. The timing of morphokinetic events, including first, second and third cleavage division and cavitation were recorded and intervals between each event determined and analysed using the Primo Vision Analyser Software (version 4.4.1.01.010). The diameters of the oocytes and embryos (measured to the outer circumference of the zona pellucida) were also automatically recorded throughout development. Therefore, the measurement included both the oocyte/embryo and its zona pellucida, and incorporated differences in perivitelline space and zona thickness. Three experimental replicates were performed, with 16 COCs per treatment group and replicate.

2.2.5 Cholesterol Staining of Oocytes and Embryos

Filipin complex from *Streptomyces filipinesis* was used to stain free cholesterol in ovulated oocytes and embryos (Day 5 blastocysts). Following cholesterol depletion or IVF, oocytes or embryos were immediately fixed in 4% paraformaldehyde in 1x phosphate buffered saline (PBS) with 1 mg/mL polyvinylpyrrolidone (PBS/PVP) for 1 h. Zona-enclosed oocytes or embryos were then washed three times in PBS, and incubated with 50 µg/mL filipin in PBS for 1 h in the dark at room temperature. This dose of filipin stain was used based on optimisation experiments which showed dose-dependent staining of free cholesterol in oocytes using filipin doses ranging from 20 µg/mL to 200 µg/mL (Supplementary Fig 2). After staining, the oocytes were washed in PBS/PVP for 5 min and mounted onto a microscope slide adhered with a secure-seal spacer (Life Technologies, Molecular Probes, Eugene, OR) in 5 µL PBS/PVP. Embryos were transferred with minimum medium onto

slides with a microdrop of glycerol (5 μL) without spacer. Images were captured using a Leica SP5 spectral scanning confocal microscope using blue filter (excitation = 405 nm, emission = 420-520 nm). Images were captured at 60x magnification and laser, sensitivity, and imaging parameters were kept constant between replicates and across experiments. Intensity of fluorescence was determined using Image J version 1.47r software by placing a circle across the oocyte image and measuring pixel intensity. The mean, area and integrated density (the sum of the pixel values in the circle) were reported and the corrected total cell fluorescence was calculated using the formula stated below. The data were then represented graphically as intensity of fluorescence over pixel widths. Three experimental replicates were performed, with 10 oocytes per treatment group.

Total fluorescence = Integrated Density- (Area of selected cell × Mean fluorescence of background readings)

2.2.6 Lipid Raft Staining of Oocytes and Embryos

Fluorescent-labeled cholera toxin subunit B (CTB-AF488, Life Technologies, Molecular Probes, Eugene, OR) staining was optimised using concentrations based on a previous study (Buschiazzo *et al.* 2013) with 20 μg/mL CTB-AF488 in 1x PBS resulting in clear visualisation of lipid rafts (data not shown). Briefly, COCs were hyaluronidase-treated by the addition of 10 μL of pre-warmed 1000 IU/mL hyaluronidase to the handling media, oocytes collected and fixed in 4% paraformaldehyde in PBS/PVP. Zona-enclosed oocytes were then washed in PBS/PVP followed by incubation in 100 μL drops with 20 μg/mL of CTB-AF488 for 30 min at 37°C in the dark. After staining, the oocytes were washed in PBS/PVP for 5 min and mounted under a coverslip in 5 μL PBS/PVP. Three experimental replicates were performed, with 6 oocytes per treatment group.

For embryos, they were permeabilised in 0.5% Triton X-100 in Cook Vitro Wash for 20 min at room temperature. To block non-specific uptake of staining reagents, embryos were incubated in 10% Normal Goat Serum (NGS) in PBS/PVP for 40 min. Embryos were then washed in PBS/PVP followed by incubation in 100 μL drops with 20 μg/mL of CTB-AF488 for 30 min at 37°C in the dark. To label cell nuclei, 1 μg/mL of Hoechst 33342 (Life Technologies, Molecular Probes, Eugene, OR) was added to the culture drop during the last

10 min of the incubation. After staining, embryos were washed in PBS/PVP for 5 min and transferred with minimum medium onto slides with a microdrop of glycerol (5 μL). Images were captured using a Leica SP5 spectral scanning confocal microscope using green filter (excitation = 473nm, emission = 490-590 nm). Images were captured at 90x magnification and laser, sensitivity, and imaging parameters were kept constant between replicates and across experiments. Four experimental replicates were performed, with 6 embryos per treatment group.

2.2.7 Mitochondrial Membrane Potential Assay

Denuded oocytes or embryos were incubated with 1.5 mM JC-1 (5,5', 6,6'-tetrachloro-1,1', 3,3', tetraethylbenzimida-zolylcarbocyanine iodide (Invitrogen, CA, USA) in handling media (oocytes)/ Cook Vitro Wash (embryos) for 15 min at 37°C in the dark. Oocytes were washed once in PBS/PVP and then imaged immediately using the confocal microscope using a narrow green filter (490-540 nm) and a narrow red filter (570-620 nm). Images were captured at 60x magnification and laser, sensitivity, and imaging parameters were kept constant between replicates and across experiments. Intensity of fluorescence was determined as described above for filipin staining. Four experimental replicates were performed for oocytes and five replicates for embryos, with 10 oocytes/blastocysts per treatment group.

2.2.8 Inner Cell Mass (ICM) and Trophectoderm (TE) Assessments

Allocation of cells to TE and ICM lineages was assessed in Day 5 blastocyst-stage embryos using a modification of the method of Handyside and Hunter (1984) (Handyside and Hunter 1984). Briefly, blastocysts were incubated with 0.5% pronase at 37°C to remove the zona pellucida, followed by 10 min incubation at 4°C in 10 mM TNBS (2,4,6-trinibrobenzene sulfonic acid) in the dark. Blastocysts were then washed with MOPS (3-(N-morpholino)propanesulfonic acid) and incubated with 0.1 mg/mL anti-DNP (Dinitrophenyl) for 10 min at 37°C. Following, blastocysts were washed again and incubated for 5-10 min in 10 μg/mL of propidium iodide (PI) in 10% guinea pig serum. Blastocysts were then transferred to 6 μg/mL bisbenzimide in ethanol overnight and washed in 100% ethanol the following day. Blastocysts were then mounted on microscopic slides in glycerol underneath

a cover slip and visualised using an epifluorescence microscope (Nikon, TE 2000-E) at 200× equipped with an ultraviolet filter (excitation, 340–380 nm; emission, 440–480 nm). ICM nuclei labeled with bisbenzimide appear blue whereas TE nuclei labeled with a combination of bisbenzimide and PI appear pink or red. Total and TE cell numbers were counted individually and ICM cell numbers were calculated by subtracting TE from the total cell number.

2.2.9 Neutral Lipid Staining in Embryos

Embryos were fixed in 4% paraformaldehyde in PBS/PVP, washed in PBS/PVP and stained with 1 μg/mL of the neutral lipid stain BODIPY 493/503 (Life Technologies, Invitrogen, CA, USA) in PBS/PVP for 1 h in the dark at room temperature. Embryos were washed in PBS/PVP for 5 min and mounted under a coverslip in 5 μL of DAKO fluorescent mounting media (Dako North America Inc, CA, USA). Images were captured using a Leica SP5 spectral scanning confocal microscope using green filter microscope using green filter (excitation = 473nm, emission = 490-590 nm). Images were captured at 90x magnification and laser, sensitivity, and imaging parameters were kept constant between replicates and across experiments. Intensity of fluorescence was determined as described above for filipin staining. Two experimental replicates were performed, with five blastocysts per treatment group.

2.2.10 *In vitro* BGP-15 Treatment

To test the effect of BGP-15 on lipid raft distribution in oocytes, preliminary experiments were conducted under different conditions. Treatment groups were i) control (α-MEM handling media supplemented with 1% FCS), ii) cholesterol depletion (15 mM MβCD), iii) 15 min 10 μM BGP-15 followed by 30 min cholesterol depletion, iv) 30 min cholesterol depletion followed by 30 min 10 μM BGP-15, and v) 30 min of cholesterol depletion + 10 μM BGP-15. Following treatment, oocytes were washed in PBS/PVP and then fixed in 4% paraformaldehyde for at least 1 h. Lipid raft staining was performed as previously described. After staining, the oocytes were washed in PBS/PVP for 5 min and mounted under a coverslip in 5 μL of DAKO fluorescent mounting media. Images were captured using the

Olympus Fluoview FV10i confocal microscope (Olympus; Tokyo, Japan). Two experimental replicates were performed, with 6 oocytes per treatment group.

2.2.11 Statistical Analyses

All measures are reported as mean ± SEM. Statistical significance was determined as indicated, by using Student's *t*-test or one-way ANOVA with Tukey's post hoc tests, as appropriate, using GraphPad Prism v008 (GraphPad Software, La Jolla, CA, USA) for Windows. Time-lapse data were analysed by using repeated measures with linear mixed-effects model in SPSS (IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.). Normality of data was confirmed by analysing the spread of residuals. A *P*-value of less than 0.05 was considered statistically significant.

2.3 Results

2.3.1. Cholesterol depletion alters oocyte lipid raft distribution but not membrane potential

The ability of MβCD to deplete mouse oocyte cholesterol levels was verified by incubating oocytes in increasing doses of MβCD (0, 5, 10 and 15 μg/mL) for 30 min followed by filipin staining of free cholesterol. Fluorescent filipin stain in oocytes was visibly reduced with increasing MβCD concentration (Figure 1A). Image analysis confirmed that MβCD significantly and dose-dependently, reduced cholesterol content in the treated oocytes ($P \leq 0.05$; Figure 1B). The reduction in cholesterol content by MβCD at doses up to 15 mM for 30 min did not impact oocyte morphology, while MβCD doses of more than 20 mM for 30 min, or more than 15 mM for 1 h were toxic to oocytes (Suppl. Fig. 1). These results, similar to others (Buschiazzo *et al.* 2013), indicate that treatment of oocytes with 15 mM MβCD for 30 min significantly decreases oocyte cholesterol content but does not affect viability.

The effect of cholesterol depletion on oocyte raft distribution was also examined. Untreated oocytes showed uniform distribution of lipid rafts throughout the cell membrane as well as punctate staining within the cytoplasm (Figure 2A). This staining pattern was specific as negative control oocytes processed identically but without CTB stain gave minimal background. Oocytes treated with MβCD exhibited disrupted lipid raft distribution, specifically a marked reduction in the plasma membrane staining (Figure 2A) although punctate cytoplasmic staining persisted.

To determine whether oocyte cholesterol depletion and lipid raft disruptions are associated with changes to mitochondrial function, MMP was examined using JC-1 potentiometric dye. Both control and MβCD-treated oocytes exhibited red punctate fluorescence localised to the pericortical region and green fluorescence concentrated in the deeper cytoplasm (Figure 2B). Reductions in MMP, determined by the ratio of red to green fluorescence, are considered a hallmark of mitochondrial damage (Vayssier-Taussat *et al.* 2002; Savitha and Panneerselvam 2006). Analysis of the ratio of red/green fluorescence intensity showed that oocytes depleted of cholesterol with MβCD exhibited no differences compared with untreated oocytes (Figure 2C).

Cumulatively these results confirm that cholesterol is essential for lipid raft formation in mouse oocytes and demonstrate that disruptions to lipid rafts in the oocyte plasma membrane is not associated with significant alterations in oocyte mitochondrial activity.

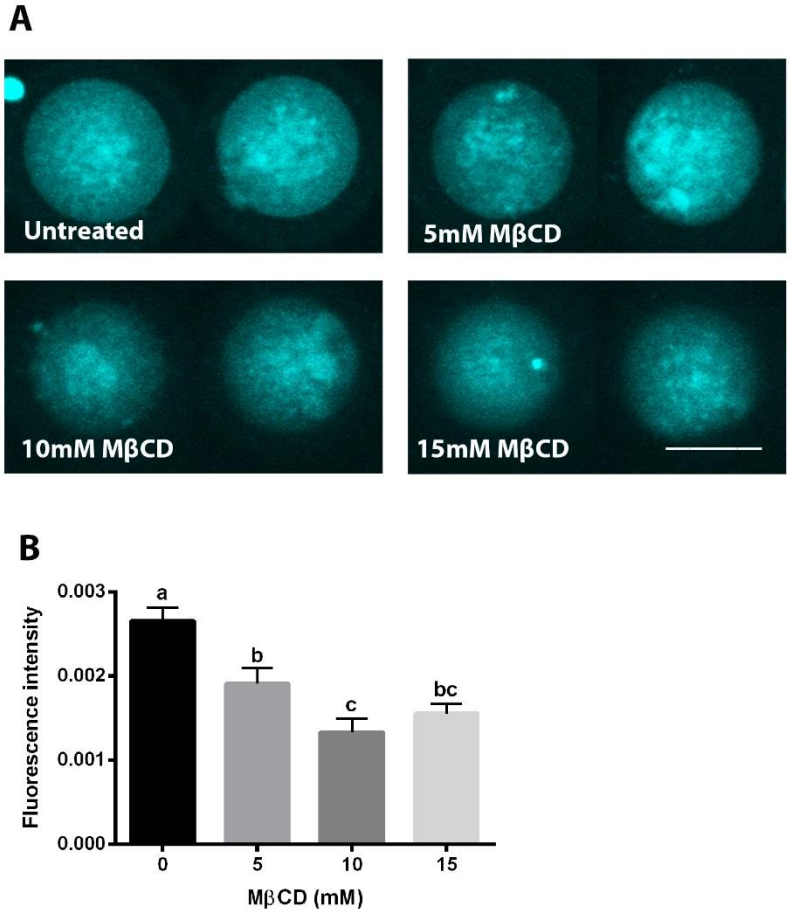


Figure 1 Effect of methyl-β-cyclodextrin (MβCD) on mouse oocyte cholesterol content. (A) Representative photos of oocytes stained with filipin after incubation in the indicated dose of MβCD for 30 min. (B) Fluorescence intensity (arbitrary units) of filipin-stained oocytes. Data presented as mean ± SEM; n = 21-32 oocytes per treatment group from at least 3 independent experiments. Groups with different superscripts differ significantly by one-way ANOVA with Tukey's post hoc test ($P \leq 0.05$). Scale bar = 50 µm.

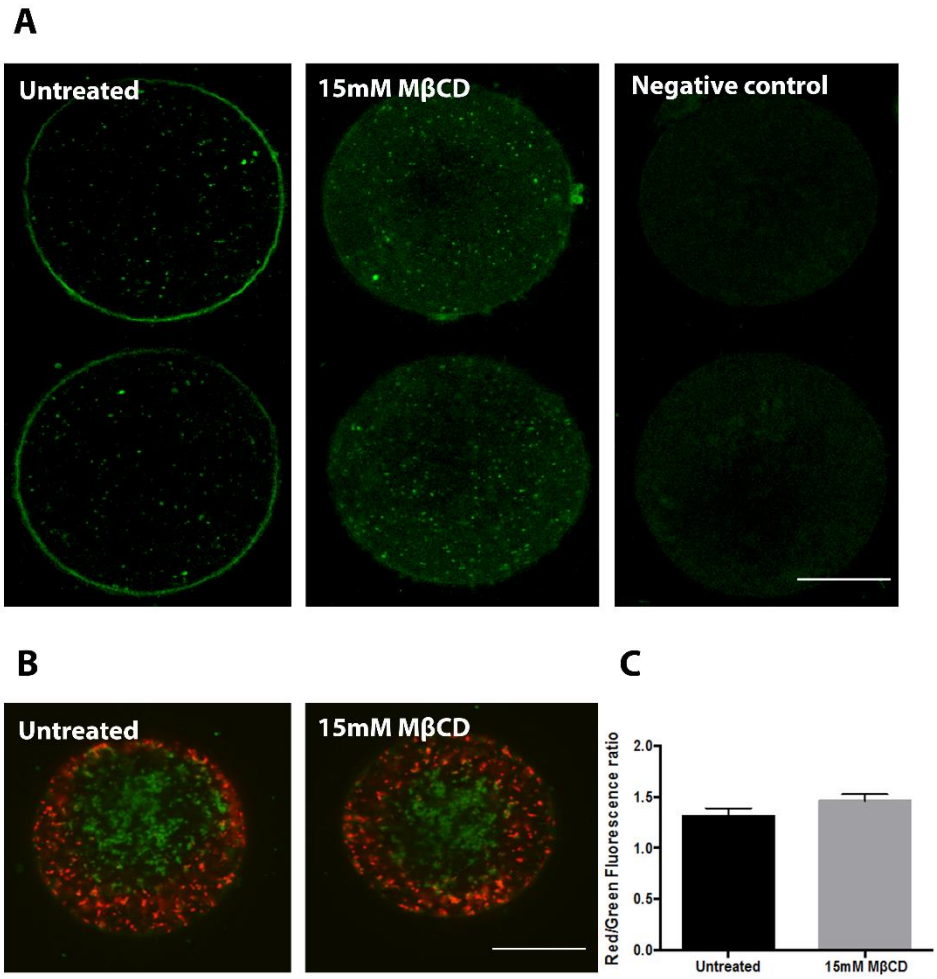


Figure 2 Effect of cholesterol depletion on mouse oocyte lipid raft distribution and mitochondrial membrane potential.

(A) Representative photos of oocyte lipid rafts detected by Cholera Toxin Subunit B (CTB-AF488 staining in untreated oocytes or those treated with MβCD. Negative control consisted of omission of the CTB stain in untreated oocytes. (B) Representative photos of untreated or MβCD-treated oocytes stained with JC-1 dye for detection of mitochondrial membrane potential. (C) JC-1 fluorescence intensity quantification presented as mean ± SEM, n = 25 (untreated) and n = 26 (MβCD) oocytes from 4 independent experiments. $P > 0.05$, unpaired *t*-test. Scale bar = 50 µm.

2.3.2 Oocyte cholesterol depletion alters developmental kinetics and embryo development

Control oocytes or those depleted of cholesterol using MβCD were subjected to IVF, followed by morphometric assessment and time-lapse imaging to closely monitor developmental kinetics and cell size. Following IVF, a significant proportion of cholesterol-depleted oocytes failed to undergo cleavage, however, those which cleaved continued to develop to blastocyst stage with no further reduction in the blastocyst development rate (Figure 3A). On average, embryos cleaved at 14 h post-insemination and reached the 3-cell stage and 4-cell stage at 43 h and 45 h, respectively (Figure 3B). Blastocyst formation ranged from 92 h to 96 h post-insemination (Figure 3B). Interestingly, the timing of the morphokinetic events revealed that the time to cleavage was longer in the cholesterol-depleted oocytes when compared to control (Figure 3B and Figure 3C). Although the cholesterol-depleted oocytes took longer to cleave, they were quicker to progress from 2-cell to 3-cell stage when compared to the control. Thus they were 'caught up' by the 3-cell to 4-cell cleavage division (Figure 3C). However, as the embryo progressed from 4-cell to morula, the time duration was again increased in the cholesterol-depleted group when compared to the control. Similarly, the timing from morula to blastocysts was slightly longer in the cholesterol-depleted group, but this was not statistically different compared to the control (Figure 3C). Furthermore, the time interval from post-insemination to blastocyst was also not different between the groups (Figure 3C). Overall, these results indicate that cholesterol depletion affects the time intervals between early developmental milestones as early as the 2-cell stage. Moreover, examination of oocyte size showed that MβCD-induced cholesterol depletion significantly reduced oocyte size (Figure 3D). Subsequently, blastomere size in the 2-cell embryos generated from the cholesterol-depleted oocytes was also significantly reduced (Figure 3E); and this reduction in embryo size was maintained through to the blastocyst stage (Suppl. Fig. 3).

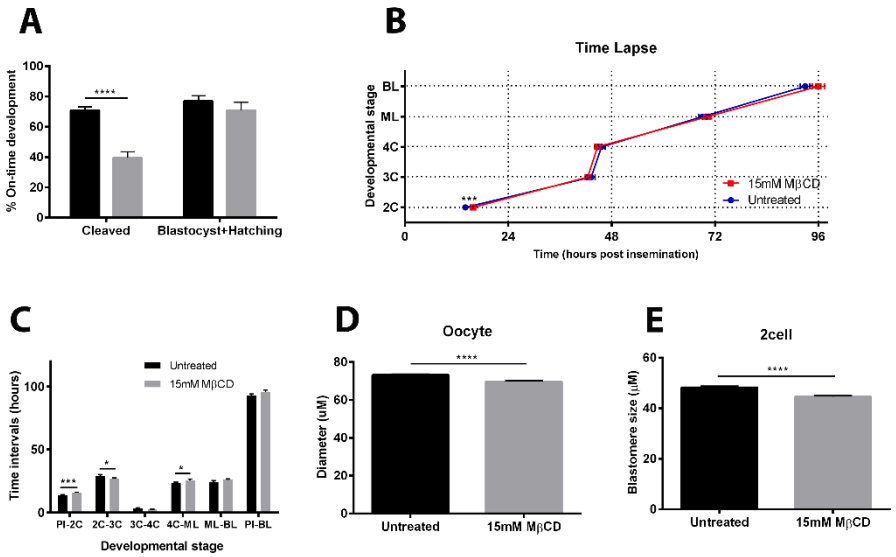


Figure 3 Effect of cholesterol depletion on developmental kinetics and cell size following time-lapse imaging.

(A) Cleavage rate assessed on Day 2 and blastocyst rate on Day 5. Data are presented as mean ± SEM; n = 23 experimental replicates. (B) Timing of developmental milestones (hours post-insemination) in control embryos and those treated with 15 mM MβCD. (C) Time intervals between each developmental stage. (D) Oocyte diameter post-insemination. (E) Blastomere size of 2-cell embryos. (B-E) Data are presented as mean ± SEM; n = 38-46 embryos from 3 independent experiments; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, unpaired *t*-test and repeated measure by linear mixed-effects model; PI- post-insemination, 2C- 2-cell, 3C- 3-cell, 4C- 4-cell, ML- morula, BL- blastocyst.

2.3.3 Oocyte cholesterol depletion affects embryo phenotypes

To determine whether oocyte cholesterol stores influence embryo health, preimplantation embryos generated by IVF of control or MβCD-treated oocytes were assayed for a number of markers of quality and differentiation, including cell number allocation, neutral lipid content, free cholesterol and lipid raft distribution. Differential staining for ICM and TE showed a significant reduction in total cell number in embryos derived from cholesterol-depleted oocytes compared to controls (Control = 47.91 ± 0.89 vs. MβCD = 43.56 ± 1.35; $P \leq 0.01$; Table 1). TE cell numbers in the embryos from the cholesterol-depleted oocytes were also significantly lower than those in the control embryos (Control = 32.98 ± 1.24 vs. MβCD = 27.83 ± 1.33; $P \leq 0.05$). No differences in the ICM cell numbers and ICM:TE cell ratio, a marker of embryo health (Van Soom *et al.* 2002), were observed between the groups (Table 1).

Embryo lipid droplet abundance and localisation were measured using BODIPY 493/503 stain which stains neutral lipids, i.e. triglycerides and cholesterol. Lipid droplets were distributed throughout control embryos (Figure 4A). Embryos from MβCD-treated oocytes showed visibly lower levels of lipid than control embryos with droplets localised mainly in the TE (Figure 4A). Quantification of total fluorescence confirmed that lipid content was significantly reduced in embryos derived from cholesterol-depleted oocytes ($P \leq 0.05$; Figure 4B). This result suggests that the reduction in cholesterol in oocytes is maintained through embryogenesis.

MMP and reactive oxygen species (ROS) levels were also assessed as markers of embryo quality. Visualised using JC-1 staining, MMP levels and distribution in embryos was similar to previous reports (Van Blerkom *et al.* 2002; Acton *et al.* 2004; Van Blerkom 2004) and no difference was found in MMP in the intermediate or outer region of the embryos (Figure 5). Similarly, intracellular ROS levels, assayed using the fluorescent probe CM-H_2DCFDA, were not different between embryos generated from cholesterol-depleted oocytes compared to controls (Suppl. Fig. 4).

To determine the impact of cholesterol depletion on embryo membrane structure, specifically lipid bilayer and raft integrity, blastocysts were stained with either filipin or

CTB-AF488. Embryos derived from oocytes depleted of cholesterol exhibited a clear disruption to membrane structure. Specifically the TE cell junctions were apparent in the control embryos but were not visible in embryos derived from cholesterol depleted oocytes (Figure 6A, Suppl. Fig. 5).

Similarly, intensive staining of lipid rafts was observed in the TE and ICM cells of embryos from untreated oocytes, but to a lesser extent in the embryos generated from MβCD-treated oocytes (Figure 6B, Suppl. Fig 6), reflective of changes to the lipid bilayer structure and disorganisation of lipid rafts. Quantification of total fluorescence confirmed that cholesterol-depletion in oocytes led to significantly reduced lipid raft staining in embryos ($P \leq 0.05$; Figure 6C). These results provide functional data showing that the cholesterol content of oocytes influences lipid content and membrane structures through to the blastocyst stage.

Table 1 Effect of oocyte cholesterol depletion on subsequent mouse blastocyst cell number.

	Untreated	**15 mM MβCD**	**P-value**
Total cell number	47.91 ± 0.8882	43.56 ± 1.352	0.0064
TE cell number	32.98 ± 1.239	27.83 ± 1.328	0.0130
ICM cell number	14.94 ± 1.116	14.86 ± 0.5706	NS
Ratio of ICM to TE	0.5276 ± 0.05412	0.5946 ± 0.04247	NS

TE: trophectoderm; ICM: inner cell mass; n = 47 untreated oocytes and n = 36 MβCD-treated oocytes. NS = not significant. P-values are from unpaired *t*-test.

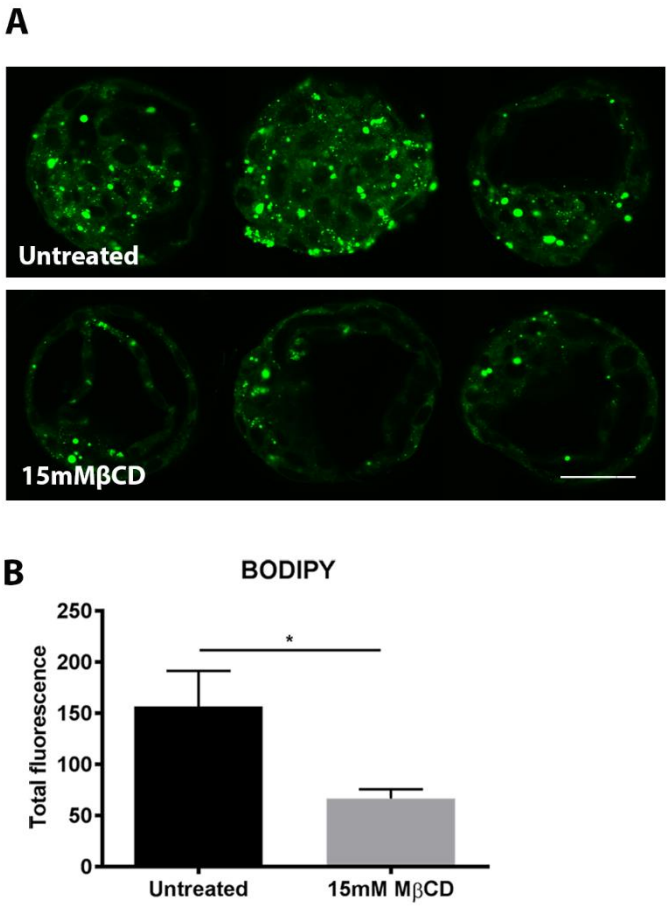


Figure 4 Effect of cholesterol depletion in oocytes on mouse embryo neutral lipid content.

COCs were untreated or cholesterol-depleted using MβCD then fertilised and cultured *in vitro* to the blastocyst stage. Blastocysts were stained with BODIPY 493/503. (A) Representative photos of BODIPY-stained blastocysts. (B) Quantification of fluorescence intensity. Data are presented as mean ± SEM, n = 8 blastocysts per group from two independent experiments. * $P \leq 0.05$, unpaired *t*-test. Scale bar = 100 µm.

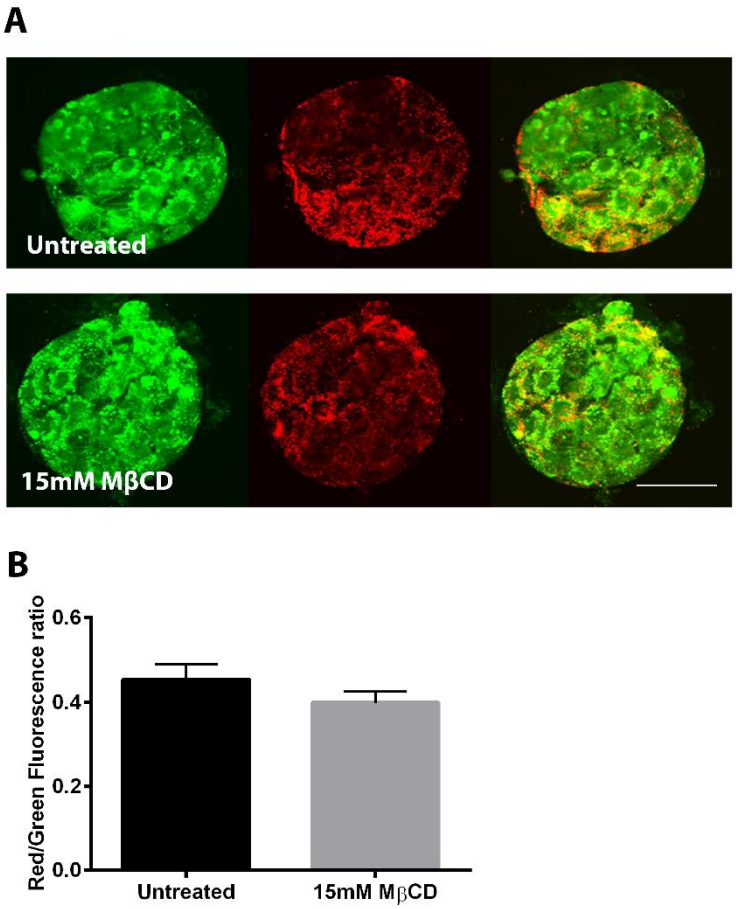


Figure 5 Effect of cholesterol depletion in oocytes on mouse embryo mitochondrial membrane potential.

COCs were untreated or cholesterol-depleted using MβCD then fertilised and cultured *in vitro* to the blastocyst. Blastocysts were stained with JC-1. (A) Representative photos of embryos stained with JC-1. (B) The ratio of red to green fluorescence quantified as an indicator of mitochondrial activity. Data are presented as mean ± SEM, n = 12 (control) and n = 15 (MβCD) embryos from 5 independent experiments. $P > 0.05$, unpaired *t*-test. Scale bar = 100 µm.

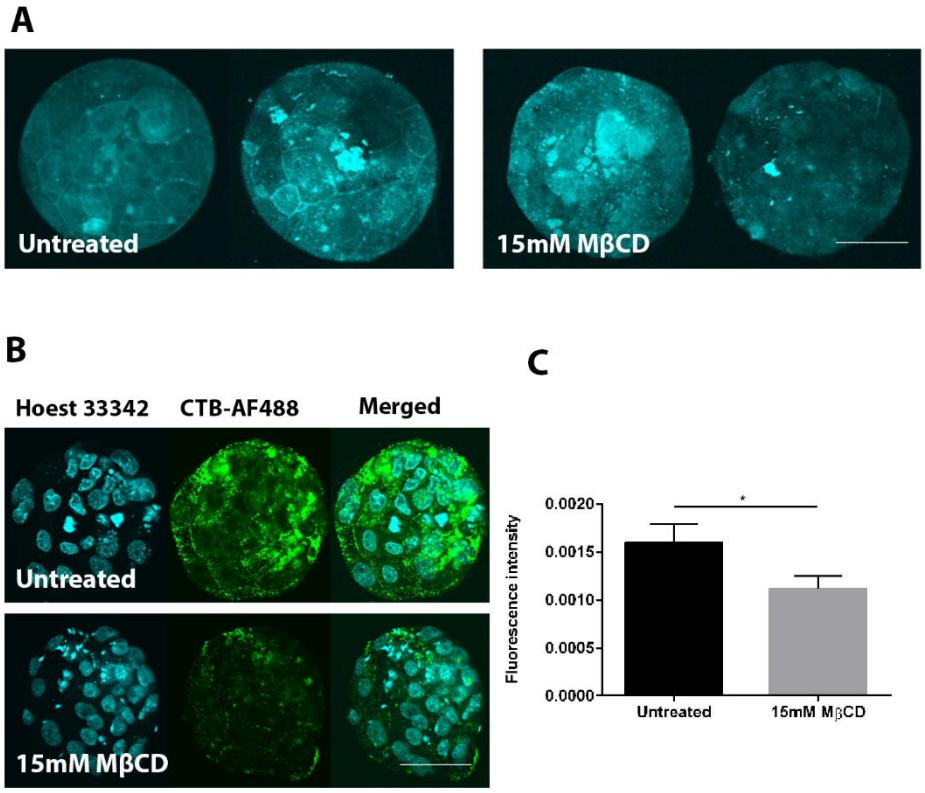


Figure 6 Effect of cholesterol depletion in oocytes on mouse embryo membrane structures.

(A) Representatives photos of blastocysts derived from oocytes depleted of cholesterol using MβCD (or controls) stained with filipin to detect cholesterol level. (B) Representative photos of CTB-stained blastocysts. Hoechst 33342 (blue) shows DNA staining; CTB-AF488 (green) shows lipid rafts staining. (C) Quantification of CTB-AF488 fluorescence intensity. Data are presented as mean ± SEM, n = 13 (untreated) and n = 14 (MβCD) blastocysts from four independent experiments. * $P \leq 0.05$, unpaired *t*-test. Scale bar = 100 µm.

2.3.4 BGP-15 does not rescue the disruption of oocyte raft distribution *in vitro*

To determine whether short exposure to BGP-15 has any effect on the cholesterol-depleted oocyte membrane structure, oocytes were exposed to 10 μM of BGP-15 before, after or simultaneously with 15 mM MβCD. Lipid raft distribution was clearly observed on the plasma membrane of control oocytes and as punctate staining intracellularly (Figure 7A). Consistent with earlier findings, short exposure to 15 mM MβCD disrupted lipid raft distribution in the oocytes particularly on the plasma membrane (Figure 7B). BGP-15 treatment for 15 min prior to exposure to MβCD did not restore lipid rafts distribution, although some patches of plasma membrane had low amounts of staining (Figure 7C). Similarly, exposure of oocytes to BGP-15 after MβCD incubation also did not rescue the raft organisation (Figure 7D), indicating that BGP-15 treatment before or after cholesterol depletion did not promote the remodelling of raft organisation. When BGP-15 was added together with MβCD**,** identical results were observed; there was no improvement in raft distribution (Figure 7E).

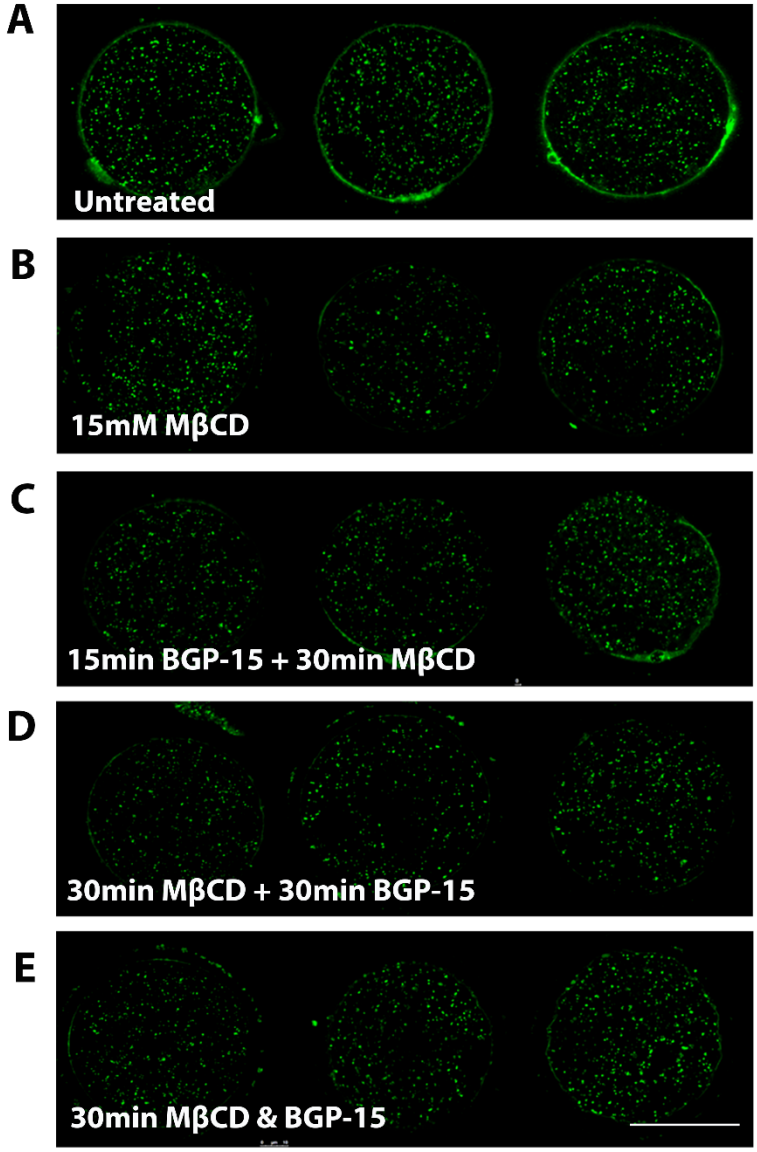


Figure 7 Effect of BGP-15 treatment on mouse oocyte membrane raft distribution *in vitro*.

(A) Representatives photos of control oocytes stained with CTB-AF488, (B) Oocytes that were exposed to 15 mM MβCD, (C) Oocytes that were incubated with 10 µM BGP-15 for 15 min followed by 30 min of 15 mM MβCD, (D) Oocytes exposed to 30 min of MβCD followed by 30 min of BGP-15 and (E) Oocytes incubated with MβCD and BGP-15 for 30 min. Scale bar = 60 µm.

2.4 Discussion

Our data confirms that lipid rafts are distributed throughout the oocyte plasma membrane and in embryonic TE and ICM. Experiments using a cholesterol-extracting reagent provided functional data showing that removal of cholesterol from the oocyte disrupted oocyte raft distributions and caused significant disruptions to embryogenesis. Interestingly, cholesterol depletion and lipid raft disruptions were not associated with alterations in mitochondrial membrane potential in either oocytes or embryos. Importantly however, we have shown that cholesterol is necessary to support normal embryo development to the blastocyst stage and the formation of normal embryonic membrane structure.

Consistent with previous studies, we saw a uniform distribution of lipid rafts on the oocyte membrane (Comiskey and Warner 2007; Buschiazzo *et al.* 2013). Our finding that treatment of oocytes with 15 mM MβCD for 30 minutes significantly decreases oocyte cholesterol content but does not affect viability was also supported by a previous study (Buschiazzo *et al.* 2013). Following cholesterol depletion, lipid raft distribution surrounding the oocyte membrane was completely disrupted demonstrating that cholesterol has a structural/stabilising role in the membrane microdomain assembly. This phenomenon has also been observed in several other cell types (Diaz *et al.* 2005; Barman and Nayak 2007; Chubinskiy-Nadezhdin *et al.* 2013). This result also suggests that signaling machinery on the oocyte surface may be altered, as many proteins involved in signal transduction processes are embedded within the plasma membrane. Consistent with this, Buschiazzo *et al.* (2013) reported that membrane localised signaling molecule, c-Src was disturbed in oocytes following cholesterol removal (Buschiazzo *et al.* 2013). In contrast, even though plasma membrane staining was disrupted, there was clear intracellular staining persistent in the oocyte cytoplasm. This accumulation of fluorescent probe has a localisation pattern that resembles MAMs, contact sites between mitochondria and ER, as judged by their shape, size, and distribution. This intracellular staining is not disrupted by MβCD suggesting that MAMs are more resistant to cholesterol depletion and possibly that communication between mitochondria and ER is not affected.

The disruption of rafts in the oocytes was maintained through embryo development; with MβCD-treated, cholesterol-depleted embryos exhibiting a disruption of lipid rafts in the

embryos as revealed by fluorescent labeling of ganglioside GM1. In early developing mammalian embryos, lipid rafts have been reported to play a key role in cytokinesis (Comiskey and Warner 2007; Sato *et al.* 2011). During cell cleavage, they were shown to play a vital role in central spindle assembly and cleavage furrow ingression (Albertson *et al.* 2005). Moreover, it was previously reported that MβCD-mediated disruption of lipid rafts in 8-cell embryos caused the release of a specific raft domain into the perivitelline space and decompaction, and suppressed cell division (Sato *et al.* 2011). In addition, cell-surface antigens that are markers of pluripotency, in particular, stage–specific embryonic antigen 4 (SSEA4), were reported to have a similar pattern of localisation with that of CTβ staining (Comiskey and Warner 2007). Therefore, the disorganisation of lipid rafts in the embryos may correlate to reduced pluripotency which would affect cell lineage differentiation. Our results extend these observations by demonstrating that manipulation of cholesterol levels even prior to fertilisation impairs zygote cleavage in a large subset of embryos, reduces proliferation in those that progress and alters in TE cell numbers.

Mitochondrial membrane potential (MMP) is a key indicator of cellular viability and is crucial to embryo viability. It reflects the pumping of hydrogen ions across the mitochondrial inner membrane during the process of electron transport and oxidative phosphorylation, the driving force behind adenosine triphosphate (ATP) production. A reduction in MMP is a hallmark of mitochondrial damage (Vayssier-Taussat *et al.* 2002; Savitha and Panneerselvam 2006), which triggers the production of reactive oxygen species (ROS) and ultimately leads to apoptosis. Furthermore, dysfunction or abnormalities of the mitochondria in oocytes can also induce apoptosis in embryos, compromising their developmental ability (Mitchell *et al.* 2009; Yu *et al.* 2010). Surprisingly, and counter to our hypothesis, cholesterol depletion, did not affect mitochondrial membrane potential. This conclusion is supported by the observation of unchanged ROS levels in embryos derived from cholesterol depleted oocytes (Suppl. Fig. 4) and may again be due to MβCD more greatly affecting plasma membrane raft structures rather than intracellular CTB-stained raft structures. Further, mitochondrial organisation is also considered an important parameter for evaluating oocyte quality. Thus, qualitative measures such as distribution of mitochondria, degree of mitochondrial clustering, and patterning of stains were also assessed in all the oocytes but no difference was observed between the treatment groups. Future studies could be directed

toward assessing the actual number, size or morphology of the mitochondria as mitochondria in oocytes are known to undergo substantial change from the oocyte to blastocyst stage embryo.

Cholesterol depletion appears to affect various aspects of the fertilisation process (Comiskey and Warner 2007; Buschiazzo *et al.* 2013). By using the same cholesterol depletion method, Buschiazzo *et al.* (2013) showed that the fertilisation index was decreased as measured by the number of sperm heads within the oocyte cytoplasm (Buschiazzo *et al.* 2013). Another study by Comiskey and Warner (2007) also reported that the presence of MβCD in the embryo culture media reduced the percentage of embryos from reaching the blastocyst stage (Comiskey and Warner 2007). In the current study, we exposed the COCs to MβCD for a short period of time prior to IVF and time-lapse video recording and image analysis were performed to determine the time of some key events during embryo development. In control embryos, cell division occurs every 18-20 hours. Embryos dividing either too slow or too fast may have metabolic and/or chromosomal defects (Leese 2002; Munne 2006; Magli *et al.* 2007; Lee *et al.* 2015). Besides the importance of timing of cleavage, the time between each division is also crucial (Scott *et al.* 2007; Lemmen *et al.* 2008; Wong *et al.* 2010; Meseguer *et al.* 2011). In our study, we observed a slower cleavage time in the cholesterol-depleted oocytes than in the controls. Studies have found that early cleavage time was a strong predictor of embryo quality and that transfer of early cleaving embryos resulted in higher implantation and pregnancy rates (Lundin *et al.* 2001; Van Montfoort *et al.* 2004). In addition, blastocysts generated from early-cleaving embryos were of higher quality than those from later-cleaving embryos such that they had a larger ICM cell number and higher percentage of developing fetuses on E13.5 (Fenwick *et al.* 2002; Salumets *et al.* 2003; Lee *et al.* 2015).

In this study, exposure of mouse COCs to MβCD had notable negative effects on embryo quality. Using BODIPY 493/503 which stains triglycerides and cholesterol, there was a clear reduction in these neutral lipid droplets in the embryos generated from the cholesterol-depleted oocytes. This implicates that availability of cholesterol is essential for lipid droplet formation or maintenance in the embryos. Lipid droplets are now understood to be involved in a range of cellular processes and interact with various organelles and in embryos may

possibly regulate lipid exchange to various membrane organelles to support further development. Cholesterol depletion also resulted in changes in the blastocyst cell numbers. Total and TE cell numbers were significantly decreased in the embryos generated from the cholesterol-depleted oocytes. This alteration in cell numbers can have long-term effects for embryo growth. Changes in the blastocyst cell numbers have been shown to positively correlate with implantation and pregnancy rates (Lane and Gardner 1997). For instance, total blastocyst cell count has been associated with hatching ability *in vitro* (Van Blerkom 1993; Lea *et al.* 1996). The overall blastocyst cell numbers reported here might be considered relatively low; even though our results are consistent with a previous study (Banwell *et al.* 2007). It is important to note that these embryos were cultured under atmospheric oxygen (i.e. 20% concentration) and studies in both mice and human embryos have reported a reduced cell number in embryos cultured under 20% oxygen when compared to those cultured under 5% or 7% oxygen (Gardner and Lane 1996; Dumoulin *et al.* 1999; Karagenc *et al.* 2004). Further, it is well known that proliferation of embryonic cells can be negatively influenced by suboptimal conditions and that TE cells, which are in closer contact with the environment (culture medium), are the first cells to differentiate in the embryo; providing likely explanations for the TE-specific reduction in cell numbers that was observed, Given that TE cells are precursor cells for the placenta and are more metabolically active (Hewitson and Leese 1993; Houghton 2006), the reduction in TE cells may have downstream effects on placental growth, which is clinically relevant since both low and high placental weight at birth in humans have been shown to correlate with the likelihood to develop coronary heart disease, hypertension, stroke and cancer in adulthood (Barker *et al.* 1990; Eriksson *et al.* 2011).

Interestingly, embryos derived from oocytes depleted of cholesterol exhibited a clear disruption to membrane structure. Specifically, the ‘tight junction-like’ permeability seals on the embryo surface were not visible. This permeability seal is thought to be important for vectorial transport of ions and water to generate the blastocoel cavity (Biggers *et al.* 1988). The presence of cholesterol-filipin complexes on the cell surface membrane suggests the likelihood of high cholesterol concentration in this region. Defects in the membrane structures of these outer, likely TE cells, further suggests that subsequent placenta development may be altered.

BGP-15 has the potential to stabilise membranes and remodel lipid rafts in some cellular contexts (Gombos *et al.* 2011) and since we have observed that BGP-15 is highly effective in improving oocyte quality (Wu *et al.* 2015) we wanted to determine whether BGP-15 action in oocytes is via membrane stabilisation. However, in the present study, *in vitro* BGP-15 treatment did not promote the remodeling of lipid rafts in the oocyte that were exposed to MβCD. In these experimental conditions, lack of cholesterol accounts for the defects observed, and thus it is possible that BGP-15 may not affect cholesterol-mediated defects in the oocytes.

In conclusion, we show that membrane raft integrity is necessary to accomplish efficient fertilisation in mouse oocytes. Functional data from embryo culture experiments supports the concept of a critical role for lipid rafts in development as removal of cholesterol from oocytes by MβCD reduced the rates of cleavage, altered morphokinetic events during embryo development and subsequently perturbed developmental competence. Taken together, our results demonstrate that changes in oocyte cholesterol level cause an effect on the oocyte and embryo membrane structure organisation and impact fertilisation success. Furthermore, obesity, a condition associated with elevated levels of cholesterol in the follicular fluid, is known to cause poor fertility outcome (Valckx *et al.* 2012). Therefore, it is speculated that high cholesterol would also disrupt rafts and this may be one of the underlying issues with obesity-mediated effects on conception rates. This study provides us a better understanding of the role of cholesterol stores and membrane organisation during preimplantation development.

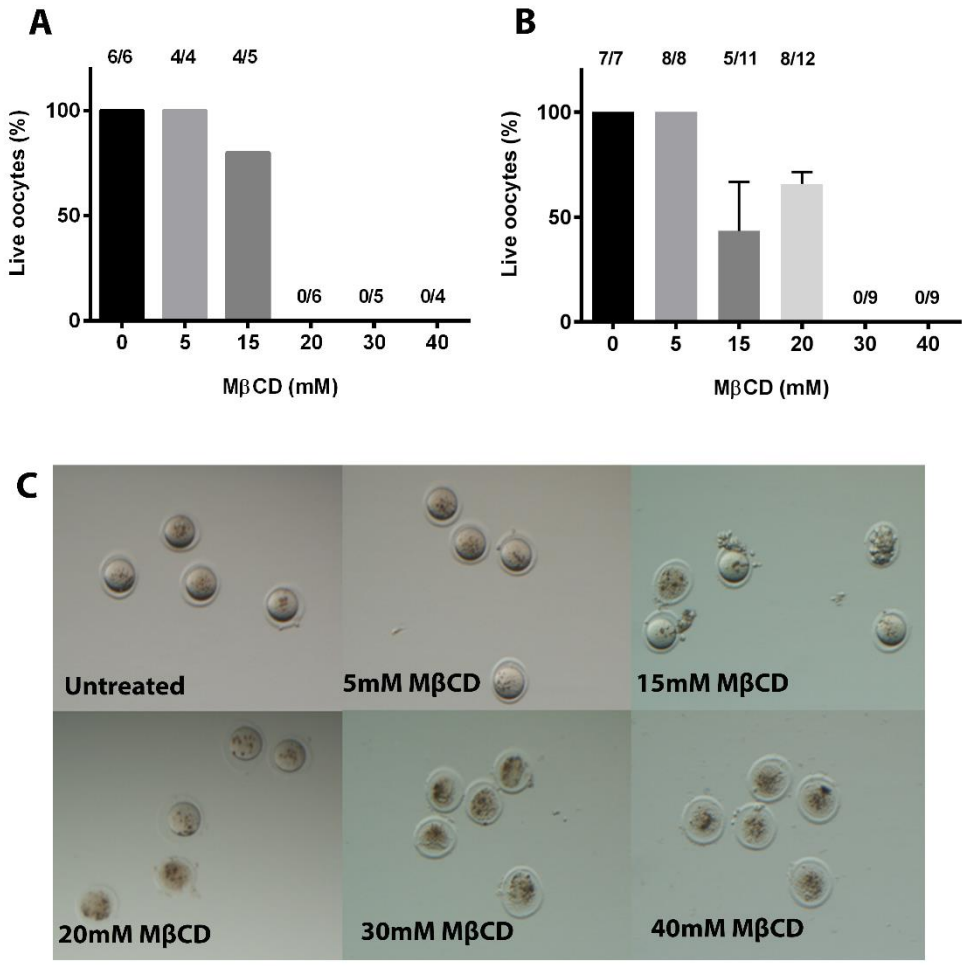
2.5 Supplementary Materials

The optimal dose of MβCD to deplete cholesterol without reducing oocyte viability was determined by examining the effect of increasing doses of MβCD on oocyte survival. MβCD doses of 0, 5, 15, 20, 30 and 40 mM were chosen based on previous reports in other cell types (Sun and Whittaker 2003; Breen *et al.* 2012; Buschiazzo *et al.* 2013).

An initial experiment (n = 1 experimental replicate with 30 oocytes) was conducted in which oocytes were incubated in HEPES-buffered α-MEM culture medium supplemented with MβCD at concentrations ranging from 0 mM to 40 mM for 1 h. Oocyte morphology was assessed as a marker of viability with live oocytes defined as having homogeneous cytoplasm and the shape is maintained (round). The oocyte survival rate in 15 mM MβCD was approximately 80% yet none survived at 20 mM (Suppl. Fig 1A). Subsequent experiments utilised a shorter incubation time (30 min; n = 2 experimental replicates with 56 oocytes). In these, oocyte viability was again 100% in the presence of 5 mM MβCD there was 100% survival rate (Suppl. Fig. 1B), decreasing to a survival rate of 0% with high MβCD concentrations. Increasing doses caused clear morphological changes in the oocytes (Suppl. Fig. 1C). Both 15 mM and 20 mM MβCD was able to elicit limited survival. Thus, the ability of oocytes to survive in the presence of MβCD was established. A dose of 15 mM was non-toxic and thus subsequent experiments used 15 mM at 30 min to deplete cholesterol, unless otherwise stated. Long incubation with MβCD induced cell death; however, it is important to note that its effect is non-cytotoxic and occurred as a non-apoptotic mechanism in other cell types (Motoyama *et al.* 2009).

2.5.1 Cholesterol depletion survival assessment

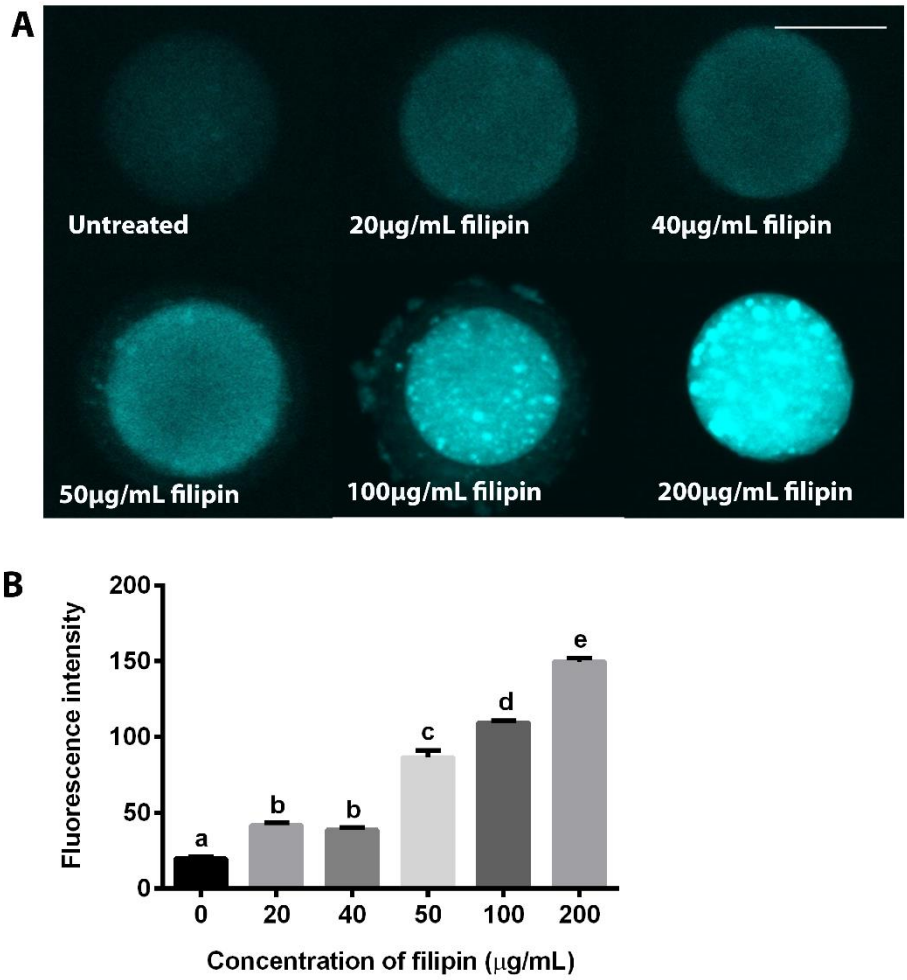
An independent assessor assessed the oocytes survival by the following criteria. In living oocytes, the cytoplasm is homogeneous and the shape is maintained (round) whereas dead oocytes are morphologically darkened, granular and with retracted cytoplasm from the zona.



Supplemental Figure 1: Increasing doses of methyl-β-cyclodextrin (MβCD) cause morphological changes in live mouse oocytes.

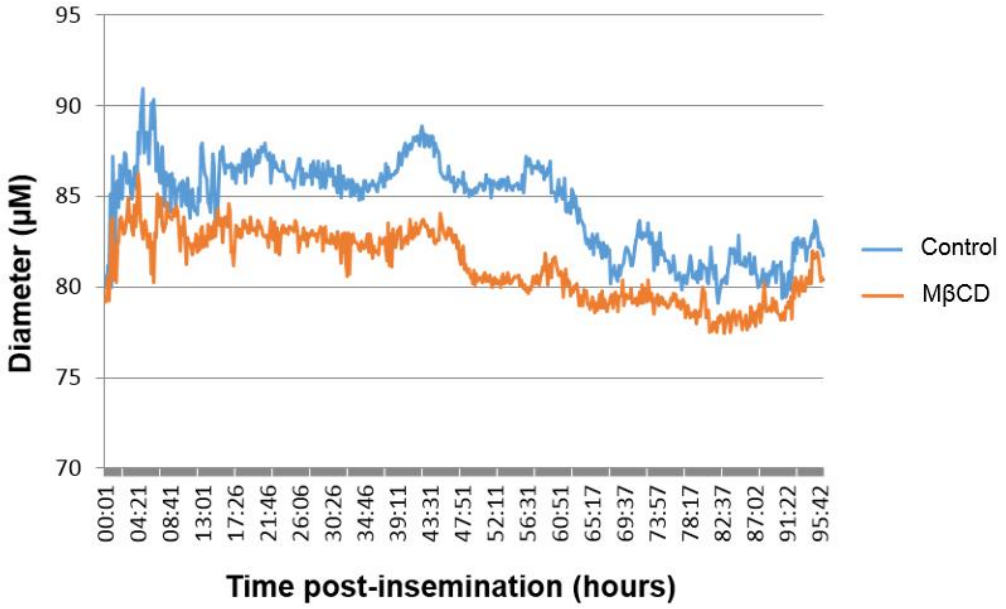
(A) Effect of cholesterol depletion mediated by MβCD on oocyte viability after 1 h and (B) 30 min. Data are presented as (A) one experiment from a total of 6 control oocytes, 4 oocytes treated at 5 mM, 5 oocytes depleted at 15 mM, 6 oocytes depleted at 20 mM, 5 oocytes depleted at 30mM and 5 oocytes depleted at 40 mM MβCD. (B) n = two experimental replicates from a total of 7 control oocytes, 8 oocytes depleted at 5 mM, 11 oocytes depleted at 15mM, 12 oocytes depleted at 20 mM, 9 oocytes depleted at 30 mM and 9 oocytes depleted at 40 mM of MβCD. Groups with different superscripts differ significantly by one-way ANOVA with Tukey's post hoc test ($P < 0.05$). (C) Representative photos of oocytes after 30 min of MβCD treatment.

To optimise staining for free cholesterol in oocytes, a dose response of filipin was conducted using concentrations of the filipin stain ranging from 20 μg/mL to 200 μg/mL. The level of fluorescence was clearly dependent on the concentration of the filipin stain in the medium (Suppl. Fig. 2.1A). In the absence of the stain, minimal positive fluorescence was detected (Suppl. Fig. 2.1B). Increased filipin concentrations resulted in significantly higher level of fluorescence ($P \leq 0.05$; Supp. Fig. 2.1B). At 200 μg/mL, the oocytes appeared to have the highest positive fluorescence than other doses. Filipin concentration at 50 μg/mL gave clear fluorescence without saturation and was selected as the concentration to be used for subsequent experiments.



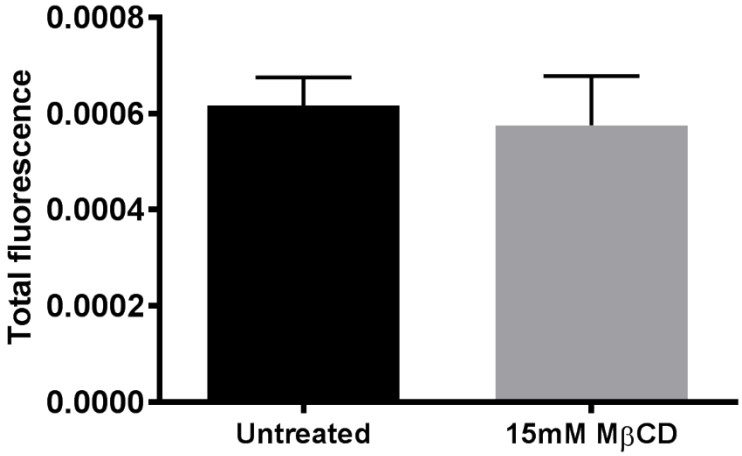
Supplemental Figure 2: Increasing doses of filipin stain resulted in higher fluorescence level.

(A) Representative photos of filipin-stained mouse oocytes. (B) Densitometry analysis of oocytes stained with increasing filipin concentrations. Data are presented as mean ± SEM fluorescence intensity where n = 22 control (0 µg/mL) oocytes, 11 oocytes at 20 µg/mL, 11 oocytes at 40 µg/mL, 28 oocytes at 50 µg/mL, 12 oocytes at 100 µg/mL and 19 oocytes at 200 µg/mL of filipin. Groups with different superscripts differ significantly by one-way ANOVA with Tukey's post hoc test ($P \leq 0.05$). Scale bar = 50 µm.



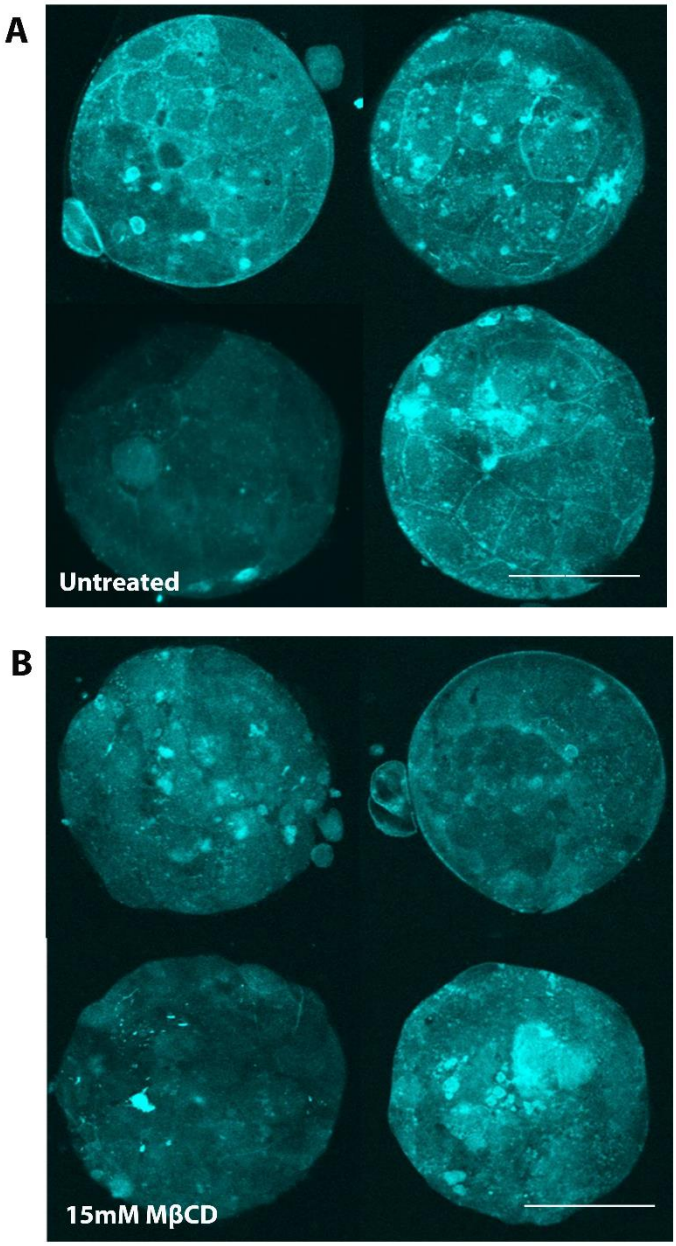
Supplemental Figure 3: Mouse embryo diameter was decreased throughout development following cholesterol depletion.

Embryo diameter was recorded using the Primo Vision time-lapse embryo monitoring system and analysed with the Primo Vision software version 4.4.1.01.010 (Vitrolife). Representative line of n = 19 embryos (control) and n = 15 embryos (MβCD).



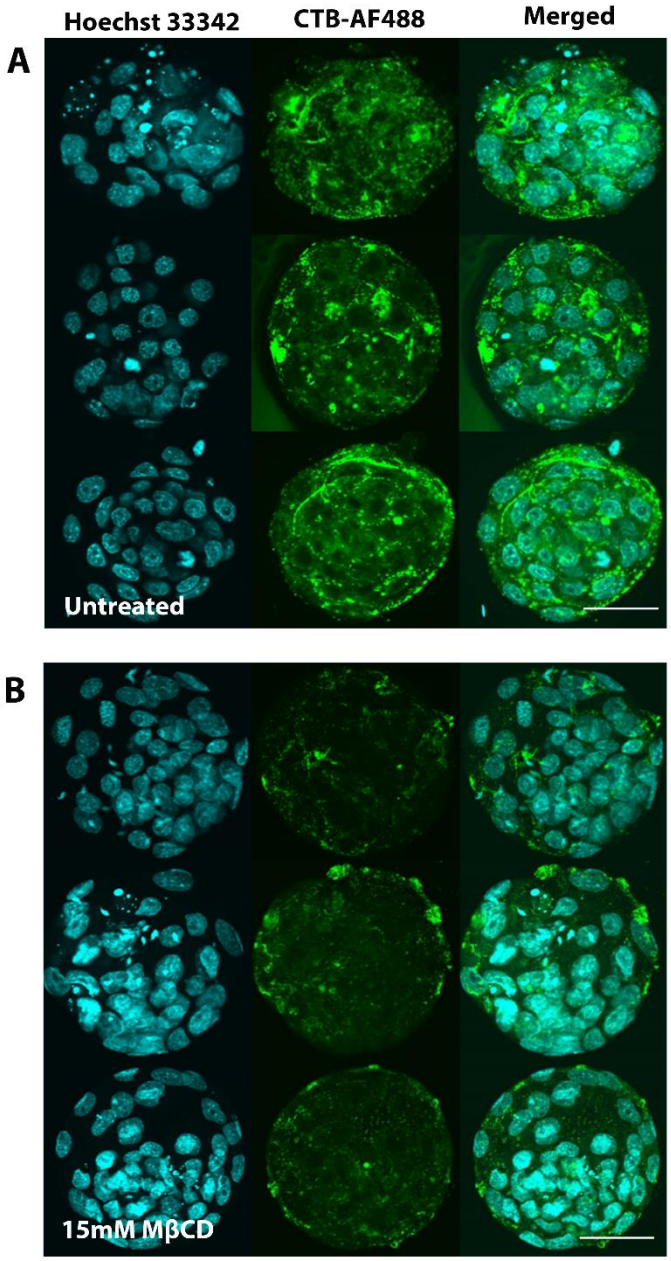
Supplemental Figure 4: Cholesterol depletion in mouse oocytes did not increase intracellular reactive oxygen species (ROS) in embryos.

COCs were untreated or cholesterol depleted using 15 mM MβCD for 30 min and subjected to IVF and *in vitro* culture until the blastocyst stage (Day 5). Stock concentration (1 mM) of CM-H_2DCFDA was prepared in 100% DMSO prior to staining. Day 5 blastocysts were incubated with 10 μM CM-H_2DCFDA for 30 min at 37°C in the dark. Embryos were washed once with PBS/PVP and images were captured using the Olympus Fluoview FV10i confocal microscope using a 10x lens (Olympus, Japan). Data are presented as mean ± SEM, n = 15 (untreated) and n = 18 (MβCD) embryos. $P > 0.05$, unpaired *t*-test.



Supplemental Figure 5: Cholesterol depletion altered mouse embryo membrane structure.

(A) Additional images of filipin-stained embryos generated from mouse untreated cumulus-oocyte complexes (COCs) and (B) 15 mM MβCD-treated COCs. Scale bar = 100 µm.



Supplemental Figure 6: Cholesterol depletion in oocytes alters lipid raft distribution in mouse embryos.

(A) Additional images of CTB-stained embryos generated from untreated cumulus-oocyte complexes (COCs) and (B) 15 mM MβCD-treated COCs. Scale bar = 100 µm.

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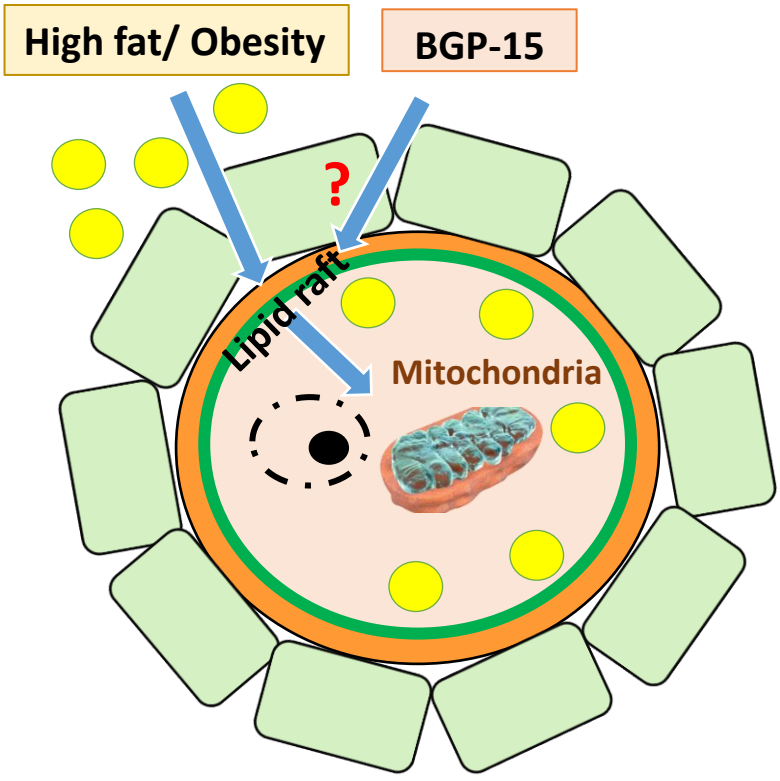
CHAPTER 3

EFFECTS OF OBESITY ON EMBRYO MEMBRANE DOMAINS

Overview

In the previous chapter, I demonstrated that cholesterol is essential for the formation of membrane domains during preimplantation development. In this chapter I now address the next aim of my research (Aim 2), by examining the consequences of a high-fat diet (HFD) and BGP-15 treatment on membrane functional domains in oocytes and embryos. I utilised a diet consisting of high fat and cholesterol in order to mimic the "Western Style" diet consumed by modern day humans. In this chapter, I considered the incidence of elevated triglycerides, cholesterol and free fatty acids, each typically perturbed in human obesity, and also incorporated the criteria of metabolic syndrome in women, and the impact these would have on embryo membrane domains. In the previous chapter short treatment with BGP-15 *in vitro* did not affect membrane rafts in cholesterol-depleted oocytes; however in this chapter, I tested whether *in vivo* BGP-15 treatment would stabilise membrane structure in the context of HFD-induced cellular defects.



Schematic illustrating the general hypothesis that perturbations in oocyte lipid rafts may develop in response to a HFD environment, and that *in vivo* BGP-15 treatment may mitigate the HFD-induced defects.

3.1 Introduction

The number of reproductive-aged women who are overweight and obese is increasing, as is the incidence of reproductive pathologies linked to female obesity (Jungheim *et al.* 2013). Obesity is a contributing factor for the development of type II diabetes, characterised by elevated blood glucose and impaired insulin signaling (Norman 2010; Dag and Dilbaz 2015; Nicholas *et al.* 2016). Obesity is also an independent risk factor for a range of metabolic complications including reproductive disorders (Malnick and Knobler 2006; Rachon and Teede 2010; Teede *et al.* 2010). The exact mechanism(s) by which obesity affects ovarian function remain poorly understood. Obese women have an increased likelihood to display signs of Polycystic Ovary Syndrome (PCOS), ovulation defects, gestational diabetes and premature and still-births, (Balen *et al.* 2006; Leddy *et al.* 2008).

The periconception period (including the final stages of oocyte development and fertilisation) is particularly sensitive to maternal metabolic environment. For instance, zygotes collected from oviducts of diabetic mice and subsequently transferred to non-diabetic pseudopregnant female recipients exhibit retarded fetal growth and increased fetal abnormalities (Wyman *et al.* 2008). Similarly, in our obese but not diabetic mice-the Blobby mice, blastocysts transferred to normal weight surrogates showed that oocytes from obese blobby mice gave rise to fetuses that were heavier than controls and had reduced liver and kidney mitochondrial DNA (mtDNA) content (Wu *et al.* 2015). It was also shown that female mice fed a high-fat diet (HFD) have a higher rate of anovulation, smaller and fewer mature oocytes with increased lipid accumulation and altered mitochondrial activity and poor developmental competence (Igosheva *et al.* 2010; Jungheim *et al.* 2010; Wu *et al.* 2010; Luzzo *et al.* 2012) compared with oocytes from control diet mice.

Yet the basis for these defects, their ability to program embryo metabolism and the potential for reversal are poorly understood. Excitingly, we have identified a compound, BGP-15, which is known to combat cellular stress by inducing chaperones (Gehrig *et al.* 2012), that act to restore oocyte mitochondrial activity and developmental potential in oocytes exposed to a high lipid environment (Wu *et al.* 2012b; Wu *et al.* 2015), providing proof-of-concept that pharmaceutical drugs can efficaciously improve oocyte and embryo quality.

Data from our lab and others indicate that mitochondria are morphologically and functionally altered in oocytes exposed to high levels of lipid (Wu *et al.* 2012a) and oocytes of obese mice (Igosheva *et al.* 2010; Wu *et al.* 2010; Reynolds *et al.* 2015; Hou *et al.* 2016). Mitochondrial complement of the oocyte has been suggested to have an impact on developmental competence (El Shourbagy *et al.* 2006; Santos *et al.* 2006; Wai *et al.* 2010) and it is well-documented that post-implantation development requires threshold amounts of mitochondria in the oocyte/embryo because the mtDNA encodes for electron transport proteins essential for cellular energy production (Wai *et al.* 2010; Babayev and Seli 2015; Fragouli *et al.* 2015). We have recently published that following fertilisation, the 'obese oocytes' have poor developmental competence and give rise to blastocysts that have less mtDNA; which is restored by BGP-15 treatment (Wu *et al.* 2015). This compelling data suggest that embryos from obese mothers are fundamentally deficient in mitochondria and this may be the basis for their metabolic reprogramming.

In the current study, mitochondria-specific fluorescent probes MitoTracker Green FM and MitoTracker Orange CM-H_2TMRos were utilised to measure mitochondrial activity. MitoTracker Green FM binds to mitochondrial membranes irrespective of respiratory status (Fernandes *et al.* 2012) whereas MitoTracker Orange CM-H_2TMRos, a non-fluorescent reduced form of tetramethylrosamine, is oxidised by molecular oxygen in actively respiring cells; thereby allowing mitochondrial oxidative activity to be estimated (Agnello *et al.* 2008).

Cellular membranes are complex structures capable of regulating numerous cell functions including cell signaling, pore formation, protein clustering, and vesiculation. Membrane lipid composition is associated with membrane-linked cellular processes that are major contributors to energy metabolism, and are influenced by high lipid diet (Hulbert *et al.* 2005). Evidence to support the concept that obesity and associated dyslipidemia modify the biomechanical properties of the cell membrane has been reported in a variety of cell types. For instance, hypercholesterolemia in humans has been shown to increase both membrane cholesterol content and mechanical shear damage in erythrocytes (Koter *et al.* 2004). In skeletal muscle, consumption of a HFD can increase the saturation of fatty acyl chains within the sarcolemma (Andersson *et al.* 2002). However, it is unknown if an excessive fatty acid

environment could exert any effect on the oocyte membrane and its correlation with intracellular organelles such as the mitochondria. Importantly, BGP-15 treatment has been reported to impact the level and size distribution pattern of cholesterol-rich membrane rafts and is likely involved in raft-associated stress sensing and signaling pathways (Gombos *et al.* 2011). However whether it is via a membrane-stabilising mechanism such that it restores oocyte mitochondria and developmental competence is not known and requires investigation. I hypothesised that the high lipid environment resulting from HFD would alter the membrane structures of oocytes and embryos, and be reflected as disruptions in lipid raft distribution. The aim of the experiments in this chapter was to investigate the perturbations that develop in response to a HFD environment, on these membrane functional domains, and whether *in vivo* BGP-15 treatment is able to reverse the HFD-induced defects. Ovulation and embryo development were measured in female mice fed HFD, and metaphase II (MII) rates, mitochondrial activity and lipid raft distribution on the oocytes were determined.

3.2 Materials and Methods

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

3.2.1 Animals

All experimental procedures were approved by the University of Adelaide Animal Ethics Committee and were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. C57BL/6 mice were obtained from the University of Adelaide Laboratory Animal Services and housed in the Animal Facility under a 14:10 hour light:dark cycle at 24°C with *ad libitum* access to food and water**.**

Upon arrival at 6 weeks of age, female mice were randomly allocated to either the control diet (CD) or the high-fat diet (HFD) group for 10 weeks. The control group was fed a diet containing 20% calories from fat, 16% calories from protein, and 64% calories from carbohydrates (SF06-105, Specialty Feeds, Glen Forrest, Australia). HFD was made in house using the same recipe as D12492 of Research Diets (New Brunswick, NJ); 60% energy from fat with 91% lard and 9% soybean oil, 20% energy from protein, and 20% energy from carbohydrates. HFD recipe is shown in Table 1 (Appendix) and nutrient composition of these diets is shown in Table 2 (Appendix).

3.2.2 Oocyte and Embryo Collections

After 10 weeks feeding, female mice were induced to ovulate by intraperitoneal (i.p.) injections of 5 IU (international units) per 12 g body weight of pregnant mare's serum gonadotrophin (PMSG; National Hormone and Peptide Program, Torrance, CA, USA) and 46 h later with human chorionic gonadotrophin (hCG; Merck, Sharp and Dohme, Kenilworth, NJ, USA). BGP-15 (kindly provided by N-Gene Research Laboratories, Budapest, Hungary) was injected i.p. at 40 mg/kg in approximately 0.1 mL saline daily for four consecutive days starting the day prior to treatment with PMSG. Mature, expanded cumulus-oocyte complexes (COCs) were obtained from oviducts 13-16 h following hCG injection.

Additional mice received ovarian hormonal stimulation and then mated overnight with males. Male mice were 8-10 weeks old at time of mating, were proven fertile, and had been maintained on normal rodent chow. Mating was confirmed the following morning with the presence of a vaginal plug. Morulae or blastocysts were obtained by flushing the oviducts 86-88 h post-hCG with pre-warmed HEPES-buffered α-MEM (Life Technologies, Invitrogen, CA, USA) supplemented with 1% (vol/vol) FCS (Invitrogen).

All COCs and embryos were collected into HEPES-buffered α-minimum essential medium (MEM) handling media supplemented with 1% fetal calf serum (FCS) (Invitrogen) and pre-warmed to 37°C prior to use.

3.2.3 Lipid Raft Staining in Mouse Oocytes and Embryos

Ovulated COCs for raft staining were hyaluronidase-treated by adding 10 μL of pre-warmed 1000 IU/mL hyaluronidase to the handling media, at the time of collection and oocytes were fixed in 4% paraformaldehyde in 1% phosphate buffered saline/ polyvinylpyrrolidone (PBS/PVP). Zona-enclosed oocytes were then washed in PBS/PVP followed by incubation with 20 μg/mL of fluorescently-labeled cholera toxin B subunit (CTB-AF488; Molecular Probes, Eugene, OR, USA), for 30 min at 37°C in the dark. After staining, the oocytes were washed in PBS/PVP for 5 min and mounted under a coverslip in 5 μL PBS/PVP.

Embryos flushed from the uteri were fixed in 4% paraformaldehyde in PBS/PVP. After fixing, the embryos were washed in PBS/PVP for 5 min and permeabilised in 0.5% Triton-X in Cook Vitro Wash (William A. Cook Australia Pty. Ltd., Queensland, Australia) for 20 min at room temperature. Embryos were placed in 10% Normal Goat Serum (NGS; Vector Laboratories) in Cook Vitro Wash for 40 min to block non-specific uptake of staining reagents. To label the cell nuclei, 1 μg/mL of Hoechst 33342 (Life Technologies, Molecular Probes, Eugene, OR, USA) was added to the culture drop during the last 10 min of the incubation. After staining, the embryos were washed in PBS/PVP for 5 min and transferred with minimum medium onto slides with a microdrop of glycerol (5 μL). Images were captured using a Leica SP5 spectral scanning confocal microscope using green filter (excitation = 473nm, emission = 490-590 nm). Images were captured at 90x magnification

and laser, sensitivity, and imaging parameters were kept constant between replicates and across experiments. Three experimental replicates were performed, with 10 embryos per treatment group. Intensity of fluorescence was determined using Image J version 1.47r software by placing a circle across the embryo image and measuring pixel intensity. The mean, area and integrated density (the sum of the pixel values in the circle) were reported and the corrected total cell fluorescence was calculated using the formula:

Total fluorescence = Integrated Density- (Area of selected cell × Mean fluorescence of background readings)

3.2.4 Dual Fluorescent Staining of Mitochondria

Stock solutions of MitoTracker Green FM (1 mM) (Life Technologies, Molecular Probes, Eugene, OR) and MitoTracker Orange CM-H_2TMRos (1 mM) (Life Technologies, Molecular Probes, Eugene, OR) were prepared in 100% dimethyl sulfoxide (DMSO) and stored in the dark at -20°C. Ovulated oocytes from hyaluronidase-treated COCs were first incubated in 500 nM of MitoTracker Orange CM-H_2TMRos for 30 min and MitoTracker Green FM (100 nM) was added during the last 15 min of the incubation. Oocytes were then washed once in PBS/PVP and mounted under a coverslip in 5 μL PBS/PVP. Images were captured using the Leica Sp5 spectral scanning confocal microscope using a narrow green filter (490-540 nm) and a narrow red filter (570-620 nm). Images were captured at 60x magnification and laser, sensitivity, and imaging parameters were kept constant between replicates and across experiments. Total fluorescence intensity was measured as above. Two experimental replicates were performed with 10 oocytes per treatment group.

3.2.5 Mitochondrial DNA (mtDNA) Copy Number Quantification

The mtDNA copy number in individual oocytes was quantified as previously described (Kameyama *et al.* 2010; Wu *et al.* 2015). Briefly, denuded oocytes were washed with PBS/PVP (1 mg/mL of PVP in PBS), collected individually into 1.5 mL siliconised low retention microcentrifuge tubes (Fisher Scientific) with 5 μL of PBS/PVP and stored at -80°C. Genomic DNA was isolated using the QIAamp DNA micro kit (Qiagen) according to manufacturer's protocol with carrier RNA (1 μg; Qiagen) added to each sample. Genomic DNA was eluted with 50 μL of water and diluted 10 times for quantitative PCR.

The quantification standard was a plasmid containing a 1186 bp fragment of the 12S ribosomal (r)RNA region of mtDNA (Wu *et al.* 2015). Plasmid copy number was calculated as: mass of plasmid (g) = plasmid size (bp)×(1.096×10^{-21} g/bp); mass of plasmid required to generate 1×10^{7} copy number standard stock = 1×10^{7}×mass of single plasmid. A standard curve was generated by using seven ten-fold serial dilutions ($10^{-1}\times10^{7}$ copies), and standard curve correlation coefficients were consistently greater than 0.98. Real-time quantitative PCR using the primer pair 5′-CGTTAGGTCAAGGTG-TAGCC-3′ and 5′-CCAGACACACTTTCCAGTATG-3′ was performed in triplicate using SYBR Green PCR master mix (Applied Biosystems) and a Rotor-Gene 6000. Primer sequences for the quantification of mtDNA were derived from (Kameyama *et al.* 2007). Standard curves were created for each run and sample copy number was generated from the equation of Ct value against copy number for the corresponding standard curve.

The PCR program employed an initial step of 95°C for 10 min followed by 40 cycles, denaturation at 95°C for 10s, annealing at 60°C for 30s and extension at 72°C for 20s. Every reaction was followed by melting curve analysis to ensure the specificity of the amplification. All reactions were performed in triplicate with total reaction volumes of 20 μL. Premix for quantitative PCR was prepared from Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA). The premix consisted of 10 μL of Power SYBR Green, 6 μL of PCR grade water, 2 μL of 50 μM primer pair; with 2 μL of DNA template then added to 18 μL of premix for PCR reaction.

3.2.6 Statistical Analysis

All measures are reported as mean ± SEM. Statistical significance was determined as indicated, by using one-way ANOVA with Tukey's post hoc tests, using GraphPad Prism v008 for Windows. A *P*-value of less than 0.05 was considered statistically significant.

3.3 Results

3.3.1 HFD affects ovulation and oocyte maturation

After 10 weeks of feeding, there was no significant difference in the total body weight of mice fed a HFD compared to controls. Treatment with BGP-15 (40 mg/kg/day) for 4 days also had no effect on body weight (Figure 1A).

Following superovulation, the number of ovulated oocytes was significantly reduced in the HFD mice compared to the control mice. Treatment of mice with BGP-15 did not significantly influence the ovulation rate ($P \leq 0.01$; Figure 1B). Inspection of the ovulated oocytes showed that the number of live oocytes ovulated by HFD mice was reduced compared to controls and that BGP-15 treatment resulted in a slight increase in number but there were no statistically significant differences between groups (Figure 1C). Oocyte nuclear maturation, assessed as the extrusion of the first polar body during progression to MII, was dramatically decreased in the HFD group ($P \leq 0.05$; Figure 1D). Treatment with BGP-15 had no significant effect on oocyte maturation to MII in the HFD mice.

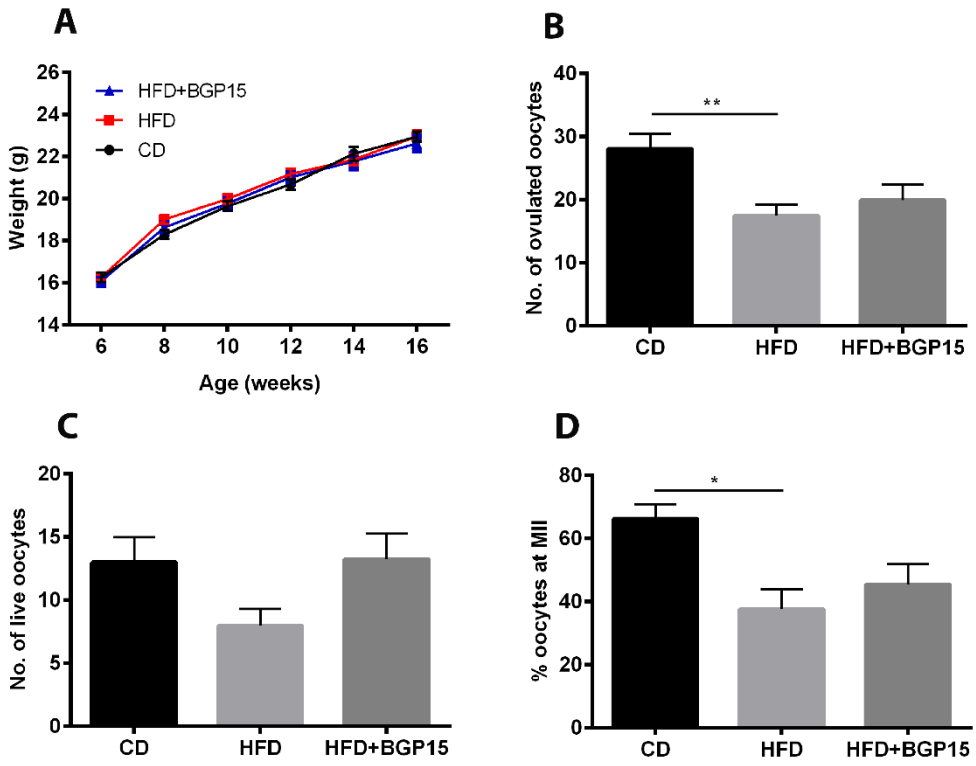


Figure 1 Effects of high-fat diet (HFD) and BGP-15 on body weight, ovulation and mouse oocyte maturation.

(A) Body weight of mice on the CD, HFD and HFD+BGP-15 treatments over 10 weeks. Data are expressed as mean ± SEM, n = 25 mice for control diet, n = 36 mice for HFD and n = 33 mice for HFD+BGP-15. (B) Ovulation rate and (C) number of live oocytes ovulated by CD, HFD, and HFD+BGP-15 mice following the 10 weeks treatment period. (D) Assessment of oocyte maturation to metaphase II (MII). Data are expressed as mean ± SEM, n= 11 (CD), n = 19 (HFD) and n = 21 (HFD+BGP-15) mice. * $P \leq 0.05$, ** $P \leq 0.01$, one-way ANOVA with Tukey's post hoc test. CD- control diet.

3.3.2 BGP-15 normalises lipid raft distributions in oocytes of mice fed HFD

Oocytes collected from the mice were stained for assessment of lipid raft distribution. Similar to previous observations (Chapter 2), lipid rafts were distributed throughout the plasma membrane of oocytes derived from the control diet mice (Figure 2). However, the oocyte rafts were visibly different in the HFD oocytes (Figure 2), namely the distinct staining on the oocyte plasma membrane was not apparent. Interestingly, *in vivo* BGP-15 treatment of the female mice was able to restore the raft distributions in the oocytes (Figure 2). In addition, all oocytes irrespective of treatment showed a homogenous distribution of positive intracellular fluorescence staining which is likely to be intracellular lipid rafts. This intracellular staining was markedly increased in the HFD oocytes when compared to the CD, and following BGP-15 treatment was more similar to those of CD oocytes. The oocytes of mice fed HFD also displayed an accumulation of the fluorescent probe in the peri-cortical region in structures that resemble lipid droplets as judged by their size, shape, and distribution (Figure 2); however these were no longer apparent following treatment with BGP-15.

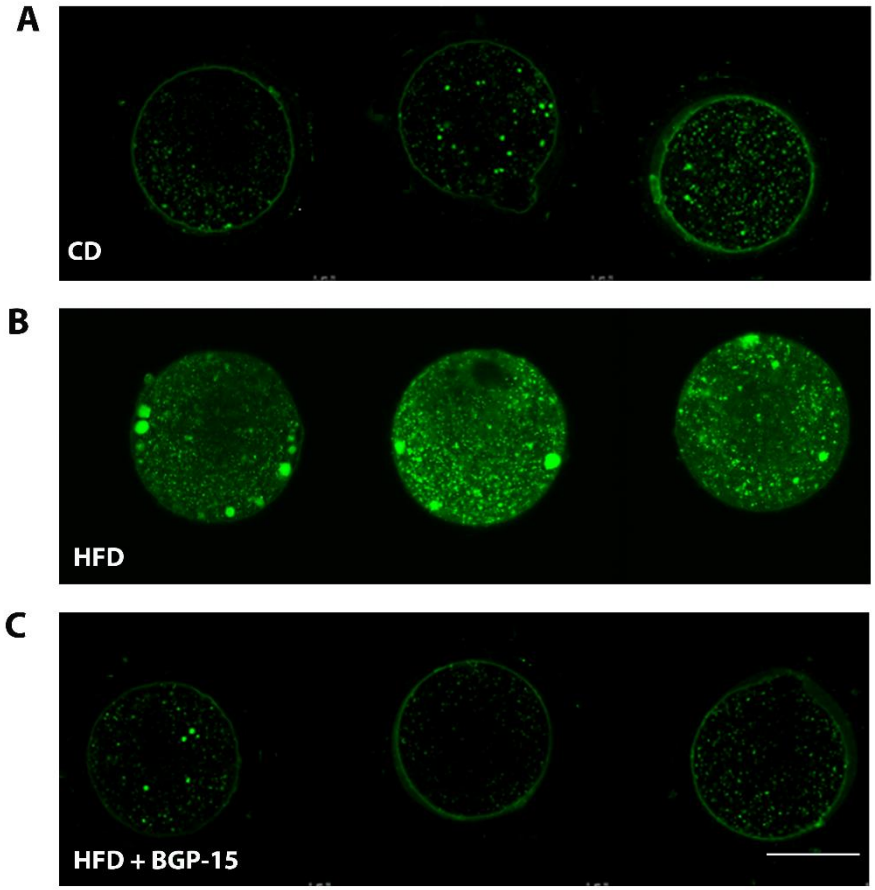


Figure 2 Lipid rafts in oocytes of mice fed control diet, or high-fat diet (HFD) or HFD followed by treatment with BGP-15.

Ovulated oocytes were stained with CTB-AF488 to visualise lipid rafts. Photos are of oocytes from three different animals. CD- control diet.

3.3.3 BGP-15 restores oocyte mitochondrial activity in mice fed HFD

Oocytes collected from mice of each treatment group were examined for mitochondrial distribution and oxidative activity using MitoTracker Green FM (MTG) and MitoTracker Orange CM-H_2TMRos (MTO) respectively. When co-localised these dyes fluoresce yellow and are reflective of the mitochondrial population consuming oxygen; and they change colour to red when mitochondrial activity is increased (Morici *et al.* 2007; Agnello *et al.* 2008). Mouse oocytes stained with MTG and MTO showed relatively uniform cytoplasmic distribution and granular clusters of various sizes at the equatorial section of the oocyte which were visualised using confocal microscopy. Interestingly, MTG fluorescence was most intense in the oocytes from HFD mice and reduced by BGP-15 treatment (Figure 3A). Following densitometry analysis, the relative intensity of MTG fluorescence was significantly increased in the HFD oocytes suggesting that there were more mitochondria distributed in those oocytes compared to the CD and HFD+BGP-15 oocytes ($P \leq 0.01$; Figure 3B).

The majority of the mitochondrial population was consuming oxygen as indicated by the MTO staining. No significant difference was detected between groups (Figure 3C). The ratio of MTO to MTG is thought to correspond to mitochondrial activity and by this measure mitochondrial activity in the HFD oocytes was significantly lower than that of the CD, and BGP-15 treatment restored the mitochondrial activity of HFD oocytes ($P \leq 0.01$; Figure 3D). These data suggest that the mitochondrial population in the oocytes of CD and HFD+BGP-15 mice were more active and had higher oxygen consumption compared to oocytes from HFD mice which had lots of inactive mitochondria.

MtDNA copy number was also determined in oocytes of each of the treatment groups. No difference in mtDNA copy number was observed between the CD and HFD oocytes. However, mtDNA copy number was markedly increased in the HFD+BGP-15 oocytes when compared to those in CD and HFD oocytes, reaching an average maximum of 400,000 copies ($P \leq 0.0001$; Figure 3E).

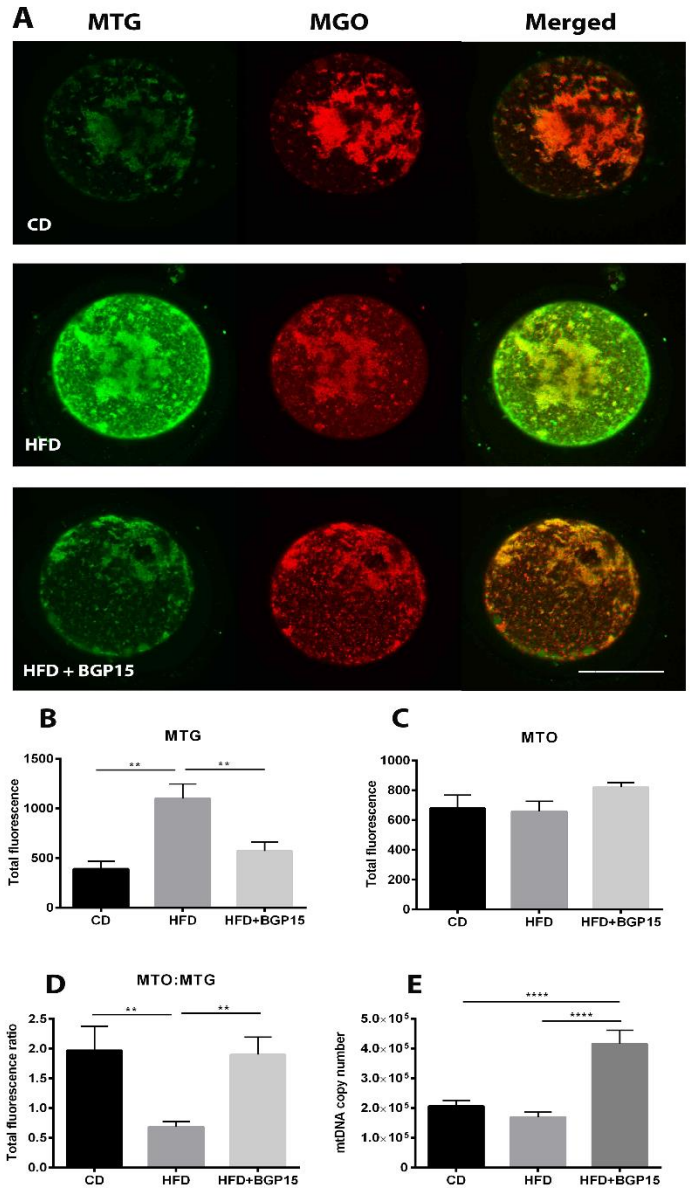


Figure 3 Effect of feeding control diet, high-fat diet (HFD) or HFD plus treatment with BGP-15 on mouse oocyte mitochondrial activity and mtDNA levels.

Ovulated oocytes were stained with MitoTracker Green FM (MTG) followed by MitoTracker Orange CM-H_2TMRos (MTO). (A) Representative photos of oocytes stained with the MitoTracker dyes. (B) Quantification of total green fluorescence, (C) red fluorescence and (D) the ratio. Data are presented as mean ± SEM, n = 5-12 oocytes per group. (E) mtDNA copy number in individual oocytes. Data are presented as mean ± SEM, n = 4-10 oocytes per group. ** $P \leq 0.01$, **** $P \leq 0.0001$, one-way ANOVA with Tukey's post hoc test. CD- control diet.

3.3.4 Effects of HFD and BGP-15 on embryo development

To determine whether or not the oocytes from HFD mice exhibited impairments later in development, embryos were collected from the mice 88 hours post-hCG treatment and their development assessed. Following superovulation and mating, the percentage of mice that yielded embryos was not statistically different between the HFD and CD groups (Figure 4A). There were fewer embryos retrieved from the uteri of HFD mice (Figure 4B) and a similar trend when the percentage of viable embryos was compared (Figure 4C) however there were no statistically significant differences between groups. Of the viable embryos, a high proportion were at the morula stage (Figure 4D).

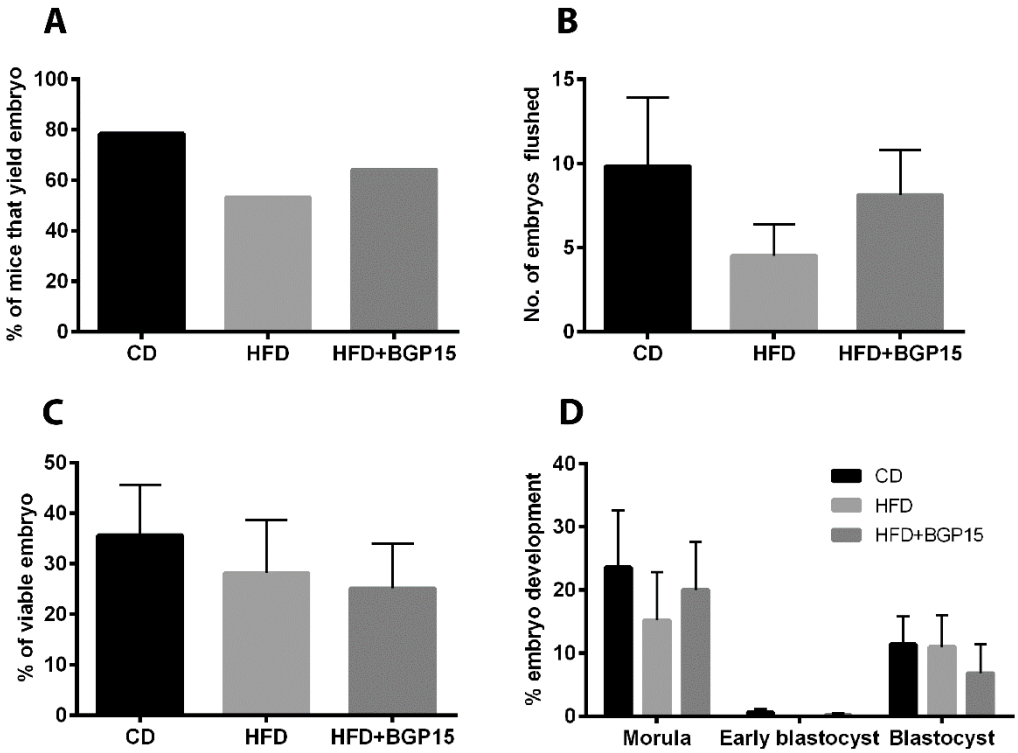


Figure 4 Effects of feeding control diet, high-fat diet (HFD) or HFD plus BGP-15 treatment on embryo development.

(A) Percentage of CD, HFD and HFD+BGP-15 mice that had embryos present in the uterus at 88 h post-hCG. (B) Number of embryos flushed from the uteri of each mouse. (C) Percentage of viable embryos assessed morphologically. (D) Developmental stage of embryos. Data are presented as mean ± SEM, n = 14 (CD), n = 15 (HFD) and n = 14 (HFD+BGP-15) mice. $P > 0.05$, one-way ANOVA with Tukey's post hoc test. CD- control diet.

3.3.5 BGP-15 partially normalises lipid raft distribution in HFD embryos

Since oocytes from mice fed a HFD exhibited a clear disruption in lipid raft abundance and a restoration following BGP-15 treatment, lipid rafts were also examined in the embryos to determine whether these modifications were maintained through development. Embryos were flushed from uteri of mice from each of the treatment groups on day 4 (86-88 h post-hCG) following mating. The morula stage embryos from CD showed lipid raft enrichment at the cell junctions at the apical edge of the blastomeres (Figure 5A). This pattern was not observed in the morulae from HFD fed mice. Interestingly, treatment with BGP-15 partially restored this organisation and punctate staining was clearly visible at the embryonic space (Figure 5A). Densitometry analysis showed a corresponding significant reduction of fluorescence intensity in the HFD morulae but not with the BGP-15 treatment ($P \leq 0.05$; Figure 5B).

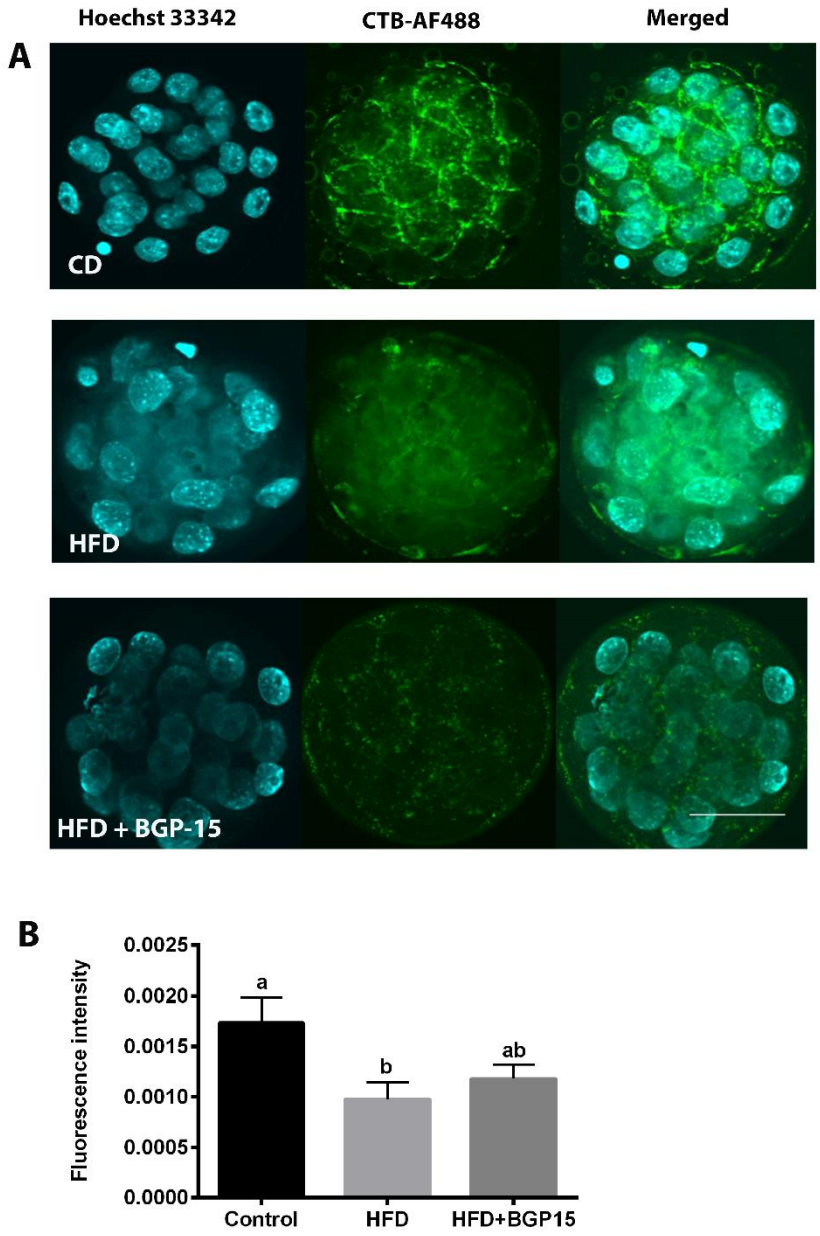


Figure 5 Effect of feeding control diet, high-fat diet (HFD) or HFD plus BGP-15 treatment on lipid raft distribution in morula stage embryos.

Embryos were flushed from the uteri, fixed and stained with CTB-AF488. (A) Representative photos of CTB-stained embryos. (B) Quantification of fluorescence intensity. Data are presented as mean ± SEM, n = 18 (CD), n = 21 (HFD) and n = 12 (HFD+BGP-15) embryos from six mice per group. Different letters indicate significant differences by one-way ANOVA with Tukey's post hoc test ($P \leq 0.05$). Scale bar = 100µm.

3.4 Discussion

Maternal obesity is well known to have negative impacts on fertility (Dag and Dilbaz 2015). In order to better understand the mechanistic basis for this negative impact, the present study determined whether maternal HFD disrupts membrane functional domains in the oocyte and embryos. The HFD used in this study largely mimics the 'Western fast food diet' and led to the onset of obesity, as well as dyslipidemia and mild hyperinsulinemia (Wu *et al.* 2010). Mice fed this diet had reduced numbers of ovulated oocytes and decreased MII rates. This disrupted physiologic environment was detrimental to oocytes, causing a disruption of membrane domains and mitochondrial activity in association with impaired embryo development and membrane raft formation. These results provide new insights into cellular alterations that might mechanistically contribute to obesity-associated impairments in oocyte developmental competence. Understanding the potential defects in membrane domains is important as alterations to the membrane structure in the oocytes of obese mice could impair or exacerbate downstream signaling pathways which may impact subsequent preimplantation processes.

Surprisingly, in this study the mice fed HFD did not gain more weight compared to those fed a standard chow diet, as is typically reported by our lab and others. One explanation for this may be due to the fact that the two diets are not isocaloric, with almost 2 kcal/g more in the HFD. Moreover, the control diet was administered as pellets as purchased from Specialty Feeds (Glen Forest, Australia), however, the HFD was made in-house. Thus, differences in the consistency of the food could have introduced differences in food consumption when compared to the control diet group. Thus, whether the results of feeding a HFD are due to the absolute increase in the fat content of the diet, or due to increased intake of cholesterol or other specific lipids are yet to be determined. Alternatively, changes in relative proportion of carbohydrate and fat between the two diets may also account for the discrepancy. Importantly, even though body weight was not increased in mice fed HFD, dissection of fat pads post-mortem showed that by the end of 10 weeks of diet, there was a visible accumulation of mesenteric fat in the HFD mice; however, this was not measured as part of this study.

Even though mice fed HFD did not have increased body weight, the numbers of ovulated oocytes were significantly lower in the HFD mice. The reduced number of ovulated oocytes observed in the HFD mice is consistent with our previous findings in obese Blobby mice and HFD mice (Minge *et al.* 2008; Wu *et al.* 2010; Wu *et al.* 2015) and reports of others (Ge *et al.* 2014; Hou *et al.* 2016). Thus it seems likely that abdominal fat tissue contributed to the observed defects in ovulation, as increased fat mass has been found to be related to infertility (Zaadstra *et al.* 1993; Kuchenbecker *et al.* 2010).

BGP-15 is an orally active compound and has been shown to have an excellent safety profile in multiple human clinical trials in healthy individuals (Literati-Nagy *et al.* 2010; Literati-Nagy *et al.* 2012) and insulin-resistant non-diabetic patients (Literati-Nagy *et al.* 2009). The initial rationale for assessing the therapeutic potential of BGP-15 in an animal model was related to its role to combat cellular stress by inducing chaperones (Literati-Nagy *et al.* 2010) and remodelling plasma membrane rafts (Gombos *et al.* 2011). It is important to note that a previous study from our laboratory showed that BGP-15 administration to obese Blobby or lean mice significantly increased the rate of ovulation but this was not observed in the current study. The most likely explanation is that these HFD mice were not severely obese, and hence the level of cellular stress was not sufficient to elicit detectable actions of BGP-15. In support of this there are other reports of BGP-15 having no effects in healthy cells and tissues (Chung *et al.* 2008; Nagy *et al.* 2010; Crul *et al.* 2013).

Our demonstration that our HFD composition of 60 kcal% from fat impairs oocyte maturation is generally consistent with previous reports that showed decreased number of MII oocytes in mice with HFD-induced obesity (Ge *et al.* 2014; Sohrabi *et al.* 2015). In addition, Sohrabi *et al*. (2015) and Hou *et al.* (2016) collected murine germinal vesicle (GV) oocytes and continued to culture them *in vitro*. Following culture, they also observed a decreased proportion of HFD oocytes that matured to MII stage compared to those oocytes from control mice.

An important finding of the current work is that following HFD, the oocytes exhibited disrupted raft distribution, however, this was restored by BGP-15 treatment. To our knowledge, this is the first study to demonstrate that HFD affects membrane rafts, and

further that BGP-15 is able to restore the defects. The mechanism behind the disruption of raft distribution however remains unknown. Cholesterol appears to be essential for the stability and functionality of lipid rafts. The obese Blobby mice have been found to have increased total lipid and cholesterol level in their blood (Wu *et al.* 2015) and this may lead to alterations in lipid composition within cells, including the oocytes. With obesity, intracellular accumulation of lipids in non-adipose tissues leads to high levels of free fatty acids, which leads to cellular stress response, specifically endoplasmic reticulum (ER) stress, mitochondrial dysfunction and ultimately apoptosis. ER is the organelle responsible for lipid membrane biosynthesis (English and Voeltz 2013) and so perhaps the alterations in lipid raft distribution are reflective of ER dysfunction. BGP-15 was previously shown to act on GRP78 (78 kDa glucose-regulated protein; a major ER chaperone) (Szabados *et al.* 2000) and here we showed that BGP-15 was able to restore lipid raft distribution in the HFD oocytes further supporting the relationship between lipid rafts and ER function. Since mouse prevoulatory oocytes synthesise little cholesterol and obtain cholesterol primarily from the surrounding cumulus cells (Su *et al.* 2008), it is also possible that in the presence of excess lipids, cholesterol biosynthesis by cumulus cells is altered by ER stress, thereby disturbing the ability of the oocyte to acquire sufficient cholesterol for raft formation. It is more likely however that oocytes of obese mice have increased intracellular cholesterol consistent with the increased levels of oocyte neutral lipids detected by BODIPY stains (Wu *et al.* 2010; Wu *et al.* 2015) and that such increased levels would also affect plasma membrane structure. Interestingly, BGP-15 treatment was able to restore raft distribution in the oocytes. In Chinese hamster ovary cell plasma membrane, BGP-15 has been found to preserve raft integrity challenged by heat stress. It is likely that the BGP-15 induced raft remodelling via the Rac1 signaling cascade (Gombos *et al.* 2011).

To examine mitochondrial function in oocytes in response to obesity, Igosheva *et al*. (2010) used a low toxicity potentiometric fluorescence dye TMRM to examine oocyte mitochondria in HFD-fed mice. They found that obesity led to a dramatic increase in inner mitochondrial membrane potential in oocytes. In contrast, our laboratory used the JC-1 potentiometric dye and found that oocyte mitochondrial membrane potential was lower in the oocytes from obese mice than those of mice on the control diet (Wu *et al.* 2010; Wu *et al.* 2015). This discrepancy may be explained by the use of different diet and strain of mice. In the current

study, we labeled mitochondria using the MitoTracker dyes which are suitable for multiple labeling. MitoTracker Green FM binds to all mitochondria irrespective of respiratory status whereas MitoTracker Orange is only taken up by actively respiring mitochondria thereby allowing us to measure the proportion of active mitochondria in relative to the total population of mitochondria present in the oocyte. This finding is supported by McPherson *et al.* (2015) who reported increased MitoTracker Green fluorescence in 2-cell embryos retrieved from HFD-induced obese mice. Interestingly, MitoTracker Green abundance becomes fluorescent only once it has accumulated in the lipid environment of the mitochondria, suggesting that oocytes generated from the HFD mice have increased lipid in their mitochondria.

Here, we also discovered that the rafts and mitochondria may have some functional association, since the disruption of rafts in the oocytes of HFD mice was accompanied by a reduction in mitochondrial activity. This suggests that mitochondria may be closely apposed to membrane rafts. Although the functional significance of such co-localisation is not known, I speculated that rafts localise certain enzymes that produce reactive oxygen species (ROS) which may control mitochondrial function or protect the plasma membrane from the damage of mitochondria-derived oxidants. Moreover, I also speculate that the contact sites between ER and mitochondria, known as the mitochondrial associated membranes (MAMs) would play a crucial role in contributing to any such functional association. MAMs were identified as essential regions for phospholipid metabolisms, trafficking of lipid to mitochondria and the regulation of mitochondrial dynamics and homeostasis (Krols *et al.* 2016). Thus under circumstances such as obesity/HFD where there is an accumulation of lipids, it is suspected that these contact sites are damaged, limiting the transfer of substrates for normal mitochondrial metabolism. Future studies are necessary to confirm this theory.

Similar to previous findings by our group (Wu *et al.* 2010) and others (Reynolds *et al.* 2015), our results indicate that HFD oocytes exhibit decreased mitochondrial activity. However, this is restored by BGP-15 treatment. In mice, oocytes with low mtDNA copy number are more likely to be compromised before and after fertilisation (Larsson *et al.* 1998; Santos *et al.* 2006). HFD mice had a small reduction in mtDNA copy number that was not statistically significant, in contrast to previous reports (Igosheva *et al.* 2010; Luzzo *et al.* 2012). This

could be due to the lack of severe obesity in mice in our experiments. However, as mitochondrial activity is decreased, we believe that this mitochondrial pool is compromised. It remains to be determined whether this depletion in activity is a consequence of changes to mtDNA or an initial event contributing to embryo arrest. mtDNA copy number increased markedly following BGP-15 treatment in the HFD oocytes suggesting that there may be increased stimulation of the mitochondria biogenic pathway by BGP-15 to compensate for impaired mitochondrial functions in the oocytes of HFD mice. Independent to HFD effects, a previous study by our lab showed that treatment of lean mice with BGP-15 had no effect on mtDNA levels in the oocytes, yet interestingly increased ovulation rate, in the absence of any effect on body weight (Wu *et al.* 2015).

Preimplantation development of the embryo relies heavily on mitochondria for generation of ATP and energy consumption, with perturbations in this critical developmental time frame having consequences for growth and health trajectory of the embryo (McPherson *et al.* 2015). Although we did not see any difference between the treatment groups in terms of embryo development, the HFD-fed mice tended to have a lower percentage of mice that yield embryos and a lower number of embryos flushed. This is probably due to anovulation, similar to that observed in our previous studies which showed reduced blastocyst survival rates and abnormal embryonic cellular differentiation in obese female mice (Minge *et al.* 2008). HFD mice also gave rise to a lower proportion of viable embryo compared to the control diet mice. Each of these parameters are moderately restored by BGP-15 treatment but not statistically significant, likely due to the relatively small number of animals used. In this study, we also observed a delayed on-time development to the blastocyst stage in the HFD mice as large proportions of morulae were flushed from the uteri after 88 hours post-hCG. This indicates that obesity is associated with poor developmental outcome and 4 days of BGP-15 treatment prior to ovulation has slightly improved this phenomenon, demonstrating that changes in HFD oocytes are readily reversible.

Our results indicate that lipid rafts are strategically located in embryonic space and time in a manner suggestive of a functional role for rafts in cleaving mammalian embryos. In morulae, we observed an enrichment of rafts that may indicate a role in the early stages of trophectoderm differentiation and in cavitation, both critical prerequisites for formation of the blastocysts (Watson 1992; Sheth *et al.* 1997). Further, embryos generated from the HFD

mice exhibited disrupted raft organisation which was partially restored by BGP-15. This compelling data suggests that alterations before conception, for instance as a result of an environmental insult, that change the phenotype of oocytes may be retained through the course of later development.

Taken together, our functional data from embryo culture experiments in Chapter 3 support the concept of a critical role of lipid rafts in development, as oocyte cholesterol depletion slows the rate of cleavage in culture and reduces blastocyst development. In this chapter, we showed that lipid rafts are susceptible to a maternal HFD which has a significant impact on embryo quality and preimplantation development. Moreover, maternal HFD is also likely to have an effect on the uterine environment and potentially influenced the embryos recovered. Our data also verify that oocytes from HFD mothers are fundamentally deficient in mitochondrial activity and that this may be the basis for the reprogramming. Even more importantly, we have found that BGP-15 restores mitochondrial activity, in conjunction with normalising embryo development, providing important benchmarks for future pre-clinical and clinical evaluations of preconception pharmaceuticals. Our findings that a short treatment with BGP-15 is able to at least partially normalise membrane reorganisation in embryos from mice fed HFD demonstrate embryo membrane domains are acutely responsive to maternal nutrition and a membrane-stabilising pharmaceutical. However whether the effects of BGP-15 on membrane rafts are related to its effects on mitochondrial activity remains to be determined.

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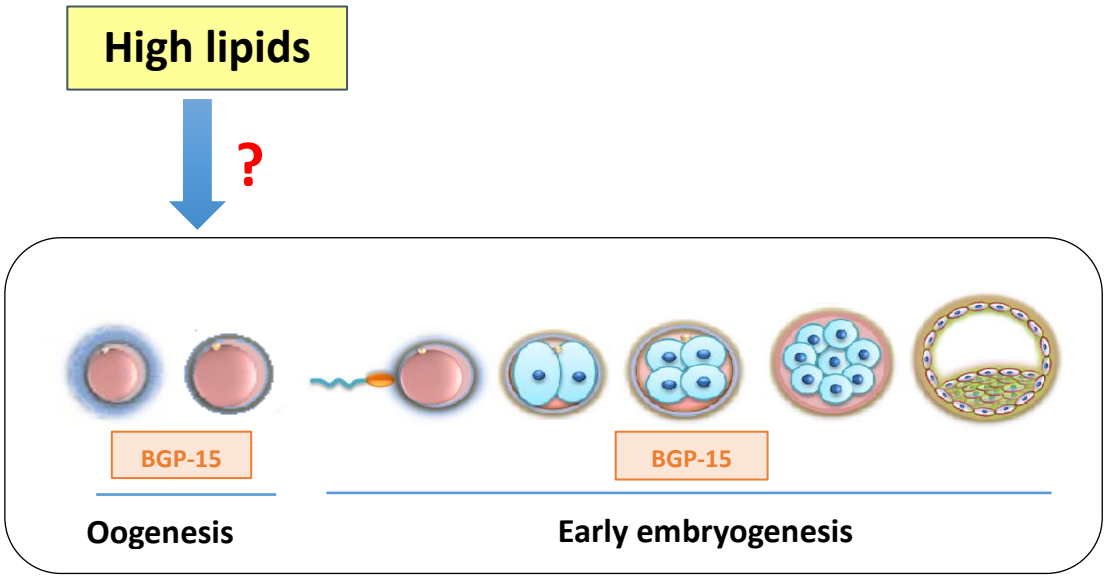
CHAPTER 4

KINETICS OF MTDNA REPLICATION IN OOCYTES AND EMBRYOS AND INDUCTION BY BGP-15

Overview

Following the findings that BGP-15 was able to restore mitochondrial activity and increase mitochondrial DNA (mtDNA) levels in oocytes following a high-fat diet (HFD), my hypothesis was strengthened that mitochondria may be the underlying basis for their metabolic reprogramming.

In addition, our laboratory also previously found that blastocysts generated following *in vitro* fertilisation (IVF) from the obese female mice have lower levels of mtDNA and gave rise to fetuses that had reduced liver and kidney mtDNA content per cell, indicating that maternal obesity before conception had altered the transmission of mitochondria to offspring (Wu *et al.* 2015). However, treatment with BGP-15 completely restored oocyte quality and the mtDNA content of fetal tissues to levels equivalent to those derived from lean mice. Therefore, I hypothesise that mitochondrial replication during oocyte maturation and embryogenesis is disrupted by exposure to excess lipid but induced by treatment with BGP-15. Thus, this chapter, summarised in the schematic below, examined the kinetics of mtDNA replication during oocyte maturation and embryogenesis in mouse, cow, macaque and humans, the effects of high lipid during oocyte maturation on mtDNA replication and the effects of BGP-15.



4.1 Introduction

Altered mitochondria activity has been proposed as a mechanism for compromised oocyte quality and poor reproductive outcomes in obese females (Igosheva *et al.* 2010; Luzzo *et al.* 2012; Grindler and Moley 2013; Wu *et al.* 2015). It is known that correct mitochondrial quality, quantity and distribution play a key role in the functional competence of oocytes, as mitochondria are maternally inherited, therefore any mitochondrial deficiencies could negatively affect the ability of the embryo to progress through preimplantation stages (Cummins 2004; Dumollard *et al.* 2007; Van Blerkom 2008; Van Blerkom 2011). Further, defects in mitochondria could have transgenerational impacts on the health of future generations. Indeed, the oocytes of obese mice have altered mitochondrial properties, uneven distribution of mitochondria throughout the cytoplasm and increased generation of reactive oxygen species. Van Blerkom *et al.* (2000) demonstrated that disproportionate inheritance of mitochondria in the cleavage stage of human embryos resulted in daughter cells with reduced adenosine triphosphate (ATP)-generating capacity and developmental competence.

Mitochondrial DNA (mtDNA) is present inside the mitochondria and codes for proteins that are essential for cellular energy production (May-Panloup *et al.* 2007). Oocyte and embryonic mtDNA content are necessary for embryonic development. Reduced mtDNA content in ovulated oocytes from heterozygous *Tfam* (transcription factor A, mitochondrial) knockout mice has been found to affect fertilisation. It was reported that even if *Tfam*+/- oocytes were successfully fertilised and proceeded normally through preimplantation development, they eventually died during organogenesis (Wai *et al.* 2010).

In most species, the oocyte has by far the largest number of mitochondria and mtDNA copies of any cell (approximately 2×10^5 copies) (May-Panloup *et al.* 2007). The enormous amount of mtDNA in oocytes and the fact that mtDNA is not replicated before implantation have led to the speculation that the number of mtDNA copies in oocytes could be used as a marker of viability (Reynier *et al.* 2001; May-Panloup *et al.* 2005a; Santos *et al.* 2006; May-Panloup *et al.* 2007). During oocyte development from the primordial to pre-ovulatory stage, processes of mitochondrial replication and expansion dramatically amplify the population of mitochondria, thereby supplying each gamete with a large copy number of both normal and potentially abnormal mtDNA. As such, the mitochondrial complement of the oocyte has

been suggested to have a correlation with developmental competence. Low mtDNA copy number has been linked to fertilisation failure and compromised development (Reynier *et al.* 2001; May-Panloup *et al.* 2005a; El Shourbagy *et al.* 2006; Santos *et al.* 2006; Wai *et al.* 2010; Ge *et al.* 2012) and absence of meiotic spindle in *in vitro* matured human oocytes (Zeng *et al.* 2007). These findings suggest that mtDNA copy number is associated with oocyte quality and subsequent fertility.

Studies in mice (Piko and Taylor 1987; Ebert *et al.* 1988), rats (Kameyama *et al.* 2007), and pigs (El Shourbagy *et al.* 2006) have shown that mtDNA replication does not occur during the cleavage stages of embryogenesis, which would suggest that the mitochondrial content of the oocytes need to be amplified to sufficient levels prior to fertilisation in order to maintain development until implantation. Therefore, it has been proposed that the increase in mtDNA during oogenesis is a mechanism to ensure that sufficient numbers of organelles and genomes are present in cells of the developing embryo once mtDNA replication restarts (Shoubridge and Wai 2007) and that sufficient mitochondria are essential to provide energy for fertilisation and embryonic differentiation (St John 2014) .

It was previously proposed that high mitochondrial content can increase the quality as well as the competence of mature mammalian oocytes (Santos *et al.* 2006), leading to the speculation that mtDNA copy number could be used as a marker of viability. A critical threshold of approximately 100,000 copies in the metaphase II (MII) oocytes has been proposed for mice (Piko and Taylor 1987), humans (Reynier *et al.* 2001) and pigs (El Shourbagy *et al.* 2006), whilst bovine oocytes possess a mean of 260, 000 copies (Michaels *et al.* 1982). However, the essential amount of oocyte mtDNA is controversial, as other studies in mice (Cree *et al.* 2008) and humans (Barritt *et al.* 2002) identified mtDNA copy number around 300,000 copies and 800,000 copies respectively. Equally, other studies show the threshold to be 50,000 (Chiaratti *et al.* 2010; Wai *et al.* 2010). In fact, studies have shown figures estimating oocyte mtDNA copy number to be hugely variable, even in oocytes collected from the same female (Barritt *et al.* 2002).

Data from human studies indicated that mtDNA in cleavage stage embryos remain stable during the first three days of preimplantation development (Steuerwald *et al.* 2000; Reynier

et al. 2001; Barritt *et al.* 2002; Lin *et al.* 2004; Chan *et al.* 2005; May-Panloup *et al.* 2005a). This implicates that the mammalian embryo inherits mitochondria (and thus mtDNA) exclusively from the population found in the oocyte just prior to fertilisation. However, a study in the porcine also indicated that the amount of mtDNA during the cleavage stages of embryogenesis declined and began to increase only near the time of implantation when the embryo begins to grow in size (Spikings *et al.* 2007). Table 1 shows the regulation of mtDNA copy numbers throughout preimplantation embryo development in different species.

mtDNA content of oocytes and embryos is sensitive to stressors, such as oxidative stress, obesity and aging (Wang *et al.* 2009; Wai *et al.* 2010; Chappel 2013; Wu *et al.* 2015; Sutton-McDowall *et al.* 2016). Processes such as *in vitro* oocyte maturation, fertilisation and preimplantation embryo development, which are used in assisted reproductive technologies, are vulnerable to environmental stress and thus have great impact on fertility outcome (Fukuda *et al.* 2007; Ellenbogen *et al.* 2014). The mtDNA replication during oocyte maturation and embryo development is not fully understood. Similarly, the impacts of various environmental stressors during oocyte maturation on mtDNA content in the oocyte and embryo also remain unknown.

High levels of palmitic acid (PA) treatment is a well-established model in our laboratory, where it caused identical effects as were seen in mice with obesity. Experiments using mouse cumulus-oocyte complexes (COCs) showed that high levels of PA induced endoplasmic reticulum (ER) stress in the COCs, mitochondrial dysfunction in oocytes and caused poor embryo development (Wu *et al.* 2012). Similarly, non-esterified fatty acids (NEFA) is a standard model of bovine that mimics high lipid. NEFA are made of a mixture of palmitic, oleic and stearic acid and when present during *in vitro* maturation of bovine COCs also induced defects to oocytes and embryos similar to those induced by palmitic acid in the mouse model (Sutton-McDowall *et al.* 2016). Our group has previously reported that the ER stress inhibitor, salubrinal, can reverse the reduced mitochondrial activity (mitochondrial membrane potential) caused by high lipid stress in mouse cumulus-oocyte complexes (COCs) during *in vitro* maturation (IVM) (Wu *et al.* 2012). Similar findings have also been reported in bovine COCs (Sutton-McDowall *et al.* 2016). In addition, similar to salubrinal, BGP-15 was also found to be able to restore mtDNA in blastocysts derived from oocytes of obese

mice. Interestingly, treatment of obese mice with salubrianl or BGP-15 before IVF also resulted in restored mtDNA levels in the tissues of the offspring derived from obese mothers to levels that are comparable to those from the non-obese control mice (Wu *et al.* 2015).

Thus, in this chapter, I examined mtDNA replication by measuring copy number, using a qPCR method, on different time courses of *in vivo* versus *in vitro* mouse oocyte/embryo development; introduced stress to mice and bovine COCs with high lipid supplementation (PA or non-esterified fatty acids (NEFA)) *in vitro* in the presence or absence of BGP-15 to investigate how lipid stress will disturb mtDNA replication, and how this stress with or without treatment affects the blastocyst outcome and the mtDNA content in the resulting embryos. Based on the previous findings in this thesis, it is well-documented that BGP-15 has some beneficial effects on mouse oocytes and embryos (partially restores membrane functional domains and normalises mtDNA copy numbers) in a high fatty acid environment. Furthermore, I sought to generate pre-clinical data on the efficacy of BGP-15 as an IVF additive by using the macaque animal model and human oocytes.

Table 1 Regulation of mitochondrial DNA (mtDNA) copy number throughout preimplantation embryo development in different species.

Species	Strain	Findings	Methods
Mouse	(CD-1 females) (Mahrous *et al.* 2012)	• There was a continuous increase in mtDNA copy number as oocytes grew in size, reaching a maximum of ~175000 copies. Thus, mtDNA steadily accumulates during oocyte growth. • No difference between immature and mature oocytes. Taken together, these results indicate that mtDNA accumulates during the growth phase of oocyte development but stops when oocytes reach full size.	• *In vivo*: compared 18 day and 24 day old mice oocytes • GV oocytes isolated from 20-22 day old mice, incubated overnight to allow meiotic maturation
	Heterozygous floxed Tfam mice (Wai *et al.* 2010)	• No significant differences in mtDNA copy number between fertilised and cleavage stage embryos in heteroplasmic floxed Tfam mice.	• 2-, 4-, and 8-cell embryos generated by natural matings
	Mouse (B6D2F1) (Thundathil *et al.* 2005)	• Number of mtDNA molecules remained stable throughout preimplantation at 256,000 on average but ranged from 97,000 to 587,500. • No significant difference in the mean number of mtDNA molecules present in MII oocytes, 2-cell, 8-cell, morula and blastocyst.	• 4-6 week old females, males: FVB, embryos (2-, 8-cell, morula, blastocyst) flushed from uteri

Porcine	Porcine (Spikings *et al.* 2007)	• Progressive decrease in mtDNA copy number form 2-cell to 4-cell and 8-cell embryo. Increase in mtDNA copy number in 16-cell embryos, morulae and then further increase in expanded blastocyst stage	• COCs matured *in vitro* then cumulus cells removed for IVF
	Porcine (Mao *et al.* 2012)	• Higher mtDNA copy number in MII oocytes than GV oocytes	• COCs matured *in vitro*
Bovine	Bovine (May-Panloup *et al.* 2005d)	• No difference between mean mtDNA copy number in MII oocytes and 2-cell embryos • mtDNA content was higher in 2-cell embryos compared to 4/8-cell embryos. • No difference in mtDNA copy number between 4/8-cell stage, 8/16-cell stage and the morula • Considerable increase in mtDNA copy number at blastocyst stage	• COCs matured *in vitro* then fertilised *in vitro* with frozen-thawed semen
Rat	Rat (6-10 week old female Sprague-dawley) (Kameyama *et al.* 2007)	• No difference in mtDNA between *in vivo* and *in vitro* derived zygotes, thus superovulation does not affect the mtDNA copy number • No difference at early 2-cell, late 2-cell and 8-cell stage, significant differences were observed at the morula and blastocyst stage between *in vivo* and *in vitro* embryos.	• *In vivo*: naturally ovulated • Oocytes: Superovulation • Zygotes: naturally ovulated vs superovulated • Embryos: *in vivo* vs *in vitro* (*in vivo* from naturally mated females, *in vitro* from superovulated females-zygotes recovered were cultured *in vitro*)

		• *In vivo*, mtDNA copy number was stable throughout early development (from zygote to blastocyst) • *In vitro*, mtDNA in 8-cell, morula and blastocyst embryos was significantly higher than in zygotes	
Human	Human (Monnot *et al.* 2013)	• The average mtDNA amount decreased as maturation stage proceeded • mtDNA amount was reduced in MII oocytes when compared to the GV oocytes • No difference in mtDNA amount between oocytes and fertilised embryos • No difference in mtDNA amount between pre-blastocyst and blastocyst embryos	• GV oocytes n = 2, MI oocytes n = 7, MII oocytes n = 9 • Pre-blastocyst n = 12, Blastocyst n = 5 • Samples from 2 different patients • Ovarian stimulation
	Human (Santos *et al.* 2006)	• Mean mtDNA copy number for fertilised oocytes was 250,454 whereas unfertilised group was 163,698 • Significant difference between fertilised and unfertilised oocytes (P <0.002)	• Fertilised oocytes = 35 zygotes from 21 patients, mean age = 32 • Unfertilised oocytes = 65 from 36 patients, mean age = 32 • Ovarian stimulation

4.2 Materials and Methods

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

4.2.1 Mouse Ovarian Stimulation and Oocyte Recovery

All experimental procedures were approved by the University of Adelaide Animal Ethics Committee and were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. CBAF1 female mice were obtained from the University of Adelaide Laboratory Animal Services and housed in the Animal Facility under a 14:10 hour light:dark cycle at 24°C with *ad libitum* access to food and water**.**

Female mice were induced to 'superovulate' by consecutive intraperitoneal (i.p.) injections of 5 IU (international units) per 12 g body weight of pregnant mare's serum gonadotrophin (PMSG; National Hormone and Peptide Program, Torrance, CA, USA) and 5 IU human chorionic gonadotrophin (hCG; Merck, Sharp and Dohme) in 0.1 mL 0.9 % saline.

For time course experiments, *in vivo* cumulus-oocyte complexes (COCs) were collected at different time points, 48 h after PMSG injection, and then 2 h, 4 h, 8 h and 16 h post-hCG injection. Three experimental replicates were performed, with 5 mice used per replicate. For *in vitro* maturation (IVM), immature COCs (isolated from mice treated with PMSG for 48 h) were cultured in groups of 30 in bicarbonate-buffered α-MEM supplemented with 5% (vol/vol) fetal calf serum (FCS; Invitrogen), 50 mIU/mL recombinant human FSH, and 10 ng/mL epidermal growth factor as control, or with the addition of indicated treatments, in drops of 100 μL overlaid with sterile paraffin oil (Merck, Darmstadt, Germany) and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air for 2 h, 4 h, 8 h and 16 h. Treatments consisted of control media supplemented with 400 μM palmitic acid and 400 μM palmitic acid with the presence of 10 μM BGP-15 (kindly provided by N-Gene Research Laboratories, Budapest, Hungary). Palmitic acid was solubilised and prepared by the method of Downs *et al*. (2009). Four experimental replicates were performed, with 5 mice used per replicate.

4.2.2 *In vivo* embryo collection

Female mice received ovarian hormonal stimulation as previously described and then were mated overnight with males which had previously been assessed for mating ability. Mating was confirmed the following morning with the presence of a vaginal plug. *In vivo* embryos were obtained by flushing the oviducts 44 h (2-cell), 60-62 h (8-cell), 72-78 h (morulae) and 86-88 h (blastocysts) post-hCG with pre-warmed HEPES-buffered α-MEM supplemented with 1% (vol/vol) FCS. Seven experimental replicates were performed, with 3 mice used per replicates.

4.2.3 Fertilisation and Embryo Culture

Male mice, which had previously been assessed for mating ability (not less than 3 days prior), were used as sperm donors for *in vitro* fertilisation (IVF). Male mice were sacrificed by cervical dislocation and the epididymides and vasa deferentia were collected into 1 mL of warm (37°C) Vitro Fertilisation medium (William A. Cook Australia Pty. Ltd., Queensland, Australia). Sperm were extracted into the medium and allowed to capacitate at 37°C in an atmosphere of 5% CO_2, 20% O_2 and 95% air for 1 h. Ten μL of capacitated sperm (35,000 sperm/mL) was added to 90 μL of fertilisation drop. Following 16 h maturation, expanded COCs were washed twice in Research Vitro Wash (William A. Cook Australia Pty. Ltd., Queensland, Australia), and co-incubated with the sperm for 4 h at 37°C in an atmosphere of 5% CO_2, 20% O_2 and 95% air (15-20 COCs per drop). All cumulus cell-free oocytes (10-15 oocytes per 20 μL drop were then transferred to Research Vitro Cleave (William A. Cook Australia Pty. Ltd., Queensland, Australia) without the presence of BGP-15. Five experimental replicates were performed, with 4 mice per replicate. Embryo development was assessed on Day 2 as the percentage of embryos meeting the on-time development criteria from starting number of oocytes; while development on Day 5 as the percentage of embryos meeting the on-time development criteria from 2-cell embryos. For time course experiments, 2-cell, 8-cell, morulae and blastocysts were collected on Day 2, 3, 4 and 5 accordingly. Three experimental replicates were performed, with 3 mice per replicate.

4.2.4 Time-Lapse Embryo Culture

In a separate series of experiments, time-lapse imaging was employed to more closely

analyse developmental kinetics in the embryos. Four hours post-insemination, presumptive zygotes were placed individually in 16-well dishes (Primovision, Vitrolife, Sweden) in 150 µL Cook Vitro Cleave in conditions described above, and monitored by Primo Vision time-lapse embryo monitoring system (Primovision, Thermo Scientific HERAcell VIOS 160i CO_2 incubator) with images of individual embryos generated every 15 min throughout culture. The timing of morphokinetics events, including first, second and third cleavage division and cavitation were recorded and intervals between each event determined and analysed using the Primo Vision Analyser Software (version 4.4.1.01.010). The diameters of the oocytes and embryos (measured to the outer circumference of the zona pellucida) were also automatically recorded throughout development. Three experimental replicates were performed, with 16 COCs per treatment group and replicate.

4.2.5 Collection and Culture of Bovine COCs

Materials and methods were derived from (Sutton-McDowall *et al.* 2016).

NEFA aliquots were prepared as previously described (Downs *et al.* 2009). Briefly, 44.85 mM stocks of oleic acid (C18:1), steric acid (C18:0), and palmitic acid (C16:0) were made with absolute ethanol. One mL of this stock was added to 0.5 g fatty acid-free (FAF) bovine serum albumin (BSA) (MP Biomedicals) in 30 mL Milli-Q water followed by 3 mL of 0.1 M Sodium Hydroxide (NaOH) and was gently stirred until the solution was clear. Aliquots were frozen, freeze-dried, and reconstituted in IVM media prior to culture.

In vitro oocyte maturation was performed using VitroMat (IVF Vet Solutions) + 6 mg/mL FAF bovine serum albumin (BSA; MP Biomedicals) + 0.1 IU/mL follicle stimulating hormone (FSH; Puregon, Organon). Cattle ovaries were transported from a local abattoir (Thomas Foods) in warm saline (30°C–35°C). Follicles were aspirated using an 18-gauge needle attached to a 10 mL syringe, and intact, unexpanded COCs surrounded by four or more cumulus cell layers and ungranulated ooplasms were selected in undiluted follicular fluid, quickly washed twice in IVM medium, and then transferred into pre-equilibrated IVM culture drops overlaid with paraffin oil (Merck, Darmstadt, Germany). Treatment groups were i) control (IVM medium), ii) control + 10 µM BGP-15, iii) NEFA (IVM medium + 150 µM palmitic acid + 200 µM oleic acid + 75 µM steric acid) (Van Hoeck *et al.* 2011),

and iv) NEFA + 10 µM BGP-15. Groups of 10 COCs were cultured in 100 µL of media for 23 h at 38.5°C with 6% CO_2 in humidified air.

4.2.6 Assessment of Cumulus Expansion Index (C.E.I.)

Cumulus expansion was assessed after 23 h culture by an independent assessor, blinded to treatments, using a scale described previously (Vanderhyden *et al.* 1990), as follows: 0, no expansion of cumulus cells; +1, the outer most layers of cumulus cells expanded; +2, expansion of the entire outer half of cumulus cells; +3, all layers expanded except the corona radiatae; and +4, maximal expansion of all layers of cumulus cells. For each treatment group, a mean cumulus expansion index (CEI; over the range 0.0–4.0) was calculated.

4.2.7 *In Vitro* Embryo Culture (Cattle)

At the completion of IVM, COCs were washed once in wash medium- VitroWash (IVF Vet Solutions) + 4 mg/mL FAF BSA—transferred into 500 µL of IVF medium-VitroFert (IVF Vet Solutions) + 4 mg/mL FAF BSA + 10 IU/mL heparin + 25 µM penicillamine + 12.5 µM hypotaurine + 1.25 µM epinephrine and overlaid with paraffin oil. Two straws of bull sperm (Semex Australia Pty Ltd.) of proven fertility were thawed, prepared using a discontinuous Percoll gradient (45%:90%; GE Healthcare), and added to IVF wells at a final concentration of 1×10^6 sperm/mL. Following 23 h of coincubation with sperm (Day 1), presumptive zygotes were mechanically denuded by repeated pipetting, washed once in wash medium, and groups of five embryos were transferred into 20 µL of pre-equilibrated Vitro Cleave (IVF Vet Solutions) supplemented with 4 mg/ml FAF BSA overlaid with paraffin oil, and cultured at 38.5°C in 6% CO_2, 7% O_2, and 87% N_2. On Day 5, five embryos were transferred into 20 µL of VitroBlast (IVF Vet Solutions) + 4 mg/mL FAF BSA and cultured at 38.5°C in 6% CO_2, 7% O_2, and 87% N_2. Fertilisation rates and on-time embryo development were assessed on Day 8.

Briefly, following 23 h IVM in the presence of NEFAs and BGP-15, COCs were mechanically denuded of their cumulus vestment by repeated pipetting. Denuded oocytes or Day 8 blastocysts were washed with 1 mg/mL polyvinylpyrrolidone (PVP) in phosphate-buffered saline (PBS), collected individually into 1.5 mL siliconised low retention

microcentrifuge tubes (Fisher Scientific) with 5 µL of PBS/PVP, snap frozen in liquid nitrogen, and stored at -80°C until use. Four experimental replicates were performed, with 30 COCs per treatment group.

4.2.8 Macaque Ovarian Stimulation and Oocyte Recovery

To examine the effects of BGP-15 on embryogenesis in a primate species, I collaborated with Prof. Jon Hennebold at the Oregon National Primate Research Center in Oregon and conducted experiments in their macaque model of IVF. The general care and housing of rhesus monkeys (Macaca mulatta) at the Oregon National Primate Research Center (ONPRC) was previously described (Wolf *et al.* 1990). The studies were conducted in accordance with the National Institutes of Health (NIH) guide for the Care and Use of Laboratory Animals and all protocols were approved by the ONPRC Animal Care and Use Committee.

Ovarian stimulation and follicle aspiration were performed by surgical staffs at the ONPRC. Briefly, females were observed for signs of menstrual activity and were subjected to follicular stimulation through intramuscular injection of recombinant human follicle-stimulating hormone (FSH; donated by Organon, 30 IU per day) over an 8-day period. On Day 8 of the stimulation protocol, the animal was injected with recombinant human chorionic gonadotropin (hCG; Ovidrel, 1100 IU). Ovarian morphology was recorded by ultrasonography two days before the last hormonal stimulation.

COCs were collected from anesthetised animals by laparoscopic follicular aspiration (approximately 36 h post hCG injection) and placed in HEPES-buffered TALP (modified Tyrode solution with lactate and albumin) containing 0.3% bovine serum albumin (TH3) with 1% heparin (Fresenius Kabi USA, LLC) at 37°C. Tubes containing follicular aspirates were placed in a portable incubator (Minitube, Verona) at 37°C for transport back to the laboratory. Hyaluronidase (0.5 mg/mL) was added to the tubes containing aspirates and were gently agitated with a serological pipette to disaggregate cumulus and granulosa cells masses and then sifted through a cell strainer (Falcon, 70 µm mesh size; Becton Dickinson). Oocytes were retained in the mesh, whereas cumulus and granulosa cells were washed through the filter. The strainer was then immediately backwashed with warmed TH3, and the medium

containing oocytes was collected. The oocytes were then sorted according to maturation (MII, MI, GV, atresia) and transferred into an appropriate well of TALP-complete medium covered with Ovoil (Vitrolife) equilibrated at 37°C in 5% CO_2. After 4 to 6 h of culture, the oocytes were again sorted according to maturation and MI and MII oocytes were split into 3 groups (control, low dose 1 μM BGP-15, high dose 10 μM BGP-15) subjected to IVF.

4.2.9 Macaque Semen Preparation and Embryo Culture

Rhesus semen was collected by electro-ejaculation. Following liquefaction for 30 min at room temperature, the sample was washed three times in TALP-HEPES by centrifugation for 7 min at 1400 rpm. Sperm count and motility analyses were performed and the spermatozoa were resuspended in TALP-HEPES; then the sperm suspension was stored at room temperature. Approximately 1 h before insemination, spermatozoa were exposed to 1 mM cyclic adenosine 3', 5'-mono-phosphate (cAMP) and 1 mM caffeine for sperm activation. For IVF, ten oocytes were inseminated with 1.8×10^5 activated spermatozoa and cultured in 100 μL drops of TALP-complete media with indicated treatments. Oocytes were examined 16 h post-insemination for the presence of two polar bodies (PB) and two pronucleic (2PN) to confirm fertilisation. Cleaved embryos (6-10 embryos) were then transferred to fresh drops (500 μL) of global media (Life Global) supplemented with 10% of protein supplement (Life Global) with or without the presence of BGP-15 equilibrated at 37°C in 6% O_2, 5% CO_2 and 89% N_2. Embryos were transferred to a fresh plate of global media every 2 days and cultured for a maximum of 10 days. Three experimental replicates were performed, with 15 oocytes per treatment group.

4.2.10 Human Oocyte Collections

All samples obtained from Fertility SA were donated to the Fertility SA BioResource and were approved by the St. Andrew's Hospital Human Research and Ethics Committee (HREC; Project Number 93). Biochemical analysis of human samples were covered by St. Andrew's Hospital HREC Project Number 80 and were approved by the Adelaide University HREC.

Women undertaking intracytoplasmic sperm injection (ISCI) antagonist cycle were treated with 125-350 IU recombinant FSH (Gonal-F; Puregon, Merck Sharp & Dohme Australia

(MSD) Pty Limited, Macquarie Park, NSW, Australia) and ovulation was blocked with a gonadotrophin-releasing hormone (GnRH) antagonist (Cetrotide; Merck Serono Australia Pty Ltd, Frenchs Forest, NSW, Australia) or Orgalutran (MSD) or proceeding GnRH analogue (Synarel; Merck Serono) or Lucrin (MSD) as a long down-regulation protocol (Hohmann *et al.* 2003). There were no differences in stimulation protocols between patients according to BMI. When at least three follicles reached 16 mm in diameter, recombinant hCG (250 µg; Ovidrel, Merck Serono Australia Pty Ltd) was administered and transvaginal oocyte retrieval was performed 36 h later under anaesthesia. COCs were collected from women via transvaginal aspiration of follicles under anaesthetic. Oocytes were subjected to ICSI according to standard clinical practice.

The mean (± SEM) age of the five women participating in the study was 32.4 ± 2.1 years. Immature oocytes (germinal vesicle to metaphase I) were discarded during routine assessment prior to ICSI and donated to research. Oocytes were then transferred into pre-equilibrated 10 µL culture drops (G-1™ Plus, Göteborg, Sweden) with or without 10 µM BGP-15 (one oocyte per drop) , overlaid with Ovoil (Vitrolife, Göteborg, Sweden) and cultured overnight in humidified MINC benchtop incubator (Cook Medical) for 16 h at 37.5°C, 6% CO_2 and 5% O_2. Following 16 h culture, oocytes were washed three times in PBS/PVP and collected individually into 1.5 mL siliconised low retention microcentrifuge tubes (Fisher Scientific) with 5 µL of PBS/PVP, snap frozen in liquid nitrogen, and stored at -20°C until transported on ice to the University of Adelaide.

4.2.11 Mitochondrial DNA (mtDNA) Copy Number Quantification

The mtDNA copy number in individual oocytes or embryos was quantified as previously described (Kameyama *et al.* 2010; Wu *et al.* 2015). Briefly, denuded oocytes or embryos were washed with PBS/PVP (1 mg/mL of PVP in PBS), collected individually into 1.5 mL siliconised low retention microcentrifuge tubes (Fisher Scientific) with 5 µL of PBS/PVP and stored at -80°C. Genomic DNA was isolated using the QIAamp DNA micro kit (Qiagen) according to manufacturer's protocol with carrier RNA (1 µg; Qiagen) added to each sample. Genomic DNA was eluted once with 50 µL of water and diluted 10 times for quantitative PCR.

To prepare the quantification standards, a 1186 bp fragment of the 12S ribosomal (r)RNA region of mtDNA was amplified from mouse liver by PCR using the primer pair 5′-ACACCTTGCCTAGCCA-3′ and 5′-TTTGCCACATAGACGAGTT-3′ with the LongRange PCR kit (Qiagen), and then purified by using the QIAquick PCR purification kit (Qiagen) and cloned using the Qiagen PCR cloning kit (Qiagen). Plasmid DNA was purified from bacteria using Plasmid Maxi kit (Qiagen), and the concentration was determined by using a Nanodrop ND1000 Spectrophotometer (Biolab). Plasmid copy number was calculated as: mass of plasmid (g) = plasmid size (bp)×(1.096×10^{-21} g/bp); mass of plasmid required to generate 1×10^7 copy number standard stock = 1×10^7×mass of single plasmid. A standard curve was generated by using seven tenfold serial dilutions ($10^{-1}\times10^7$ copies), and standard curve correlation coefficients were consistently greater than 0.98. Real-time quantitative PCR using the primer pair 5′-CGTTAGGTCAAGGTGTAGCC-3′ and 5′-CCAGACACACTTTCCAGTATG-3′ was performed in triplicate using SYBR Green PCR master mix (Applied Biosystems) and a Rotor-Gene 6000. Primer sequences for the quantification of mtDNA were derived from (Kameyama *et al.* 2007). Standard curves were created for each run and sample copy number was generated from the equation of Ct value against copy number for the corresponding standard curve.

The PCR program employed an initial step of 95°C for 10 min followed by 40 cycles, denaturation at 95°C for 10s, annealing at 60°C for 30s and extension at 72°C for 20s. Every reaction was followed by melting curve analysis to ensure the specificity of the amplification. All reactions were performed in triplicate with total reaction volumes of 20 µL. Premix for quantitative PCR was prepared from Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA). The premix consisted of 10 µL of Power SYBR Green, 6 µL of PCR grade water, 2 µL of 50 µM primer pair; following which 2 µL of DNA template was added to 18 µL of premix for PCR reaction.

4.2.12 Statistical Analyses

All measures are reported as mean ± SEM. Statistical significance was determined as indicated, by using Student's *t*-test or one-way ANOVA with Tukey's post hoc tests, as appropriate, using GraphPad Prism v008 for Windows. Time-lapse data were analysed by

using repeated measures with linear mixed-effects model in SPSS (IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.). Normality of data was confirmed by analysing the spread of residuals. A *P*-value of less than 0.05 was considered statistically significant.

4.3 Results

4.3.1 Kinetics of mtDNA during oocyte maturation and embryogenesis in the mouse

To examine the dynamics of mtDNA copy number throughout preimplantation embryo development, *in vivo* derived oocytes and embryos were collected from the mice following superovulation. During oocyte maturation, mtDNA copy number remained constant from GV oocyte to MII oocytes isolated at 16 h post-hCG. Similarly, mtDNA did not increase following fertilisation through the 18-32 cell stage but significantly increased at morula and blastocyst stage ($P \leq 0.01$; Figure 1A). The average mtDNA copy number was 2.3×10^5 for GV oocytes and 4.1×10^5 for MII oocytes, increasing to an average value of 8.8×10^5 and 1.3×10^6 for morula and blastocyst respectively. Next, in order to determine whether there was biological variability in mtDNA content in the oocytes of the mice that were matured *in vivo* or *in vitro*, oocytes were collected at defined times after PMSG treatment. There was a continuous gradual increase in mtDNA copy number as oocytes progress to maturation, reaching a maximum of 5.0×10^5 copies in the IVM oocytes (Figure 1B). Identical kinetics were observed in both *in vitro* and *in vivo* matured oocytes. Thus, mtDNA slightly but steadily accumulates during oocyte growth.

To further characterise the mtDNA replication during embryogenesis, developing preimplantation embryos (*in vivo* flushed and IVF-derived) were collected at different time points. Absolute mtDNA copy number was measured in 2-, 4-, 8-, 18-32 cell embryos, morula and blastocysts. mtDNA levels were gradually increased from a 2-cell embryo (5.7×10^5 copies for *in vivo* and 6.8×10^5 copies for IVF embryos) to a blastocyst (1.3×10^6 copies for *in vivo* and 1.2×10^6 copies for *in vitro*) (Figure 1C). An identical pattern was observed between *in vivo* and IVF-derived embryos clarifying that our methods of *in vitro* culture have no significant influence on mitochondrial biogenesis.

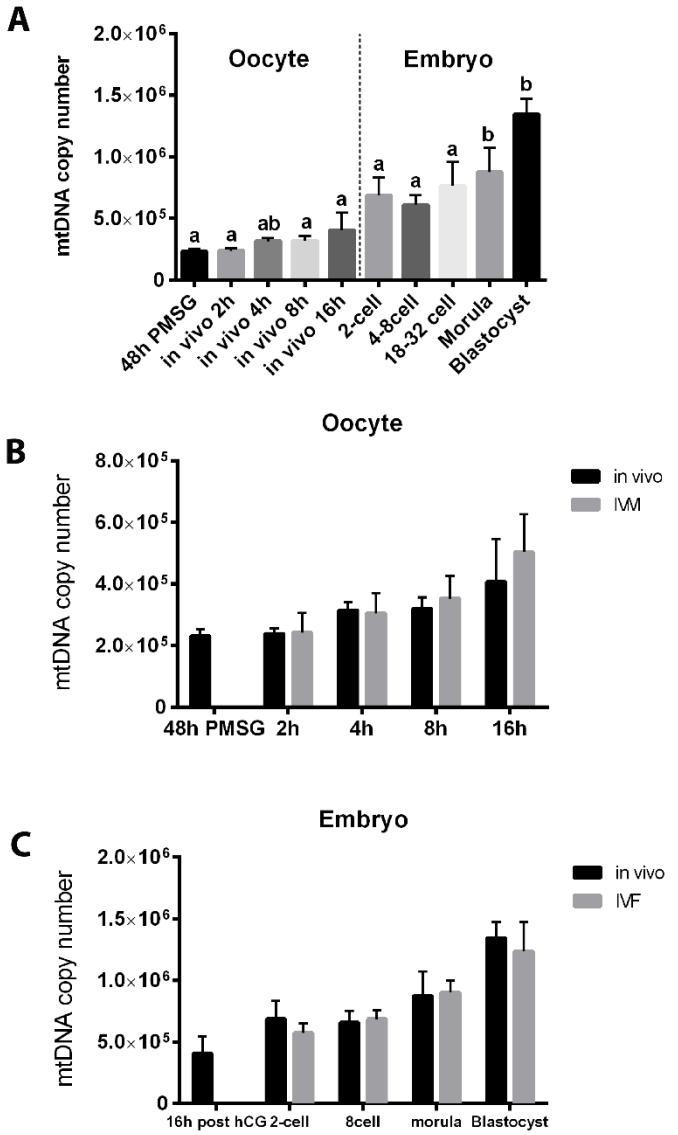


Figure 1 Mitochondrial DNA (mtDNA) copy number during mouse preimplantation embryo development.

(A) Quantification of mtDNA copy number in individual oocytes and embryos collected at different time points following ovulatory hCG administration and mating. Data are presented as mean ± SEM, n = 10-12 oocytes and n = 9-23 embryos per group. (B) mtDNA copy number in *in vivo* and *in vitro* matured (IVM) oocytes. Data are presented as mean ± SEM, n = 10-12 oocytes per group. (C) mtDNA copy number in *in vivo* flushed and *in vitro* fertilised (IVF) embryos, as determined by real time PCR. Data are presented as mean ± SEM, n = 9-23 embryos per group. Groups with different superscripts differ significantly by one-way ANOVA with Tukey's post hoc test ($P \leq 0.01$).

4.3.2 BGP-15 normalises mtDNA levels in high lipid-stressed oocytes

To understand how mtDNA is impacted by exposure to high lipids, immature unexpanded COCs were cultured in the presence of high PA and/or BGP-15 throughout maturation. At 2 h and 4 h of culture, PA-treated oocytes had increased mtDNA number (~5.3×10^5 copies), which was no longer higher than controls by 8 h and significantly decreased compared to controls by 16 h of maturation (1.85×10^5 copies) ($P \leq 0.01$; Figure 2). The presence of BGP-15 with PA resulted in mtDNA levels comparable to those in the control oocytes at 2 h and 4 h of IVM. However, oocyte mtDNA levels in PA+BGP-15 treated COCs were dramatically increased to a level much higher (7.7×10^5 copies) than the PA-treated oocytes by the end of maturation ($P \leq 0.01$; Figure 2). No control IVM+BGP-15 group was undertaken because a previous study by our lab showed that lean mice treated with BGP-15 had no change to mtDNA levels in the oocytes (Wu *et al.* 2015).

4.3.3 Presence of high lipid during mouse IVM affects embryo development and mtDNA levels

In a separate series of experiments, expanded COCs following 16 h of culture were subjected to IVF followed by morphometric assessment and time-lapse imaging to closely monitor developmental kinetics. The time range over which embryos reached every developmental event increased as embryo development progressed and there was no significant difference in these timings between each treatment group (Figure 3A). On Day 2, the cleavage rate was lower in the PA-treated oocytes when compared to the control (Control = 48 ± 6.66% vs. PA = 17.08 ± 4.96%; $P \leq 0.05$) and it was partially normalised with BGP-15 treatment (PA+BGP-15 = 37.25 ± 6.17%) but that was not statistically significant (Figure 3B). Only zygotes that were successfully cleaved on Day 2 were further analysed. On Day 5, the blastocyst development rate in the PA-treated oocytes was reduced (8.26 ± 5.13%) when compared to the control (21.38 ± 3.64%) and partially restored with BGP-15 treatment (16.62 ± 2.78%), however, these rates of development were not significantly different between groups (Figure 3C). Subsequently, the duration of interval between each cell division also showed no significant difference between the groups except at 3-cell to 4-cell stage (Figure 3D). The time duration was significantly shorter in the PA+BGP-15 group when compared to the PA (Figure 3D).

Blastocysts that were collected on Day 5 were quantified for mtDNA levels. There was a significant reduction in mtDNA copy numbers in the blastocysts that were derived from high-lipid treated oocytes (2.5×10^5 copies) when compared to the control blastocysts (5.0×10^5 copies). BGP-15 supplementation during IVM did not significantly recover the mtDNA copies (3.8×10^5 copies) in the blastocysts. (Figure 4). Thus, the presence of high lipid during mouse IVM reduced mtDNA copy number in both the oocytes and the resulting blastocysts and BGP-15 in IVM media was able to at least normalise the mtDNA levels in the PA-treated oocytes and to a lesser extent in the blastocysts.

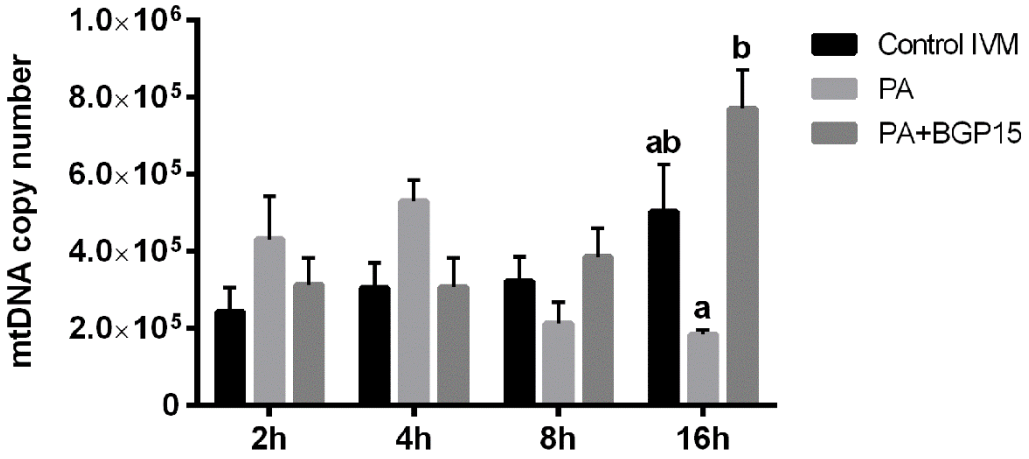


Figure 2 Effect of palmitic acid (PA) and/or BGP-15 in *in vitro* maturation (IVM) media on mitochondrial DNA (mtDNA) levels in mouse oocytes.

mtDNA copy number in oocytes that underwent IVM in the presence of 400 µM Palmitic acid (PA) with or without BGP-15. Data are presented as mean ± SEM, n = 10-12 oocytes from 4 mice per group. Groups with different superscripts differ significantly by one-way ANOVA with Tukey's post hoc test ($P \leq 0.01$).

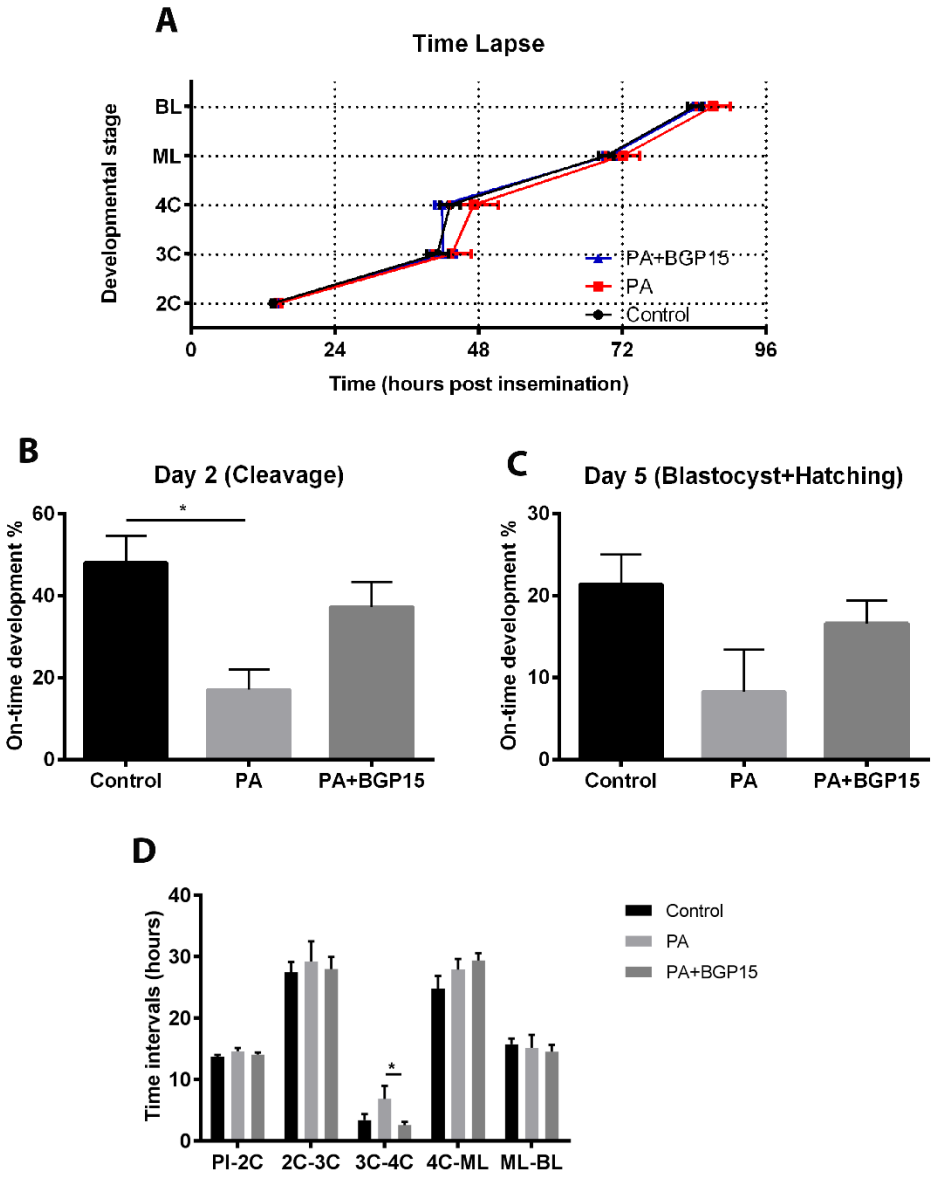


Figure 3 Mouse embryo developmental kinetics and developmental competence following *in vitro* maturation (IVM) in high palmitic acid (PA) and/or BGP-15.

(A) Timing of developmental milestones in hours post-insemination in control, palmitic acid (PA) and PA+BGP-15 treated IVM groups. (B) Cleavage rate was assessed on Day 2 and (C) blastocyst rate on Day 5. Data are presented as mean ± SEM, n = 3 experimental replicates. (D) Time intervals between each developmental stage. Data are presented as mean ± SEM, n = 26-37 embryos from 4 mice per group. Groups with different superscripts differ significantly by one-way ANOVA with Tukey's post hoc test and repeated measure by linear mixed-effects model; * $P \leq 0.05$. 2C- 2-cell, 3C- 3-cell, 4C- 4-cell, ML- morula, BL- blastocyst.

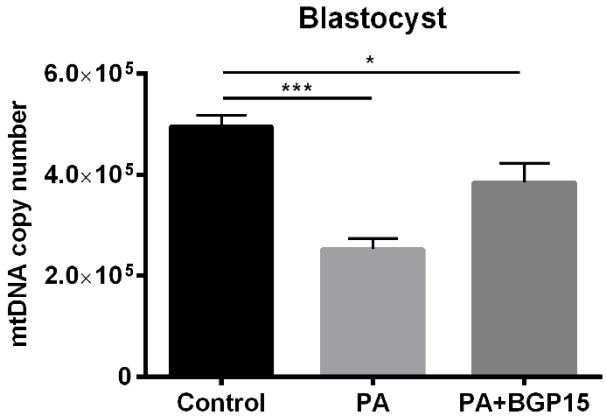


Figure 4 Effect of high palmitic acid (PA) and/or BGP-15 during *in vitro* maturation (IVM) on mitochondrial DNA (mtDNA) levels in mouse blastocysts.

mtDNA copy number in individual embryos was quantified. Data are presented as mean ± SEM, n = 7-18 embryos from 3 experimental replicates. Groups with different superscripts differ significantly by one-way ANOVA with Tukey's post hoc test. * $P \leq 0.05$, *** $P \leq 0.001$.

4.3.4 Effects of high lipid during bovine IVM on embryo development and mtDNA levels.

To further examine the effects of high lipid and BGP-15 on oocyte maturation and whether the findings in mouse are common to other species, we further utilised a well-characterised *in vitro* bovine model of lipotoxicity during IVM. Here, we supplemented the maturation medium with NEFA as in (Sutton-McDowall *et al.* 2016) but in the presence or absence of BGP-15. After IVM, COCs were evaluated morphologically for cumulus expansion. No significant difference was observed between the groups, however, the COCs that were exposed to NEFA have poor expansion when compared to the control COCs (Figure 5A). Cumulus cells were then removed and the oocytes were quantitatively assessed for mtDNA copy number. The oocytes that were cultured in the presence of NEFA had lower mtDNA copy number (1.4×10^7 copies) when compared to the control (1.9×10^7 copies) but this was not statistically significant. Supplementation of the IVM culture media with BGP-15 normalised the mtDNA copy number in the NEFA-exposed oocytes (2.2×10^7 copies) but this increase from the NEFA-only treated oocytes was not statistically significant (Figure 5B).

Maturation in the presence of NEFA had no significant effect on oocyte cleavage or blastocyst yield (Figure 6A). The blastocyst development rate was lower for the oocytes matured in the presence of NEFA (23.25 ± 7.49%) and tended to be normalised by BGP-15 supplementation in the culture media (37.75 ± 9.63%) but there were no statistically significant differences between groups (Figure 6B). However, when blastocyst rate in the NEFA and NEFA+BGP-15 was analysed as independent experiments, it is clear that supplementation of BGP-15 in NEFA IVM culture media improves developmental rate (Figure 6C). Embryos generated from the IVF experiments were quantified for mtDNA copy number and no significant difference was observed between the groups. Nevertheless, the mtDNA copy number of blastocysts from the NEFA-treated group showed a slightly lower mtDNA copy number (6.1×10^8 copies) than the other groups (Control = 9.3×10^8 copies; Control+BGP-15 = 8.8×10^8 copies) and BGP-15 supplementation in the NEFA culture medium partially restored the mtDNA copy number in the embryos (9.2×10^8 copies) to levels identical to controls (Figure 6D).

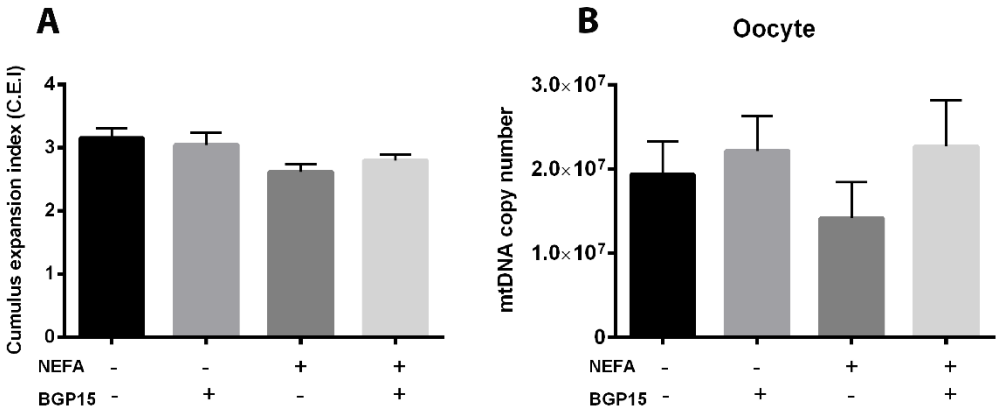


Figure 5 Effect of non-esterified fatty acid (NEFA) and/or BGP-15 supplementation on bovine cumulus oocyte complex maturation *in vitro*.

(A) Cumulus expansion index (CEI) was assessed 23 h following culture. Data are presented as mean ± SEM from 4 experimental replicates with approximately 40 COCs per experimental replicate per treatment group. (B) mtDNA copy number in individual oocytes collected from the indicated treatment groups. Data are presented as mean ± SEM, n = 18-19 oocytes per group. $P > 0.05$, one-way ANOVA with Tukey's post hoc test.

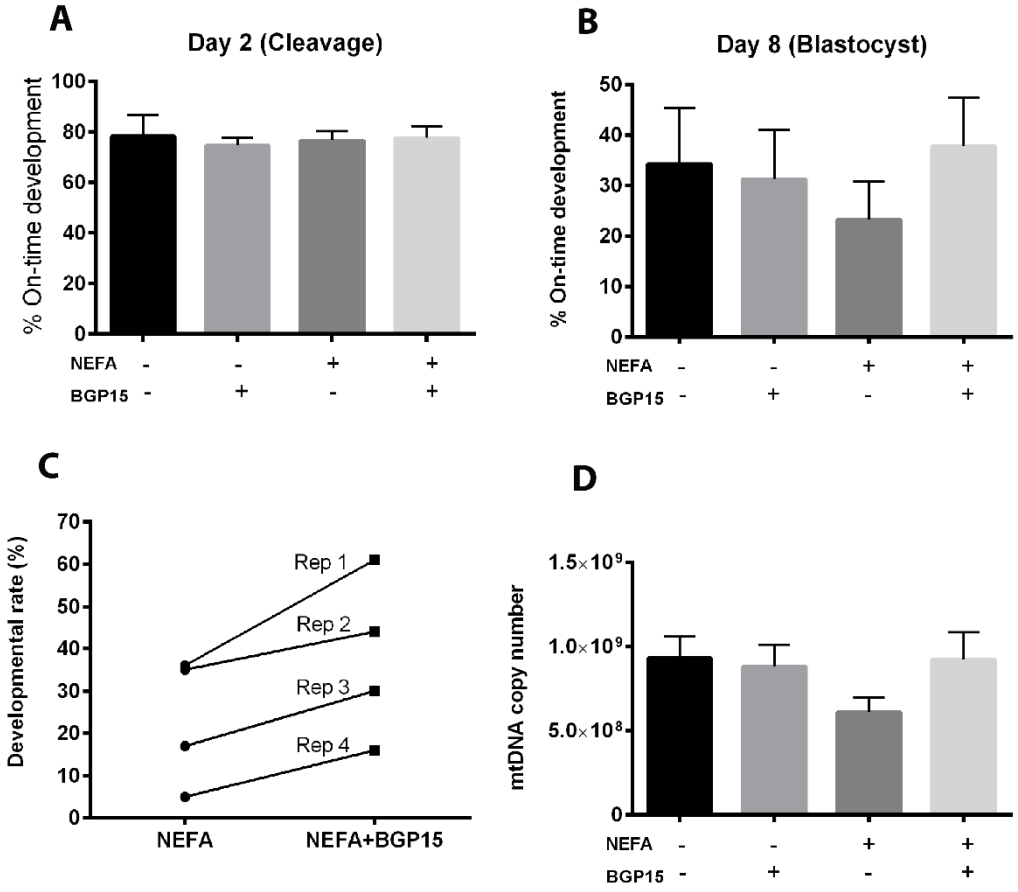


Figure 6 Effect of non-esterified fatty acid (NEFA) and/or BGP-15 supplementation during *in vitro* maturation (IVM) on bovine oocyte developmental competence.

(A) Cleavage and (B) Blastocyst development rate following IVF of oocytes from cumulus-oocyte complexes (COCs) cultured in the presence of NEFA and BGP-15 as indicated. (C) Developmental rate in NEFA and NEFA+BGP-15 in the four independent experiments. (D) Quantification of mtDNA copy number in individual embryos collected following *in vitro* fertilisation (IVF). Data are presented as mean ± SEM, n = 18-22 embryos per group. $P > 0.05$, one-way ANOVA with Tukey's post hoc test.

4.3.5 Effect of BGP-15 in IVF media on macaque embryo mtDNA levels

A total of 146 oocytes were retrieved from three ovarian stimulation of three different animals. Of these, 22.04 ± 5.073% were at MII stage, 49.77 ± 3.34% at the MI stage, and 22.47 ± 3.181% at GV stage. 5.72% of oocytes were classified as degenerate/atretic or not fully grown and therefore were excluded (Figure 7A). Thus, a large proportion of the oocytes were at MI stage at the time of aspiration and this is typical for the controlled ovarian stimulation protocol in the macaque. Following culture, MI and MII oocytes were randomly allocated to different IVF culture media with or without BGP-15 for fertilisation and embryo culture. The treatment groups were: standard (control) media, media containing low dose BGP-15 (1 µM) or media containing high dose BGP-15 (10 µM). These doses were selected based on effects we observed in mouse and bovine which utilise 10 µM during IVM and IVF.

Fertilisation was assessed visually at 12-15 h post-fertilisation by the presence of 2PN (Figure 7B). There was no significant difference in the 2PN rate between the groups (Figure 7C). Embryos that were successfully cleaved were assessed daily. By Day 3 of culture, most embryos in the three groups were at the 6-8 cell stage and by Day 5 most were at the compact morula stage. The number of embryos that reached the morula stage were recorded on day 5. By day 7, most embryos had developed to the blastocyst stage (Figure 8) and blastocyst development rate was determined. A lower cleavage rate was observed in the group treated with a low dose of BGP-15 when compared to the control and high-dose BGP-15 treatment, but was not statistically significant (Figure 9A). However, the group that was treated with a high dose BGP-15 had a lower percentage of embryos that reached the morula stage when compared to the control and low dose BGP-15 treatment, however, this was not statistically significant (Figure 9B).

GV oocytes that were not matured were collected and quantified for mtDNA copy number. mtDNA levels did not increase between the oocyte and morula stage in the rhesus macaque (Figure 9C). There was a dramatic increase in mtDNA copy number as the embryos progressed to the blastocyst stage (Figure 9C). Morulae and blastocysts that were cultured from the low dose BGP-15 had a slightly higher level of mtDNA compared to the control and high dose BGP-15 treatment, but again, was not statistically significant (Figure 9C).

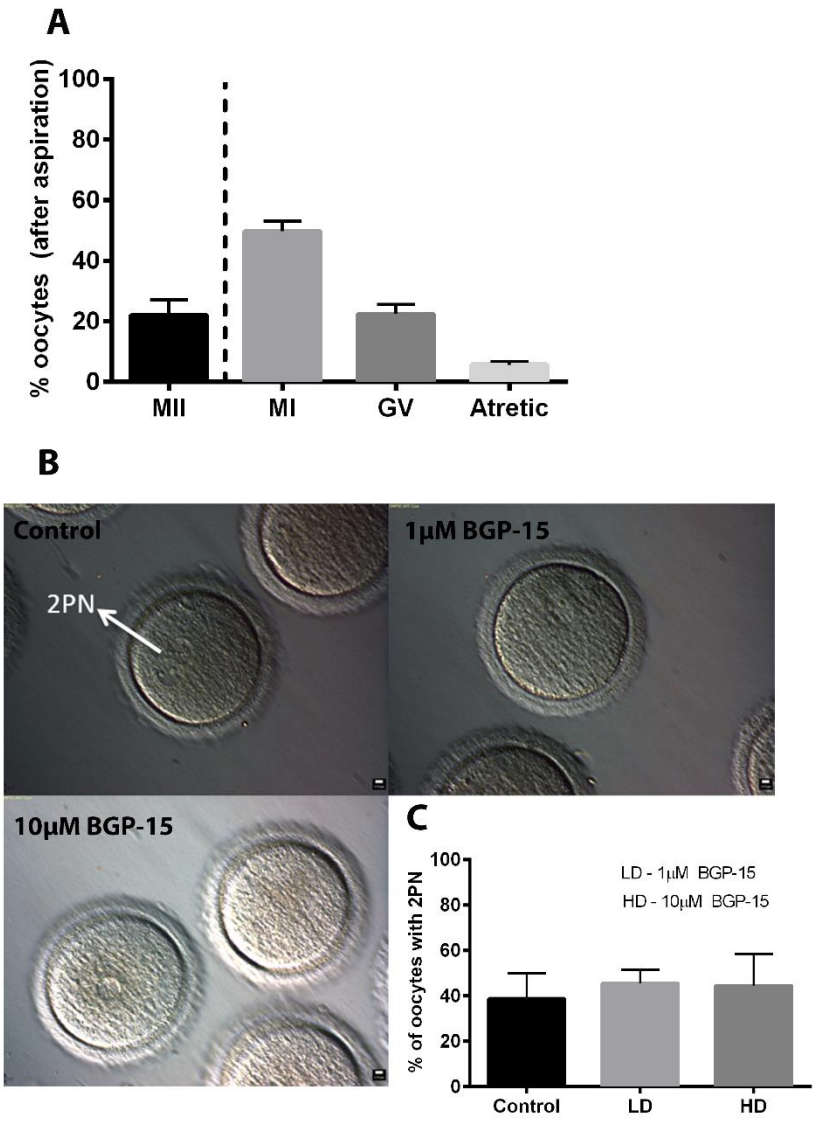


Figure 7 Proportion of Rhesus macaque oocytes at different phases throughout maturation and presence of 2 pronuclei (2PN) after fertilisation.

(A) The percentage of MII, MI, GV and atretic oocytes after aspiration. (B) Representative images of oocytes with 2PN on day 2 from each of the treatment groups. (C) The percentage of oocytes with 2PN. Data are presented as mean ± SEM, n = 3 experimental replicates. $P > 0.05$, one-way ANOVA with Tukey's post hoc test. LD- low dose; HD- high dose

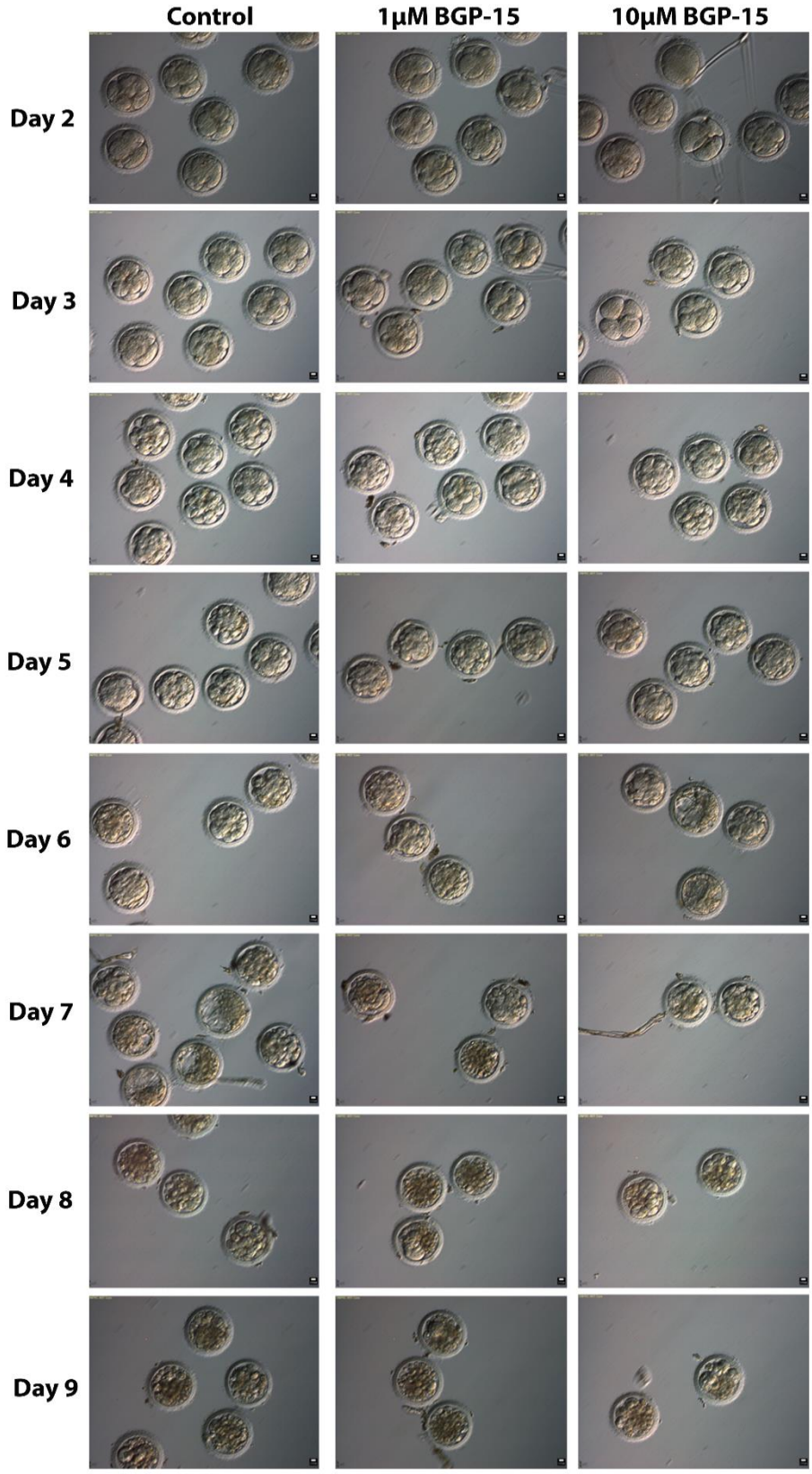


Figure 8 Representative photos of Rhesus macaque embryos from day 2 to day 9 *in vitro* with the indicated treatments.

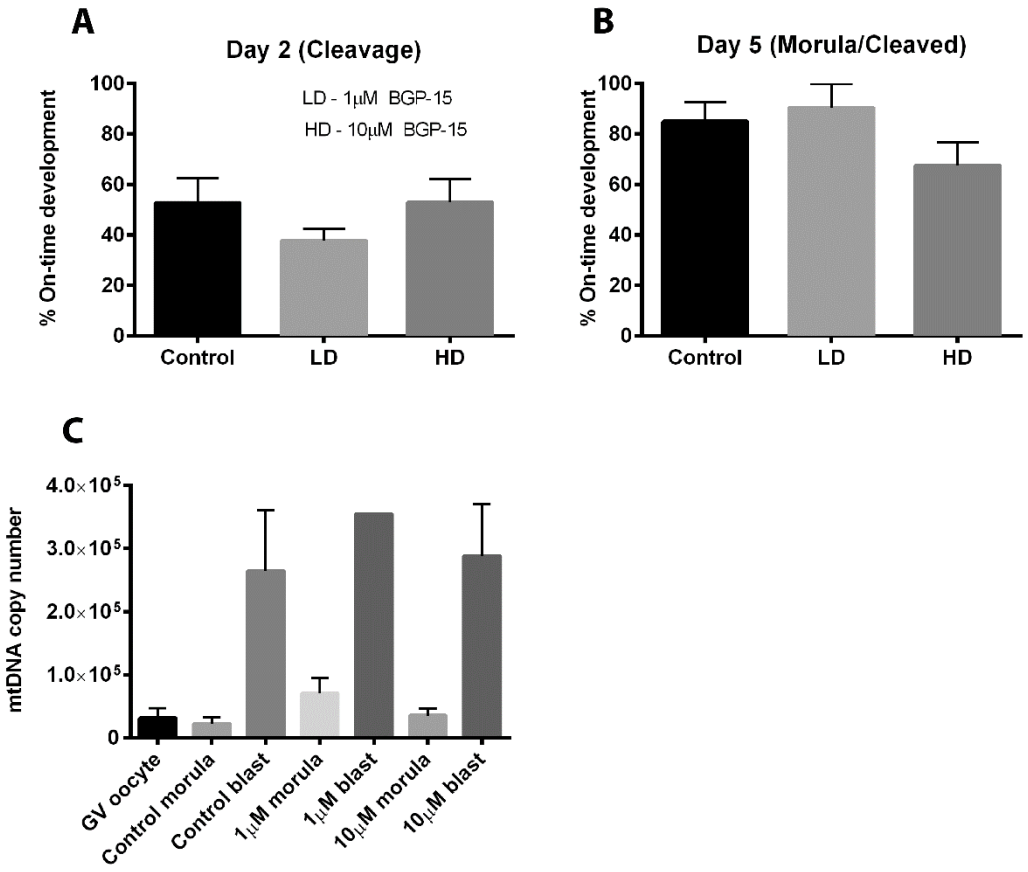


Figure 9 Effect of BGP-15 on macaque embryo development and mitochondrial DNA (mtDNA) levels.

(A) Cleavage rate was assessed on Day 2 and (B) morula rate was examined on Day 5. Data are mean ± SEM, n = 3 experimental animals. (C) mtDNA copy number was assessed in individual oocytes and embryos using real-time PCR. Data are presented as mean ± SEM, n = 1-11 oocyte/embryo. $P > 0.05$, one-way ANOVA with Tukey's post hoc test. LD – low dose (1 μM) BGP-15, HD = high dose (10 μM) BGP-15.

4.3.6 Effect of BGP-15 on human immature oocytes

Human immature oocytes (germinal vesicle to metaphase I) were cultured overnight (~16 h) in the presence or absence of BGP-15 (10 μM) and then collected for quantitative analysis of mtDNA. There was a great deal of variation between oocytes, even within individual patients. The average mtDNA in oocytes (with and without BGP-15) of patient 9176 was 7.2×10^4 copies but there were marked differences between oocytes with 2 oocytes having much higher values than the other two. The average oocyte mtDNA level in patients 10680 and 20153 increased with BGP-15 treatment, but this was not statistically significant. In patient 10680, average mtDNA copy number increased from 1.6×10^4 to 5.2×10^4 copies, and in patient 20153 from 3.7×10^4 to 7.4×10^4 copies. However, patient 8314 had lower mtDNA levels in the oocytes following BGP-15 treatment (from 2.5×10^4 to 9.9×10^3 copies) (Figure 10). There was no relationship between mtDNA copy number in oocytes and patient age or BMI. These results however are preliminary data and this experiment is ongoing.

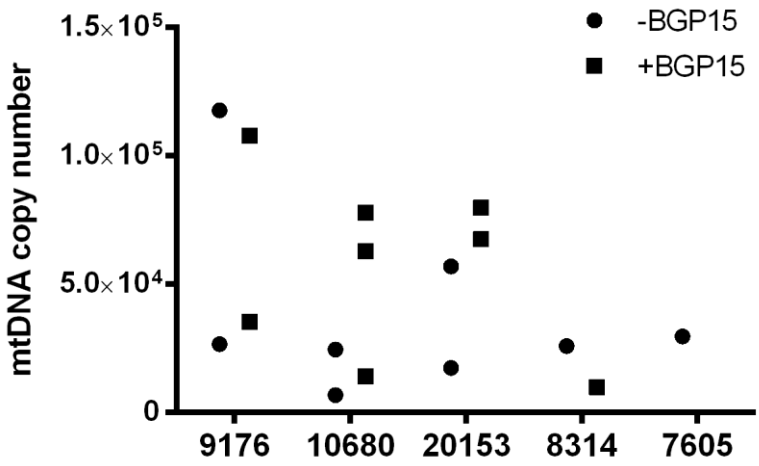


Figure 10 Mitochondrial DNA (mtDNA) copy number in human germinal vesicle (GV) and metaphase I (MI) oocytes.

The X-axis shows the patient number with 1-5 immature oocytes donated by each woman with half treated with BGP-15 as indicated. mtDNA level in individual oocytes was determined by real-time PCR. $P > 0.05$, unpaired *t*-test.

4.4 Discussion

The importance of mitochondria and its DNA genome in oocytes is well-established. Mitochondria supply the majority of adenosine triphosphate (ATP) in a cell and mtDNA has been proposed as a key marker of viability and might be a key determinant of preimplantation embryo development. However, little is known about its regulation during oocyte maturation and preimplantation development and how lipid stress and BGP-15 will disturb and/or restore its regulation. I investigated this in a number of models and contexts, particularly in the mouse, cattle, macaque and human during preimplantation development.

Firstly I show that in mouse mtDNA copy number was relatively stable throughout oocyte maturation, but with higher mtDNA copy number in MII oocytes. The number of mtDNA molecules also remained remarkably stable throughout group and single cultured preimplantation development, beginning to increase only at the morula stage. To date, there is little information on mtDNA copy number during mouse oocyte maturation. It was found that the average mtDNA copy number in GV oocytes was 2.3×10^5 and in MII oocytes was 4.1×10^5 which were higher than those reported previously at (Mahrous *et al.* 2012) (~1.75×10^5 copies in GV and MII oocytes). The discrepancy might be due to different culture conditions and mouse strain used. Our results showing that mtDNA remained stable throughout oocyte maturation and reaches its maximum at MII agrees with the data reported by others (Mahrous *et al.* 2012; Mao *et al.* 2012). A similar lack of increase in mtDNA copy number from *in vivo*-derived GV and MII oocytes has also been shown in humans (Reynier *et al.* 2001; Barritt *et al.* 2002), suggesting that the majority of mtDNA replication had occurred by the completion of oocyte growth. It is well-established that there was an increase in ATP content in the oocyte in cattle (Stojkovic *et al.* 2001), pig (Brevini *et al.* 2005) and mouse (Yu *et al.* 2010) during the final oocyte maturation, suggesting our observation of an accumulation in mtDNA at the end of maturation is to coordinate with the ATP demands during oocyte growth and possibly to ensure successful fertilisation and survival to the later stages of preimplantation development.

There is some variability among reports in relation to the mtDNA copy number during embryo development. Our results that mtDNA levels increased significantly at the blastocyst stage are consistent with findings in porcine (Spikings *et al.* 2007) and bovine (May-Panloup

et al. 2005d) but not with those in mice (Thundathil *et al.* 2005) and rats (Kameyama *et al.* 2007) where there was no difference in mtDNA levels throughout preimplantation development. The discrepancies might be due to different species and different strain of mice used. The quantification standard used may also contribute to the variation observed. A study by (Thundathil *et al.* 2005) used a 189 bp fragment of the *mt-Nd2* gene whereas the current study employed a 1186 bp fragment of the 12S ribosoman(r)RNA region of mtDNA as quantification standards. In different species, mtDNA varies greatly in size, structure and gene organisation therefore its kinetics of regulation may vary with species. Another explanation for this variation might be the different implantation patterns among species. Moreover, the method of DNA extraction used in the current study is robust, producing large amount of DNA from a single cell compared to previous methods reported in (Thundathil *et al.* 2005; Kameyama *et al.* 2007). In addition, mtDNA copy number remained constant during early embryogenesis and began to increase only at the morula stage highlighting the general belief that mtDNA replication does not occur until after later stage of embryo development. A strength of my observation is that, a comparison in mtDNA copy number was also made between IVM and *in vivo*-derived oocytes and between IVF and *in vivo*-derived embryos. No significant difference was observed between the groups indicating that IVM and IVF conditions in this experiment mimic *in vivo* development and had no impact on mtDNA replication progression during oocyte maturation and preimplantation development. Following fertilisation, mitochondria are thought to be randomly segregated between blastomeres, therefore it is expected that embryos will have a reduction in the numbers of mtDNA copies per cell. Thus, future studies will require measurement of a nuclear reference gene so that embryo cell number could be adjusted for and mtDNA copy number per cell can be calculated.

When exposed to high levels of PA lipid, oocyte mtDNA was significantly reduced by the end of maturation (16 hours). It is known that the cumulus cells which surround the oocyte nourish the developing oocyte through gap junctional cell-to-cell contacts and COCs are known to increase β-oxidation lipid metabolism for energy during maturation (Dunning *et al.* 2010). However, in the presence of excessive saturated fatty acids, COCs undergo lipotoxicity responses (Wu *et al.* 2012). Further, the cumulus cell layer is the first metabolic area that is affected by altered fatty acids in the culture media. Indeed, exposure to saturated

fatty acids *in vitro* induces apoptosis in cumulus cells but not in the oocyte (Mu *et al.* 2001; Leroy *et al.* 2005; Lolicato *et al.* 2015). Thus, future studies are necessary to determine this. In bovine, the cumulus cell layer also acts as a barrier, protecting oocytes from *in vitro*-induced lipotoxicity (Lolicato *et al.* 2015). Thus, at the end of maturation, it is reasonable to speculate that the gap junctions between the oocytes and cumulus cells are disrupted, hence limiting the transfer of nutrients between the cells and ultimately altering mitochondrial dynamics. Furthermore, a previous study from our laboratory also found that exposure of COCs to high levels of PA resulted in ER stress and impaired mitochondrial activity (Wu *et al.* 2012), suggesting that the oocytes might be undergoing extensive unfolded protein response and protein folding to restore ER function and cellular homeostasis rather than mtDNA replication to maintain cell survival. It was interesting that mtDNA levels were initially increased by PA at 2 h and 4 h post-hCG and I speculate that this elevated mtDNA levels are a consequence of a compensatory mechanism, aimed at normalisation of ATP generation in the face of compromised organelles of reduced function. Importantly, BGP-15 supplementation was able to normalise the decrease in mtDNA in oocytes exposed to PA to levels similar to the controls, indicating that BGP-15 might be enhancing mtDNA biogenesis under stress conditions or that it acts to prevent a lipid-induced degradation of mtDNA. Further investigation is required to biochemically dissect these two possibilities and to determine whether either involves a restoration of gap junction communication between oocytes and cumulus cells or modulation of apoptotic signals.

We had previously showed that exposure to high levels of palmitic acids impaired cleavage and blastocyst formation rate (Wu *et al.* 2012). In the current study, the reduction in mtDNA levels in the oocytes is associated with lower cleavage rate following IVF. This finding is supported by a previous study in porcine (Mao *et al.* 2012) and human embryos (Reynier *et al.* 2001) where they showed that lower mtDNA levels may affect oocyte fertilisability and developmental competence. Conversely, other studies indicated that key checkpoints for cleavage are not strictly regulated by mtDNA copy number as very low mtDNA copy number (as few as 4000) did not inhibit embryo preimplantation development (Wai *et al.* 2010). Importantly, the reduced mtDNA levels observed in the PA-treated oocytes were also reflected subsequently in the embryos; and BGP-15 treatment during IVM slightly improve the mtDNA levels in the embryos generated following exposure to high PA. This result

suggests that decreased and/or compromised mtDNA in the embryo is possibly reversible by the blastocyst stage, implicating that low levels of mtDNA in mature oocytes can be sensed and adjusted upward in the preimplantation embryos.

The effect of BGP-15 in IVM medium was further tested in a bovine model. NEFA supplementation during oocyte maturation resulted in a decreased trend in mtDNA copy number in both mature oocytes and embryos. However, the effect was not statistically significant, most probably due to relatively low numbers of oocytes and embryos analysed within the groups and the high degree of variability in embryo development rates between experimental replicates. Nevertheless, the results were consistent with previous findings (Sutton-McDowall *et al.* 2016). In bovine, oocytes with higher mtDNA copy numbers also have better blastocyst development (Hua *et al.* 2007; May-Panloup *et al.* 2007). Similar to earlier findings in the mice, BGP-15 was able to normalise mtDNA levels in both the oocytes and embryos that were generated following exposure to high lipids, but this was not statistically different. The lack of statistical differences between groups in both embryo development and mtDNA levels is very likely due to variability across the experimental runs which is typically due to inconsistencies between the abattoir tissues. Overall however these results clearly implicate that mtDNA are vulnerable to external stressors and that mtDNA replication/biogenesis is altered in response to high lipids environment. Further, the findings that BGP-15 is able to at least partially normalise mtDNA in response to high lipids condition demonstrate that mtDNA levels can be enhanced by a drug currently in human clinical trials, which may lead to improved embryo development.

From the mice and bovine studies, it is clear that addition of BGP-15 to IVM medium is able to slightly improve mtDNA levels and embryo development following exposure to high lipids. Therefore another goal of this study was to generate pre-clinical data on this compound's effectiveness in Rhesus macaque assisted reproductive technology (ART). BGP-15 showed no significant impact on the macaque cleavage, blastocyst development and also mtDNA copy number in the blastocyst. Interestingly, there was a massive increase in mtDNA levels in the macaque blastocysts when compared to the GV oocytes and morula, further confirming that mtDNA replication does not take place prior to blastocyst stage in primates. This is the first study that looked at mtDNA levels in the macaque model and

importantly it shows that BGP-15 is not detrimental to development. Besides that, human oocytes that were not successfully fertilised were cultured overnight in the absence or presence of BGP-15. There were two out of five patients (10680 and 20153) whose oocytes had higher mtDNA following BGP-15 culture. The variable mtDNA copy number observed between patients and between samples is similar to the variability in mtDNA copy number previously reported in human oocytes (Steuerwald *et al.* 2000; Reynier *et al.* 2001; Barritt *et al.* 2002). It has been suggested that the biological variation in mtDNA in human oocytes is indicative of oocyte quality and attainment of a threshold of mitochondrial activity and/or ATP production necessary for fertilisation and embryogenesis (Reynier *et al.* 2001; Santos *et al.* 2006; Zeng *et al.* 2007). From the macaque and human studies, BGP-15's effects remained inconclusive due to low experimental replicates and patient samples.

Based on the findings in this chapter, although the results are promising, there are limitations in the current study. For instance, in the cattle studies, the use of abattoir material prevents the ability to identify whether oocytes were from obese or lean females, so variation would be expected in the ability of BGP-15 to modulate oocyte/embryo characteristics. Furthermore, differences in mtDNA replication across the different species may be due to difference in systemic lipid profiles and/or oocyte lipid content. Differences in lipid content between mouse, human, pig, sheep and cow have been previously reported (Dunning *et al.* 2014). One additional confounding factor in the data presented in the current chapter is the use of different stimulation regimens in different species. For instance, gonadotropin stimulation is known to result in decreased oocyte mtDNA content in the cattle (Cree *et al.* 2015). Moreover, unlike mice, humans, cows and macaques are not litter-bearing, so the mechanisms that regulate the generation of multiple follicles/oocytes in mice may also affect mtDNA copy number; with mice presumably having a higher number of oocytes with the 'required' mtDNA copy number, rather than one or two dominant follicles/oocytes in the other species. It is also important to note that a different strain of mouse was used in this chapter compared to previous chapters which may also account for minor differences observed. Regardless, we have used the C57BL/6, the Blobby mouse (which is a C57BL/6J strain with an ENU-induced mutation) and the CBAF1 (C57 x CBA cross) (Wu *et al.* 2015).

In conclusion, the present study shows that there was an accumulation of mtDNA levels during oocyte maturation from GV to MII oocytes in the mice. There was also relatively abundant levels of mtDNA during embryo development in the mice, bovine and macaque. mtDNA replication/biogenesis is very sensitive to environmental stressors such as high lipids. This is demonstrated by my results in both mouse and bovine showing supplementation of IVM medium with high lipids suppressed mtDNA levels and this was partially prevented by BGP-15. Thus, supplementation of maturation medium with BGP-15 may provide a means to increase mtDNA in both the oocytes and embryos, and thereby improve oocyte viability and developmental competence.

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CHAPTER 5

IMPAIRED EMBRYO DEVELOPMENT DUE TO OXIDATIVE STRESS IS NORMALISED BY BGP-15

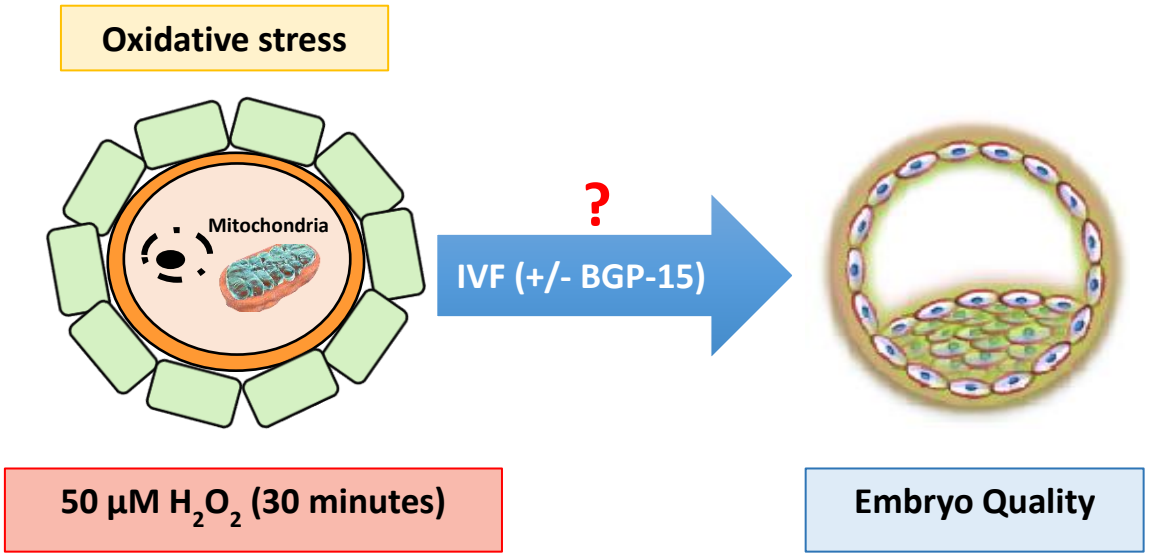
Overview

In the previous chapter, I showed that presence of BGP-15 in *in vitro* maturation (IVM) culture media restored mitochondrial DNA (mtDNA) levels in embryos that were conceived from lipid-stressed oocytes. In this chapter, I sought to further understand BGP-15 mechanism of action, particularly its beneficial effects on oocytes affected by high lipid levels or obesity by testing its efficacy in a general model of cellular stress, namely oxidative stress.

Approximately 90% of cellular reactive oxygen species (ROS), a natural by-product of cellular respiration, are produced in the mitochondria (Balaban *et al.* 2005). Under homeostatic stress-free conditions, ROS are required for many physiological processes and in intracellular signal transduction regulation (Mittler *et al.* 2011). However, deficient management of ROS results in oxidative stress and prolonged oxidative stress is linked to pathological ailments such as cancer, arthritis, and neurological diseases (Hayes and McLellan 1999). Most of these diseases involve ROS and mitochondrial dysfunction, which can both be linked to glutathione (GSH) homeostasis imbalance (Schulz *et al.* 2000). Oxidative stress also has broad effects on the reproductive system and has been reported to impair function of ovarian steroidogenesis and ovulation (Agarwal *et al.* 2005), embryogenesis (Bedaiwy *et al.* 2004), and implantation and maintenance of pregnancy (Myatt and Cui 2004). In particular, oxidative stress plays a crucial role during ovulation by inducing apoptosis of luteal cells within the ovary, which perturbs the area inside and around ova and granulosa cells (Schallreuter *et al.* 1999) which could potentially decrease oocyte and embryo viability (Van Blerkom *et al.* 1997; Yang *et al.* 1998). Moreover, it was also previously reported that repeated exposure of oxidative stress in the ova and other cells during ovulation might trigger ovarian aging (Miyamoto *et al.* 2010).

Several biomarkers of oxidative stress are increased in the blood of obese, non-pregnant women (Iyer *et al.* 2010), and oxidative stress has been implicated in obese pregnancies (Jarvie *et al.* 2010). In fact, women with obesity have high circulating levels of catalase and glutathione peroxidase enzyme activities, indicating systemic oxidative stress (Bausenwein *et al.* 2010). Maternal diet-induced obesity has contributed to oxidative stress in the oocytes and zygotes (Igosheva *et al.* 2010) and also in other tissues such as the liver and adipose

tissues (Matsuzawa-Nagata *et al.* 2008). Thus, I hypothesise (see schematic below) that oxidative stress during oocyte maturation impairs oocyte mitochondria and subsequent embryogenesis similar to obesity but that these effects are reversible with BGP-15 treatment.



To test this hypothesis, I utilised a brief treatment with hydrogen peroxide (H_2O_2) to induce oxidative stress in oocytes and then determined whether the changes in ROS, mitochondrial activity, mtDNA and embryo quality are reversible by supplementing the *in vitro* fertilisation (IVF) media with BGP-15.

5.1 Introduction

A hallmark of mitochondrial dysfunction is oxidative stress. Oxidative stress is considered an enhanced state of oxidants or a lack of antioxidants in cells, a situation in which the concentration of reactive oxygen species (ROS) increases above its biological values (Gonzalez-Flecha *et al.* 1993; Sikka 2001). Some amount of ROS is needed in the ovarian follicle (Attaran *et al.* 2000) as well as for normal sperm-oocyte interaction and sperm capacitation (de Lamirande *et al.* 1997). However, raised levels of ROS have a deleterious effect on cell membranes, cellular DNA, and mitochondria and ultimately accelerate cell death by apoptosis or necrosis (Alvarez 2003). Mitochondria are a major source of ROS and ROS production can be triggered by dysfunctional mitochondrial oxidative phosphorylation; as well as increases or decreases in ROS-related enzymes (Kamogashira *et al.* 2015). Oxidative stress can also result from impaired cellular antioxidant defense mechanisms (Rahal *et al.* 2014). Mitochondria are themselves also sensitive targets for the damaging effects of oxygen radicals. In fact, mitochondrial DNA (mtDNA) is highly susceptible to damage because it is not protected by histones and is directly exposed to ROS generated by the respiratory chain (Alexeyev 2009).

In multiple contexts of physiological stress, particularly aging and obesity, oxidative stress is a common feature in the gametes as in other cells. In mice it has been shown that oocytes from females with diet-induced obesity exhibit oxidative stress, with increased ROS levels (Igosheva *et al.* 2010; Zhang *et al.* 2015; Hou *et al.* 2016). Furthermore, similar to obese females, high-fat diet-induced obesity in males resulted in increased oxidative stress in sperm (Bakos *et al.* 2011; Palmer *et al.* 2011; Fullston *et al.* 2012). Moreover, the role of oxidative stress in maternal aging has also been suggested (Tarin *et al.* 1996; Takahashi *et al.* 2003; Bartmann *et al.* 2004; Thouas *et al.* 2005; Lord *et al.* 2013). Aged oocytes generated either *in vitro* or *in vivo* have been reported to have increased levels of ROS, an indicative of oxidative stress, as determined by fluorescence staining (Mitchell *et al.* 2009; Yamada-Fukunaga *et al.* 2013).

In human and animal oocytes, oxidative stress has been correlated with abnormal chromosome segregation and reduction of pole-to-pole distance of the metaphase II (MII) spindle and spindle alignment during meiotic division, and with increased fragmentation and

aberrant morphology in embryos originating from aging oocytes (Tarin 1996; Hall *et al.* 2007; Miao *et al.* 2009; Selesniemi *et al.* 2011). Oocytes exposed to high ROS exhibit subsequent defective embryo development, apoptosis and embryonic arrest (Goto *et al.* 1993; Yang *et al.* 1998; Hashimoto *et al.* 2000; Guerin *et al.* 2001). For instance, bovine oocytes that were exposed to high oxygen tension had increased ROS contents and resulted in poor developmental competence (Hashimoto *et al.* 2000). In addition, a direct relationship was also observed between increased ROS concentration and apoptosis in human fragmented embryos, suggesting that ROS may induce apoptosis in embryos (Yang *et al.* 1998). Apart from that, it has been noted that bovine embryos cultured in 20% oxygen conditions sustain a 10-fold increase in intracellular ROS levels and a 2-fold increase in the frequency of permanent embryo arrest at the 2-to 4-cell stage, compared with embryos cultured in 5% oxygen tensions (Favetta *et al.* 2007).

Oxidative stress is detrimental to cells because ROS and their metabolites can damage DNA, lipids, and proteins; alter enzymatic systems; produce irreparable alterations; and cause cell death (Agarwal *et al.* 2014). For instance, a classic inducer of oxidative stress is hydrogen peroxide (H_2O_2) which has been well-characterised in a number of *in vitro* systems and documented to affect cell membrane lipids, affect DNA content, accelerate apoptosis, and cause mitochondrial dysfunction (Tatsumi and Kako 1993; Ballinger *et al.* 2000; Li *et al.* 2003; Zhu *et al.* 2005). There are several reports detailing the detrimental effects of H_2O_2 treatment on oocytes and embryos. H_2O_2 treatment of MII oocytes *in vitro* has been shown to cause a decline in levels of the anti-apoptotic molecule Bcl-2 (Takahashi *et al.* 2009), induce expression of pro-apoptotic Bax and caspase-3, and precipitate both DNA fragmentation (Chaube *et al.* 2005) and cytochrome *c* release (Liu *et al.* 2000). Moreover, exposure to H_2O_2 also attenuates spindle abnormalities and chromosome misalignment in oocytes (Choi *et al.* 2007; Shaeib *et al.* 2013) and increases cumulus cell death (Shaeib *et al.* 2016). However, the exact mechanisms by which increased ROS in oocytes impacts subsequent embryo development and health is not entirely clear.

Cumulatively, studies examining the phenotypes of oocytes from obese females and oocytes subjected to oxidative stress demonstrate that they have a lot of similarities in terms of cellular abnormality; specifically in terms of their mitochondrial dysfunction which can be

indicative of high levels of ROS (Zhang *et al.* 2006a; Igosheva *et al.* 2010; Lord and Aitken 2013; Zhang *et al.* 2015). Mitochondria are also vulnerable targets of ROS. Free oxygen radicals can damage the mtDNA of quiescent oocytes and lead to the loss of their intrinsic mitochondrial function (Brenner *et al.* 1998; Barritt *et al.* 1999; Hsieh *et al.* 2004), such as accumulation of mtDNA mutations, decreased mtDNA copy number and mRNA expression of mitochondrial genes. Mitochondrial damage induced in oocytes by oxidative stress would have detrimental consequences for oocyte viability and developmental competence because mitochondrial function plays a major role in fertility. In particular the activity of mitochondria in both oocytes and preimplantation embryos is correlated with embryo development (Van Blerkom *et al.* 1995; Wilding *et al.* 2001). For instance, lower mitochondrial membrane potential (MMP) in disordered mitochondria leads to abnormalities of oocyte meiotic apparatus, resulting in chaotic mosaicism (where chromosomes segregate randomly) of embryos (Wilding *et al.* 2003). In addition, the interactions of mtDNA, proteins or lipids with ROS cause a decline of glutathione (GSH)/glutathione disulphide ratio (Tarin 1996), which may affect the stability of oocyte spindles. Furthermore, oxidative phosphorylation within mitochondria provides a major source of adenosine triphosphate (ATP) needed for mature MII oocytes that consume high levels of ATP (Dumollard *et al.* 2004). Mitochondrial over-production of ROS, however, does not generate ATP but rather consumes it, resulting in a decrease in ATP levels within oocytes and further exacerbating mitochondrial dysfunction.

Importantly, we (Wu *et al.* 2015) have found that BGP-15, a nicotinic acid derivative is able to normalise the mitochondrial defects that occur in oocytes from obese mice. Others (Chung *et al.* 2008; Literati-Nagy *et al.* 2009; Literati-Nagy *et al.* 2010; Henstridge *et al.* 2014a; Henstridge *et al.* 2014d) have also been conducting experiments with BGP-15 and reporting that it is able to restore mitochondrial functions in stressed cells. More specifically, we found that treatment of obese mice with BGP-15 improved oocyte developmental competence by restoring mitochondrial activity and inducing mtDNA in oocytes; an effect that led to restored mtDNA levels in the blastocysts and fetal offspring tissues (Wu *et al.* 2015). Further, in the previous chapter, I showed that the presence of BGP-15 in mouse and cattle *in vitro* maturation (IVM) media was able to restore or maintain the mtDNA copy number in oocytes exposed to high lipid conditions. Others have also found that BGP-15 has beneficial effects

on mitochondria (Szabados *et al.* 2000; Halmosi *et al.* 2001; Sarszegi *et al.* 2012). It improves insulin sensitivity and inflammation in a genetic mouse model of insulin resistance (Chung *et al.* 2008), likely via increasing mitochondrial volume, and improves metabolic homeostasis in a rat model of type II diabetes (Henstridge *et al.* 2014a). Thus numerous reports indicate that BGP-15 is likely to confer protection against disturbed metabolic homeostasis via multiple modes of action including, but not limited to improving cellular function via its action on mitochondria.

Although the mechanisms of BGP-15 action are not entirely understood and are likely to be cell-type specific, BGP-15 may be acting via reducing oxidative stress (Szabados *et al.* 2000; Halmosi *et al.* 2001). It was previously shown to have antioxidant properties in the kidney during cisplatin-induced nephrotoxicity (Racz *et al.* 2002). Previous reports have also attributed the beneficial effects of BGP-15 (nicotinic amidoxime derivatives) in pathological conditions with severe oxidative stress to their poly(ADP-ribose) polymerase (PARP)-inhibitory and heat shock protein 72 (Hsp72) chaperone inducing effects (Vigh *et al.* 1997; Halmosi *et al.* 2001; Ray *et al.* 2001; Chung *et al.* 2008; Song *et al.* 2008).

Thus I sought to determine the efficacy of BGP-15 in reversing oxidative stress in oocytes, and thereby further understand its mechanisms of action, particularly its beneficial effects in oocytes affected by high lipid conditions or obesity. Therefore, the experiments both investigated the direct effect of oxidative stress and increased oocyte intracellular ROS on oocyte developmental competence and differentiation; and examined the ability of BGP-15 to alleviate any cellular defects, particularly mitochondrial defects.

To mimic oxidative stress conditions, *in vivo* matured (ovulated) cumulus-oocyte complexes (COCs) were incubated with hydrogen peroxide (50 μM, H_2O_2) for 30 min. The COCs were then fertilised *in vitro* using standard *in vitro* fertilisation (IVF) culture medium or medium containing BGP-15; to determine its ability to alleviate any cellular defects caused by oxidative stress. The results demonstrated the efficacy of BGP-15 in reversing oxidative stress-induced alterations in oocytes, and thereby allowed us to further understand its mechanisms of action in oocytes, particularly its beneficial effects in oocytes affected by high lipid conditions or obesity.

5.2 Materials and Methods

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

5.2.1 Animals

All experimental procedures were approved by the University of Adelaide Animal Ethics Committee and were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. C57BL/6 mice were obtained from the University of Adelaide Laboratory Animal Services and housed in the Animal Facility under a 14:10 hour light:dark cycle with *ad libitum* access to food and water**.**

Female mice (6 weeks of age) were hormonally induced to ovulate by intraperitoneal (i.p.) injection of 5 IU (international units) per 12 g body weight of pregnant mare's serum gonadotrophin (PMSG; National Hormone and Peptide Program, Torrance, CA, USA) followed 48 h later by 5 IU per 12 g body weight human chorionic gonadotrophin (hCG; Merck, Sharp and Dohme), each in 0.1 mL 0.9 % saline. Mice were humanely killed by cervical dislocation 13 h to 16 h post-hCG and COCs obtained from oviducts and collected in HEPES-buffered ∝-minimum essential medium (MEM) handling media (Life Technologies, Invitrogen, Carlsbad, CA, USA) supplemented with 1% fetal calf serum (FCS) (Life Technologies, Invitrogen, Carlsbad, CA, USA) and pre-warmed to 37°C prior to use. Male mice (8 weeks of age) of proven fertility were humanely killed by cervical dislocation and vas deferens/epididymis dissected for isolation of sperm for IVF.

5.2.2 Short-term Hydrogen Peroxide (H_2O_2) Exposure

For all experiments, immediately before fertilisation, COCs were incubated in 500 µL of 50 µM of H_2O_2 for 30 min. Meanwhile, for the control group, ovulated COCs were kept in α-MEM handling media without H_2O_2. COCs were then washed in α-MEM handling media and denuded with hyaluronidase by the addition of 10 µL of pre-warmed 1000 IU/mL hyaluronidase to the handling media, for live cell staining (JC-1 staining, detection of reactive oxygen species and autophagy assay). For IVF, COCs were first washed in α-MEM

handling media and then transferred to Cook wash media (William A. Cook Australia Pty. Ltd., Queensland, Australia) before fertilisation with sperm.

5.2.3 *In vitro* Fertilisation (IVF) and Embryo Culture

Fertilisation, wash and embryo culture media were Research Vitro Fertilisation, Research Vitro Wash and Research Vitro Cleave respectively from Cook Medical (William A. Cook Australia Pty. Ltd., Queensland, Australia). Stock concentration (1 mM) of BGP-15 (kindly provided by N-Gene Research Laboratories) was prepared in MilliQ water prior to use. Ovulated COCs cultured with or without H_2O_2 were used for (IVF). Epididymides and vasa deferentia from male mice were collected into 1 mL of warm (37°C) fertilisation medium. Sperm were extracted into the medium and allowed to capacitate at 37°C in an atmosphere of 6% CO_2, 20% O_2, nitrogen balance for 1 h. Ten μL of capacitated sperm (35,000 sperm/mL) was added to 90 μL of fertilisation drop in the presence or absence of BGP-15 (10 μM), which contained the washed COCs (15-20 COCs per drop), followed by co-incubation 4 h at 37°C in an atmosphere of 6% CO_2, 20% O_2, nitrogen balance. BGP-15 treatment was applied on Day 1 of IVF in the fertilisation medium through to Day 5 (cleave medium also contained 10 μM of BGP-15).

On Day 2, fertilisation was assessed and 2-cell embryos were transferred into a fresh 20 μL drop of Cook cleave medium (10-15 embryos per drop) and cultured at 37°C in an atmosphere of 5% CO_2, 20% O_2 and 95% air. . Embryo morphology was classified as appropriately developed ('on-time') using the following criteria; on Day 2, embryos at the 2-cell stage and on Day 5, blastocysts or hatching blastocysts. Development rate was assessed on Day 2 as the percentage of embryos meeting the on-time development criteria from starting number of oocytes; while development on Day 5 as the percentage of embryos meeting the on-time development criteria from 2-cell embryos.

In a separate series of experiments, time-lapse imaging was employed to more closely analyse developmental kinetics and cell size of oocytes. A Control + BGP-15 group was not included in the time-lapse experiments as no effect of BGP-15 was observed when compared to the untreated control group in the initial experiments. Oocytes were fertilised as

previously described and presumptive zygotes placed in 16-well dishes (Primovision, Vitrolife, Sweden), in culture medium and conditions as described above, and monitored by Primo Vision time-lapse embryo monitoring system (Primovision, Thermo Scientific HERAcell VIOS 160i CO_2 incubator) with images of individual embryos generated every 15 min throughout culture. The timing of morphokinetics events, including first, second and third cleavage division and cavitation were recorded and intervals between each event determined and analysed using the Primo Vision Analyser Software (version 4.4.1.01.010).

5.2.4 Detection of Intracellular Reactive Oxygen Species (ROS)

Stock concentration of CM-H_2DCFDA (1 mM) was prepared in dimethyl sulfoxide (DMSO) prior to staining. Denuded oocytes or embryos (10-15 oocytes/embryos in 500 µL drop) were incubated with 10 µM CM-H_2DCFDA for 30 min at 37°C in the dark. Oocytes or embryos were washed once with α-MEM handling medium and images were captured using the Olympus Fluoview FV10i confocal microscope using the green filter (excitation = 488 nm, emission = 519 nm). A single optical scan was acquired through the centre of the oocyte. Images were captured at 10x magnification and laser, sensitivity, and imaging parameters were kept constant between replicates and across experiments. Intensity of fluorescence was determined using Image J version 1.47r software by placing a circle across the oocyte image and measuring pixel intensity. The mean, area and integrated density (the sum of the pixel values in the circle) were reported and the corrected total cell fluorescence was calculated using the formula stated below. The data were then represented graphically as intensity of fluorescence over pixel widths. Three experimental replicates were performed, with 15 oocytes per treatment group.

Total fluorescence = Integrated Density- (Area of selected cell × Mean fluorescence of background readings)

5.2.5 Autophagy Assay

Autophagic vacuoles in live denuded oocytes were visualised using the Cyto-ID Autophagy Detection Kit (Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instructions. Briefly, oocytes were washed twice in 1x assay buffer followed by incubation

with dual-detection solution at 37°C in the dark for 30 min. Then oocytes were washed once with 1x assay buffer and imaged immediately in the green filter (excitation = 488 nm, emission = 519 nm) using the Olympus Fluoview FV10i confocal microscope. A single optical scan was acquired through the centre of the oocyte. Images were captured at 40x magnification and laser, sensitivity, and imaging parameters were kept constant between replicates. Intensity of fluorescence was determined as described above for ROS staining. Two experimental replicates were performed, with 10 oocytes per treatment group.

5.2.6 Mitochondrial Membrane Potential (MMP) Assay

MMP was examined using the mitochondrial probe JC-1 (5,5', 6,6'-tetrachloro-1,1', 3,3', tetraethylbenzimida-zolylcarbocyanine iodide (Invitrogen, CA, USA). Denuded oocytes or embryos were incubated with 1.5 mM JC-1 in handling media (oocytes)/Cook Vitro Wash (embryos) for 15 min at 37°C in the dark. Oocytes or embryos were washed once in 1x phosphate buffered saline (PBS) with 1 mg/mL polyvinylpyrrolidone (PBS/PVP) and then imaged immediately using the Olympus Fluoview FV10i confocal microscope. Images were captured at 40x magnification and laser, sensitivity, and imaging parameters were kept constant between replicates. Intensity of fluorescence was determined as described above for ROS staining.

5.2.7 Tetramethyl rhodamine methyl ester (TMRM) Staining

Mitochondrial staining was performed using a modified method as previously described (Igosheva *et al.* 2010). To determine the distribution of active mitochondria in the oocytes were incubated with TMRM (Molecular Probes, Eugene, OR, USA). Mitochondrial distribution pattern is categorised as in (Ou *et al.* 2012b; Hou *et al.* 2016). TMRM is a fluorescent lipophilic cation sequestered by active mitochondria commonly used as a fluorescence indicator of MMP (Zhang *et al.* 2006b; Esteves *et al.* 2012). A stock concentration of 12.5 µM was prepared in DMSO and stored in the dark at -20°C. Working concentration (25 nM) was obtained by diluting stock in pre-warmed α-MEM handling media. Ovulated oocytes from hyaluronidase-treated COCs were incubated at 37˚C for 30 min in the dark. Oocytes were then washed once in PBS/PVP and mounted under a coverslip in 5 µL PBS/PVP and then imaged using the Olympus Fluoview FV10i confocal microscope.

Images were captured at 40x magnification and laser, sensitivity, and imaging parameters were kept constant between replicates. Intensity of fluorescence was determined as described above for ROS staining.

5.2.8 Mitochondrial DNA (mtDNA) Copy Number Quantification

The mtDNA copy number in individual oocytes or embryos was quantified as previously described (Kameyama *et al.* 2010; Wu *et al.* 2015). Briefly, denuded oocytes or embryos were washed with PBS/PVP (1 mg/mL of PVP in PBS), collected individually into 1.5 mL siliconised low retention microcentrifuge tubes (Fisher Scientific) with 5 μL of PBS/PVP and stored at -80°C. Genomic DNA was isolated using the QIAamp DNA micro kit (Qiagen) according to manufacturer's protocol with carrier RNA (1 μg; Qiagen) added to each sample. Genomic DNA was eluted with 50 μL of water and diluted 10 times for quantitative PCR.

To prepare the quantification standards, a 1186 bp fragment of the 12S ribosomal (r)RNA region of mtDNA was amplified from mouse liver by PCR using the primer pair 5′-ACACCTTGCCTAGCCA-3′ and 5′-TTTGCCACATAGACGAGTT-3′ with the LongRange PCR kit (Qiagen), and then purified by using the QIAquick PCR purification kit (Qiagen) and cloned using the Qiagen PCR cloning kit (Qiagen). Plasmid DNA was purified from bacteria using Plasmid Maxi kit (Qiagen), and the concentration was determined by using a Nanodrop ND1000 Spectrophotometer (Biolab). Plasmid copy number was calculated as: mass of plasmid (g) = plasmid size (bp)×(1.096×10^{-21} g/bp); mass of plasmid required to generate 1×10^7 copy number standard stock = 1×10^7×mass of single plasmid. A standard curve was generated by using seven ten-fold serial dilutions ($10^{-1}\times10^7$ copies), and standard curve correlation coefficients were consistently greater than 0.98. Real-time quantitative PCR using the primer pair 5′-CGTTAGGTCAAGGTGTAGCC-3′ and 5′-CCAGACACACTTTCCAGTATG-3′ was performed in triplicate using SYBR Green PCR master mix (Applied Biosystems) and a Rotor-Gene 6000. Primer sequences for the quantification of mtDNA were derived from (Kameyama *et al.* 2007). Standard curves were created for each run and sample copy number was generated from the equation of Ct value against copy number for the corresponding standard curve.

The PCR program employed an initial step of 95°C for 10 min followed by 40 cycles, denaturation at 95°C for 10s, annealing at 60°C for 30s and extension at 72°C for 20s. Every reaction was followed by melting curve analysis to ensure the specificity of the amplification. All reactions were performed in triplicate with total reaction volumes of 20 μL. Premix for quantitative PCR was prepared from Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA). The premix consisted of 10 μL of Power SYBR Green, 6 μL of PCR grade water, 2 μL of 50 μM primer pair, with 2 μL of DNA template added to 18 μL of premix for PCR reaction.

5.2.9 Inner Cell Mass (ICM) and Trophectoderm (TE) Assessments

Allocation of cells to TE and ICM lineages was assessed in blastocyst-stage embryos using a modified method of Handyside and Hunter (1984). Briefly, blastocysts were incubated with 0.5% pronase (Sigma-Aldrich, St. Louis, MO, USA) at 37°C to remove the zona pellucida, followed by 10 min incubation at 4°C in 10 mM TNBS (2,4,6-trinibrobenzene sulfonic acid) in the dark. Blastocysts were then washed and incubated with 0.1 mg/mL anti-DNP for 10 min at 37°C. Following, blastocysts were washed again and incubated for 5-10 min in 10 μg/mL of propidium iodide (PI) in 10% guinea pig serum. Blastocysts were then transferred to 6 μg/mL bisbenzimide in ethanol overnight and washed in 100% ethanol the following day. Blastocysts were then mounted on microscopic slides in glycerol underneath a cover slip and visualised using an epifluorescence microscope (Nikon, TE 2000-E) at 200× equipped with an ultraviolet filter (excitation, 340–380 nm; emission, 440–480 nm). ICM nuclei labeled with bisbenzimide appear blue whereas TE nuclei labeled with a combination of bisbenzimide and PI appear pink or red. Total and TE cell numbers were counted individually and ICM cell numbers were calculated by subtracting TE from the total cell number.

5.2.10 Oct4/Cdx2 Staining of Blastocysts

Expanded blastocysts were fixed in 4% paraformaldehyde (in PBS/PVP) overnight at 4°C and then kept in PBS/PVP until needed. After fixation, blastocysts were incubated for 5 min in 0.1 M glycine (in PBS/PVP) at room temperature and permeabilised in 0.5% triton X-100 (in PBS/PVP) for 30 min. Then blastocysts were incubated in blocking solution (10%

donkey serum in PBS/PVP) for 1 h, followed by incubation in primary antibodies (Oct3/4 goat polyclonal IgG, Santa Cruz Biotechnology Sc-8628; Cdx2 rabbit monoclonal, Abcam ab76541) at a concentration of 1:500 in PBS/PVP overnight at 4°C. On the following day, blastocysts were transferred through several washes of PBS/PVP and placed in secondary antibodies diluted in PBS/PVP at 1:1000 concentration (Donkey anti-goat Alexa Fluor 488, Invitrogen A11055; Donkey anti-rabbit Alexa Fluor 594, Abcam ab150076) for 1 h at room temperature. Hoechst 33342 in 1:250 concentration (Life Technologies H1399) was added during the last 10 min of incubation. Blastocysts were transferred onto glass slides with a small amount (~5 µL) of glycerol under a coverslip for imaging. ICM nucleic labeled with Oct3/4 appear green, whereas TE nucleic labeled with Cdx2 appear red. Similar to differential staining, total and TE cell numbers were counted individually and ICM cell numbers were calculated by subtracting TE from the total cell number.

5.2.11 Statistical Analyses

All data were tested for normality of distribution prior to analyses. All measures are reported as mean ± SEM. Statistical significance was determined as indicated, by using Student's *t*-test or one-way ANOVA with Tukey's post hoc tests, as appropriate, using GraphPad Prism v008 (GraphPad Software, La Jolla, CA, USA) for Windows. Time-lapse data were analysed by using repeated measures with linear mixed-effects model in SPSS (IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.). Normality of data was confirmed by analysing the spread of residuals. A *P*-value of less than 0.05 was considered statistically significant.

5.3 Results

5.3.1 H_2O_2 increases intracellular ROS in the oocytes

To verify the effect of H_2O_2 on oocytes, ROS level was measured using 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA). Oocytes of COCs exposed to ROS clearly exhibited increased ROS levels compared to untreated oocytes (Figure 1A). Quantification verified that there was a significant increase in intracellular ROS in the H_2O_2-treated oocytes ($P \leq 0.01$; Figure 1B).

Active autophagy in live oocytes was measured using the Cyto-ID Autophagy Detection Kit. Confocal imaging revealed that both the control and H_2O_2-treated oocytes showed multiple green puncta throughout the oocyte cytoplasm (Figure 1C). Quantification showed no significant difference in fluorescence levels between the two groups (Figure 1D).

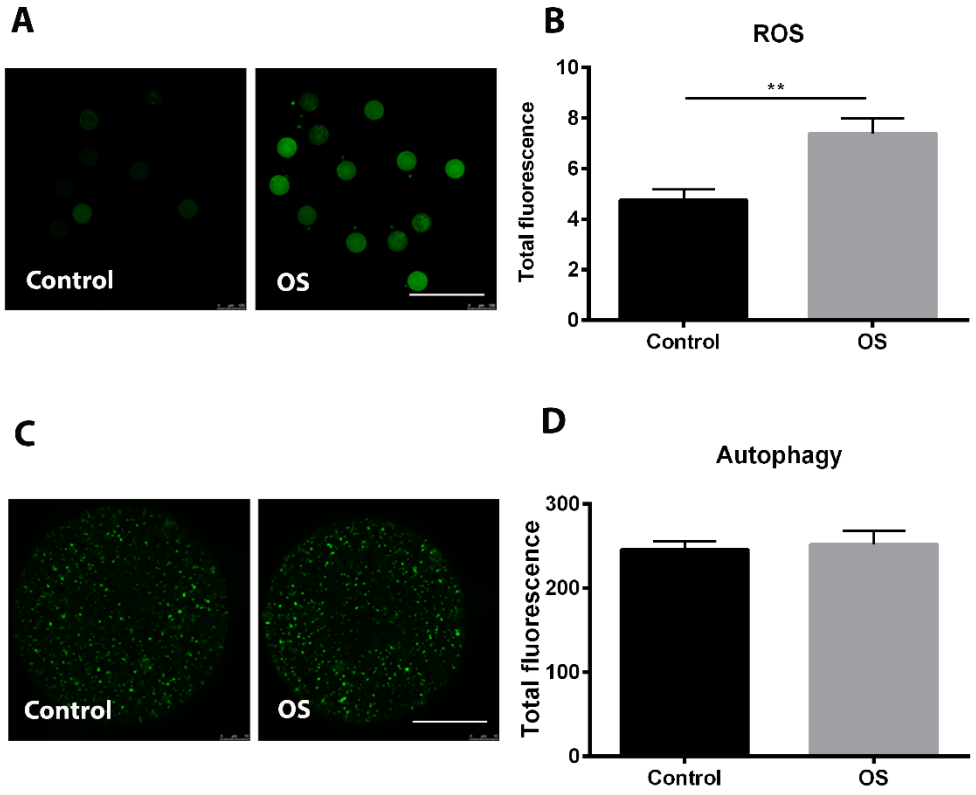


Figure 1 Effect of short exposure of mouse oocytes to hydrogen peroxide (H_2O_2) on intracellular reactive oxygen species (ROS) and autophagy levels in the oocytes

(A) Representative photos of oocytes stained with CM-H_2DCFDA for detection of ROS. (B) Fluorescence intensity analysis of CM-H_2DCFDA-stained oocytes. Data are presented as mean ± SEM, n = 25-40 oocytes from four mice per group. (C) Live oocytes were assessed for autophagic vacuoles, visualised as green fluorescence. (D) Autophagy levels were quantified as the sum of total green fluorescence within each oocyte. Data are presented as mean ± SEM, n = 10-17 oocytes from three mice per group. ** $P \leq 0.01$, unpaired *t*-test. OS-oxidative stress. Scale bar = 50 µm.

5.3.2 H_2O_2 alters oocyte mitochondrial activity and distribution

The effects of oxidative stress (OS) imposed by H_2O_2 exposure resulting in excessive intracellular ROS levels on oocyte mitochondria were examined next. Firstly it was determined whether the MMP was altered. The oocytes exhibited red punctate fluorescence around the pericortical region, indicating a high membrane potential, whereas green fluorescence, an indication of low membrane potential, was confined to the cytoplasm of the oocytes (Figure 2A). In oocytes treated with H_2O_2, the red punctate fluorescence was visibly higher compared to that of oocytes from the control group. Analysis of the red to green fluorescence ratio, an index of mitochondrial activity, showed that the H_2O_2-treated oocytes have significantly increased mitochondrial activity compared to the control oocytes ($P \leq 0.001$; Figure 2B). The mtDNA copy number was also assessed in individual oocytes with no difference between the control and H_2O_2-treated oocytes was observed (Figure 2C).

To further examine the distribution of active mitochondria, oocytes were stained with TMRM. Quantitative analysis of TMRM fluorescence intensity revealed that the H_2O_2-treated oocytes exhibited significantly higher signal, an indicator of higher MMP ($P \leq 0.05$; Figure 2E).

The distribution of mitochondria in the oocytes was also examined. Based on a previous report (Ou *et al.* 2012a), MII oocytes were categorised into three groups based on their mitochondrial distributions: i) Homogeneous distribution (normal); ii) Surrounding chromosome (normal); and iii) clustered in the cytoplasm (abnormal) (Figure 2D). The majority of the control oocytes (77%) displayed homogeneous mitochondrial distribution pattern, while 13% showed clump mitochondrial distribution (Figure 5.2F). However, in the H_2O_2 treated group, the homogeneous mitochondrial distribution was less prevalent (29%) which was a significant reduction compared to the control group (77% versus 29%, $P < 0.05$) (Figure 2F). Interestingly, the distribution of heterogeneous mitochondria in large clumps was increased in the H_2O_2-treated oocytes compared with the control oocytes (53% versus 13% in controls) (Figure 2F).

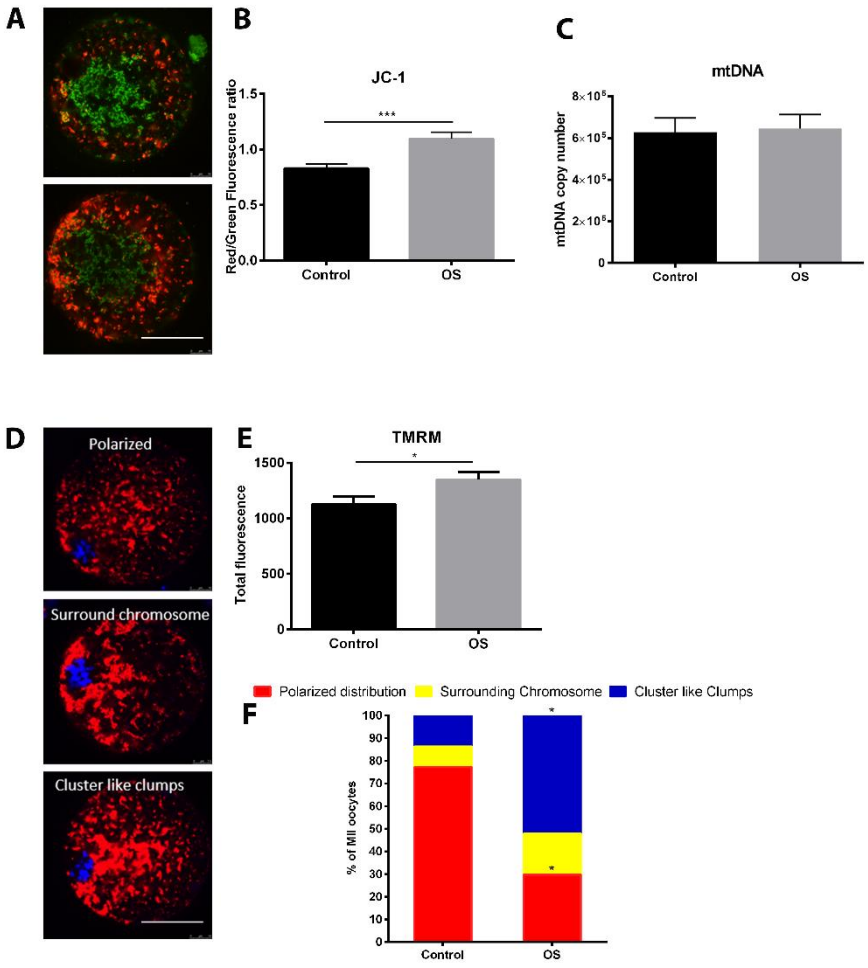


Figure 2 Mitochondrial activity and distribution in hydrogen peroxide (H_2O_2)-treated mouse oocytes.

(A) Representative images of live mouse oocytes stained with JC-1, where red fluorescence indicates high mitochondrial membrane potential (MMP), and green indicates low MMP. (B) Ratio of red to green fluorescence, an indicator of mitochondrial activity. Data are presented as mean ± SEM, n = 17-19 oocytes from three mice per group. (C) mtDNA copy number in individual oocytes collected from the indicated group. Data are presented as mean ± SEM, n = 15 oocytes from three mice per group. (D) Representative images of live mouse oocytes stained with TMRM, where red fluorescence indicates the distribution of active mitochondria. (E) Quantitative analysis of TMRM fluorescence intensity. Data are presented as mean ± SEM, n = 23 oocytes from four mice per group. (F) Proportion of MII oocytes with each distribution pattern. * $P \leq 0.05$, *** $P \leq 0.001$, unpaired *t*-test. OS- oxidative stress, TMRM- tetramethyl rhodamine methyl ester. Scale bar = 50 µm.

5.3.3 BGP-15 restores developmental competence of oocytes exposed to oxidative stress

Ovulated COCs that had been exposed to H_2O_2 were fertilised *in vitro*, and oocyte developmental competence was compared to those of control untreated COCs. In addition a separate cohort of COCs exposed to H_2O_2 were subsequently treated with BGP-15 during IVF and embryo culture. Time-lapse imaging was carried out to closely monitor developmental kinetics of the ovulated COCs. Embryos cleaved at 14 h post-insemination and reached the 3-cell stage, 4-cell stage, morula and blastocyst at 40 h, 42 h, 68 h and 86 h respectively (Figure 3A). Interestingly, the timing of morphokinetic events revealed that the time to reach 4-cell was shorter in the OS+BGP-15 group when compared to the control ($P \leq 0.05$; Figure 3A). Similarly, the time duration for OS and OS+BGP-15 groups to reach blastocyst stage was shorter when compared to the controls ($P \leq 0.05$; Figure 3A). On Day 2 after fertilisation, the percentage of putative zygotes that cleaved was significantly lower in the H_2O_2-treated oocytes. When BGP-15 was added, no difference between the controls and OS group was observed (Figure 3B). Exposure of COCs to H_2O_2 did not impair subsequent blastocyst formation on Day 5. However, BGP-15 treatment improved blastocyst development, particularly in those where oocytes had been exposed to H_2O_2 (Figure 3C). The zygotes that were successfully cleaved on Day 2 were further analysed. The time to cleavage showed no significant difference between the groups. Interestingly, the transition time from 2-cell to 3-cell embryos was shorter in the H_2O_2 treated group compared to the control. However, embryos that were treated with BGP-15 took a shorter time to cleave from 3-cell to 4-cell compared to the H_2O_2 treated group. Furthermore, BGP-15 treated embryos also took less time to develop from morula to blastocyst when compared to both the control and H_2O_2-treated group (Figure 3D).

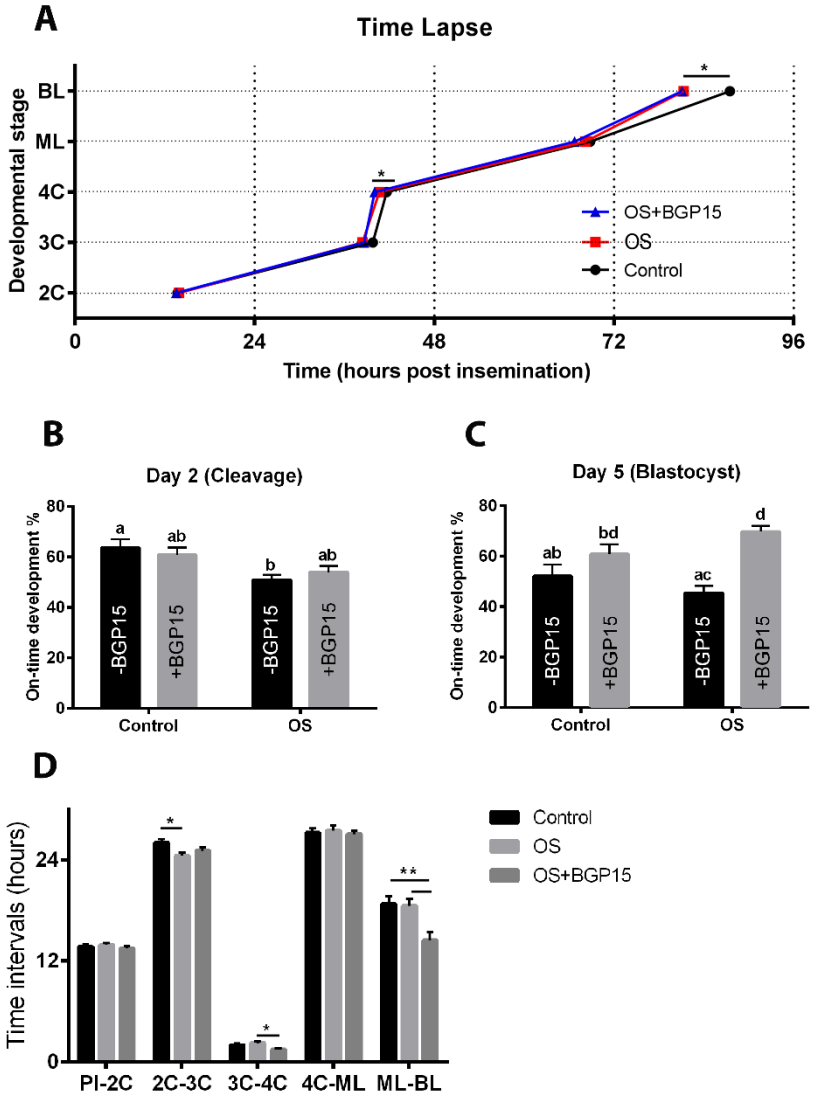


Figure 3 Developmental kinetics and developmental competence in oxidative stress (OS) mouse oocytes.

(A) Timing of developmental milestones in hours post-insemination (PI) in control, OS and OS+BGP-15 groups. Data are presented as mean ± SEM, n = 24-30 embryos from four mice per group. (B) Cleavage rate was assessed in Day 2 and (C) blastocyst rate on Day 5. Data are presented as mean ± SEM, n = 11 experimental replicates. (D) Time intervals between each developmental stage. Data are presented as mean ± SEM, n = 24-30 embryos from four mice per group. Groups with different superscripts differ significantly by one-way ANOVA with Tukey's post hoc test ($P \leq 0.01$). * $P \leq 0.05$, ** $P \leq 0.01$, one-way ANOVA with Tukey's post hoc test. 2C- 2-cell, 3C- 3-cell, 4C- 4-cell, ML- morula, BL- blastocyst.

5.3.4 Embryo mitochondrial activity and mtDNA biogenesis is impaired by oxidative stress but restored by BGP-15

To examine the health of the resulting embryos, they were examined for mitochondrial activity, mtDNA replication and ROS levels. MMP in the embryos was assessed by JC-1 staining. A significant reduction in the red:green fluorescence intensity of the JC-1 probe was observed in embryos derived from the OS group compared to control ($P \leq 0.05$; Figure 4A). This difference indicates a reduced mitochondrial activity in the embryos generated from the H_2O_2-treated COCs. However, this was not normalised with the BGP-15 treatment. Interestingly, BGP-15 supplementation caused a dramatic increase in the MMP in the control embryos ($P \leq 0.0001$; Figure 4A).

We next determined whether the intracellular ROS level in these embryos was altered by staining with the fluorescent probe CM-H_2DCFDA. There was no increase in intracellular ROS in the OS embryos compared to control embryos. BGP-15 supplementation in IVF media resulted in a decrease in embryo ROS level in only the H_2O_2 treated embryos, but not the control embryos ($P \leq 0.05$; Figure 4B).

As another measure of mitochondrial capacity, mtDNA copy number was quantitatively assessed in individual embryos. The mtDNA copy number was reduced in the blastocysts from COCs that were treated with H_2O_2 compared with control blastocyst ($P \leq 0.01$; Figure 4C). Blastocyst mtDNA copy number was normalised by BGP-15 supplementation in the IVF medium ($P \leq 0.05$; Figure 4C). In the control group, mtDNA levels were significantly up-regulated in the blastocyst-stage embryos. There was a 78% increase in mtDNA levels in the blastocysts when compared to the oocytes ($P \leq 0.01$; Figure 4D). This phenomenon did not occur in the H_2O_2 group (Figure 4D).

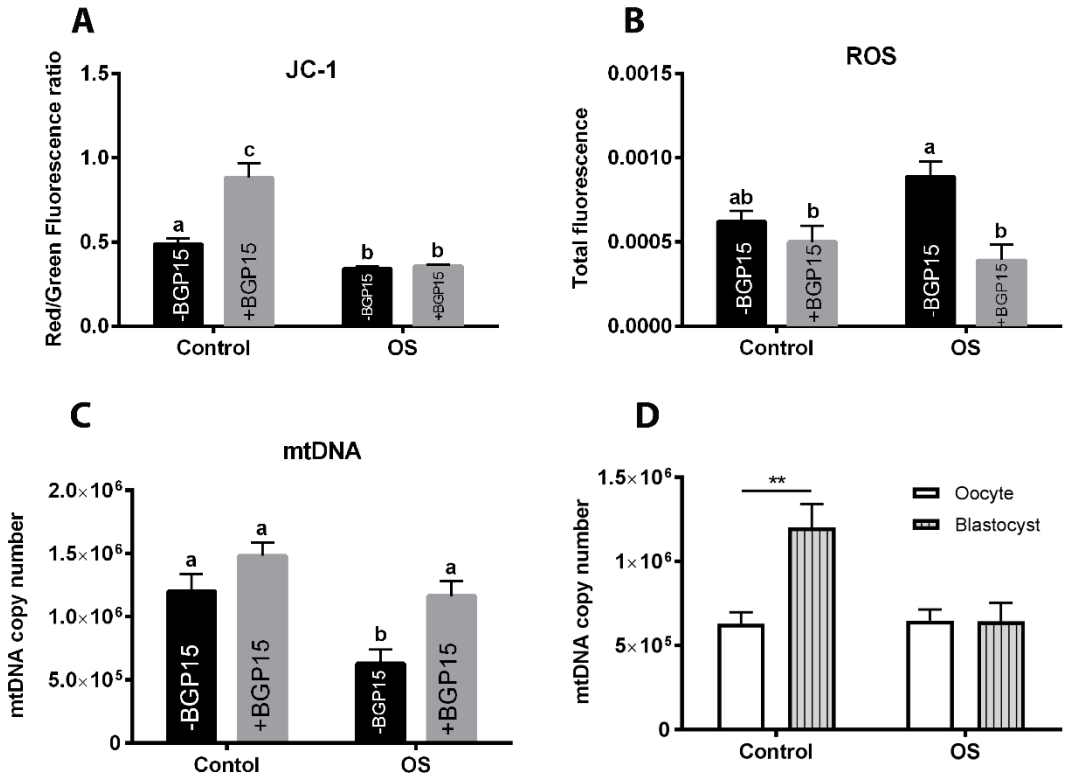


Figure 4 Effect of oxidative stress (OS) and BGP-15 on mitochondrial activity, reactive oxygen species (ROS) and mitochondrial DNA (mtDNA) levels in mouse embryos.

(A) Ratio of red to green fluorescence, an indicator of mitochondrial activity, in live embryos. Data are presented as mean ± SEM, n = 10-17 embryos from four mice per group. (B) Fluorescence intensity analysis of CM-H_2DCFDA-stained embryos. Data are presented as mean ± SEM, n = 11-28 embryos from four mice per group. (C) mtDNA copy number in individual embryos collected from the indicated groups. Data are presented as mean ± SEM, n = 17-28 embryos from four mice per group. Groups with different superscripts differ significantly by one-way ANOVA with Tukey's post hoc test ($P \leq 0.01$). (D) The mtDNA copy numbers in mouse oocytes and blastocysts, as determined by real-time PCR. Data are presented as mean ± SEM**,** n = 15-28 embryos. ** $P \leq 0.01$, unpaired *t*-test.

5.3.5 Embryo cell lineage allocation is affected by oxidative stress but restored by BGP-15

To examine cell number embryos were stained for ICM and TE using two different methods; differential staining using propidium iodide (PI) and bisbenzimide stains and Oct4 and Cdx2 immunohistochemistry. Following differential staining for ICM and TE, a significant difference in total cell number was seen between control and OS blastocysts (control= 47.915 ± 0.888 vs OS= 40.34 ± 1.138, $P < 0.0001$, Figure 5A). This was attributable to blastocysts derived from OS oocytes having a significantly smaller ICM compared with control blastocysts (Figure 5C). However, there was no significant difference in the allocation of cells to the TE between the groups (Figure 5B) and, no difference was observed in the ICM:TE ratio between the control and OS groups (Figure 5D). Interestingly, BGP-15 intervention in the IVF medium was able to restore the total cell number and ICM cell numbers in the OS embryos to a level that was comparable to the controls (Figure 5A and 5C). In addition to that, BGP-15 supplementation also increased the ICM:TE ratio in the OS embryos (Figure 5D).

Immunofluorescence for Oct4 and Cdx2 proteins was also performed to examine embryo cell number and lineage differentiation in the ICM and TE (Figure 6A). Based on previous reports, Oct4 distribution is restricted to the ICM by the mature blastocyst stage (Okamoto *et al.* 1990; Rosner *et al.* 1990; Scholer *et al.* 1990) whereas Cdx2 is found only in the TE (Beck *et al.* 1995; Niwa *et al.* 2005). Similar to previous findings (Szczepanska *et al.* 2011; Madeja *et al.* 2013), a strong signal of Oct4 staining was present in the nuclei of ICM but was much less intense in the nuclei of TE cells and the presence of Cdx2 protein was restricted to the nuclei of TE cells only (Fig 6A). Consistent with differential staining, total cell numbers in the embryos were much lower in the OS embryos when compared to those in the controls (Figure 6B). Similarly, no difference in TE cell number was observed (Figure 6C) but there was a reduction in ICM cell numbers in the OS embryos (Figure 6D). Importantly, there was also an increase in the ICM:TE cell ratio in the OS embryos following BGP-15 supplementation (Figure 6E). BGP-15 supplementation also normalised the ICM numbers in the OS embryos but not their total cell numbers (Figure 6B and 6D). These results indicate that BGP-15 has some beneficial effect on cell proliferation and/or differentiation in the embryos.

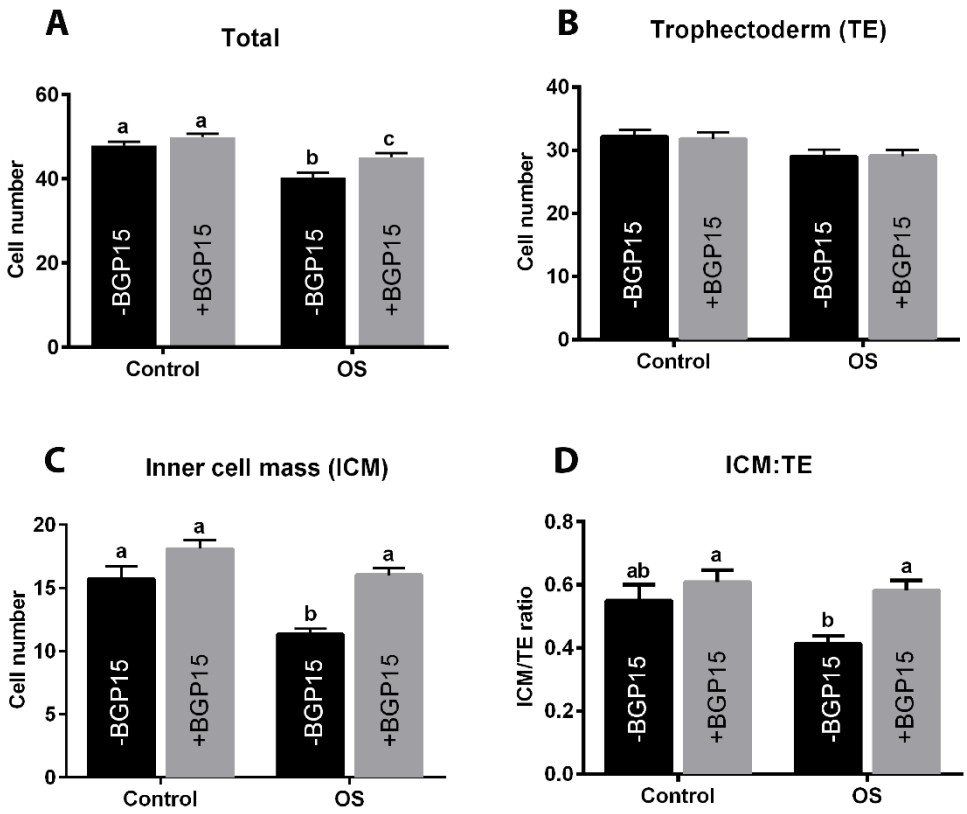


Figure 5 Effect of oxidative stress in mouse oocytes and presence of BGP-15 in *in vitro* fertilisation (IVF) media on embryo lineage allocation.

(A) Total cell number in embryos that reached blastocyst stage. (B) Trophectoderm (TE) cell numbers in the embryos. (C) Inner cell mass (ICM) cell numbers in the embryos. (D) Proportion of ICM to TE in the embryos. Data are presented as mean ± SEM, n = 37-40 embryos per group. Groups with different superscripts differ significantly by one-way ANOVA with Tukey's post hoc test ($P \leq 0.01$).

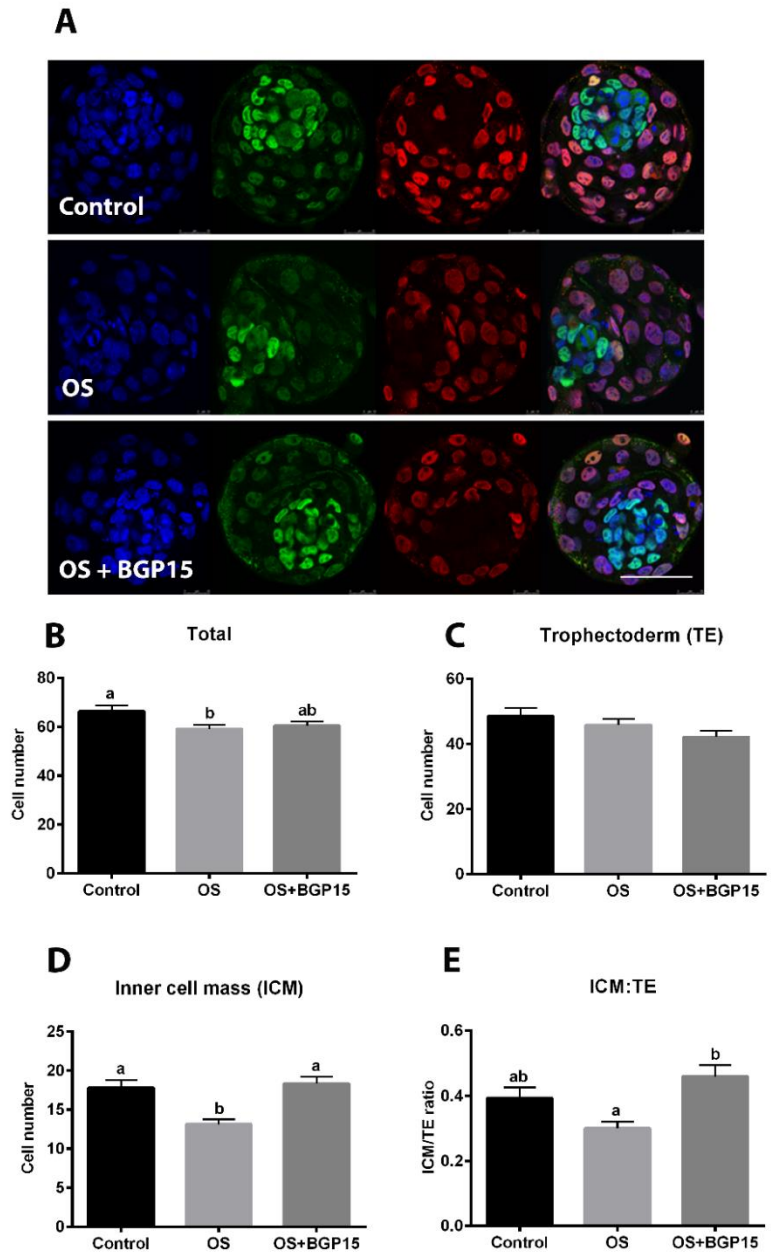


Figure 6 Immunofluorescence of Oct4 and Cdx2 in expanded mouse blastocysts.

(A) Representative blastocysts stained for Oct4 ICM marker (green), Cdx2 TE marker (red) and Hoechst 33342 DNA marker (blue). (B) Total cell numbers detected by Hoechst 333342. (C) Trophectoderm (TE) cell numbers determined by Cdx2+ staining. (D) Inner cell mass (ICM) cell numbers determined by Oct4+ staining. (E) Proportion of ICM to TE in the embryos. Data are presented as mean ± SEM, n = 20-21 embryos per group. Groups with different superscripts differ significantly by one-way ANOVA with Tukey's post hoc test ($P \leq 0.01$). Scale bar = 100 µm.

5.4 Discussion

The data in this chapter show that acute exposure of COCs to H_2O_2 increases ROS levels in the oocytes and alters mitochondrial localisation and activity and causes an impaired cleavage rate. BGP-15 treatment improved blastocyst development only in embryos from H_2O_2-treated COCs. Embryo differentiation was altered as shown by a reduction in blastocyst cell numbers, particularly ICM cells, in the embryos generated from the H_2O_2-treated COCs; and this was normalised with BGP-15 treatment. ROS levels in embryos from H_2O_2-exposed oocytes were not different compared to control embryos, but were reduced by BGP-15 treatment. Both MMP and mtDNA copy number in the embryos generated from the H_2O_2-treated COCs were decreased compared to the control. BGP-15 did not alter MMP in embryos from H_2O_2-treated oocytes, but was able to restore mtDNA copy number in these embryos.

It is currently unclear what mechanisms underlie such differences in mitochondrial activity between control and oxidative-stressed oocytes. It is possible that as ROS production is increased, rapid mitochondrial respiration took place in order to maintain redox homeostasis within the oocytes and it is possible that the distributional change of mitochondria that we observed may contribute to this process. Whether oocytes that show increased MMP also have abnormally higher ATP content remains to be determined.

Cleavage rate, but not blastocyst development, was impaired following exposure to H_2O_2. This finding may be due to the fact that the first cleavage corresponds to the critical phase in which genomic activation occurs, transitioning from maternal to embryonic control of development. Analysis of the embryos that were generated with H_2O_2-treated COCs showed that embryo development time was shorter as they progressed from 2-cell to 3-cell stage and that BGP-15 treatment resulted in embryos reaching blastocyst stage at a faster rate when compared to the controls and embryos generated from H_2O_2-treated COCs. It is important to highlight that it is unclear if early cleavage between developmental stages is an independent predictor of pregnancy or if it is correlated with other variables such as embryo morphology and cell number. Furthermore, preimplantation genetic screening (PGS) has revealed that embryos with abnormal cleavage can develop to blastocysts with chromosomal abnormalities yet appear morphologically indistinguishable from normal embryos

(Sandalinas *et al.* 2001; Somfai *et al.* 2010; Chavez *et al.* 2012; Dal Canto *et al.* 2012; Campbell *et al.* 2013a; Campbell *et al.* 2013b).

BGP-15 in the IVF medium improved embryo development in the H_2O_2-treated COCs. In our previous obese mouse model, we also observed significant improved blastocyst development in obesity-stressed oocytes when treated with BGP-15 *in vivo*. Although the precise molecular mode of action of BGP-15 is not yet completely clear, our results clearly implicate its effectiveness in the presence of cellular stress, similar to previous reports (Chung *et al.* 2008; Nagy *et al.* 2010; Crul *et al.* 2013). In the present study, BGP-15 reduced ROS levels in the embryos generated from H_2O_2-treated COCs indicating its antioxidant effect. However, it was previously reported to induce mitochondrial replication factors Tfam (transcription factor A, mitochondrial) and Drp1 *(*dynamin related protein 1) in oocytes *in vivo* (Wu *et al.* 2015) and increase mitochondrial numbers in the muscle (Henstridge *et al.* 2014a). Thus, it is likely that BGP-15 improves embryo development by enhancing mitochondrial biogenesis.

Although a source of ROS, mitochondria are also a target of ROS and their responses to oxidative stress have been investigated in various fields. A reduced MMP was observed in the embryos generated from the H_2O_2-treated COCs but was not normalised with BGP-15 treatment. Our present results demonstrate that dysfunction of embryo mitochondrial activity, which is directly caused by accumulation of ROS in the oocytes, may further impair mitochondrial biogenesis and normal cell proliferation in the embryos. Measurement of mtDNA is often used as a proxy for mitochondrial number, however there is often little correlation between mtDNA copy number and the number of mitochondria or mitochondrial function. For instance, expression of human transcription factor A in mouse embryos significantly increases mtDNA copy number, but with no detectable effect on mitochondrial number, measured by mitochondrial mass or maximal respiratory capacity (Ekstrand *et al.* 2004). Similarly in the present study, even though there was a restoration in mtDNA copy number in the presence of BGP-15 in the stressed embryos, this was not accompanied by an increased MMP. In human embryos, as well as in mice, lower mtDNA copy number in embryos correlates with reduced viability (Stojkovic *et al.* 2001; Santos *et al.* 2006; Ge *et al.* 2012), suggesting some benefit to increased copy number in the presence of BGP-15 in

the IVF culture media. The increase in mtDNA copy number in response to BGP-15 may be indicative of increased mitochondrial biogenesis. Importantly the current results cannot discern whether mtDNA copy number (or number of mitochondria) is differentially affected in cells of the ICM versus TE.

Because the exposure of COCs to H_2O_2 caused a reduction in ICM cell numbers, oxidative stress in oocytes is likely to influence cell lineage and numbers required for normal growth and thereby have long-term effects for the embryos into adulthood. Analysis of blastocyst differentiation revealed a reduction in total and inner cell mass (ICM) cells in the embryos generated from the H_2O_2-treated COCs compared to the control embryos. The ICM cells are relatively quiescent compared with the TE cells, so the reduction in ICM cells may indicate that cells giving rise to the ICM are more susceptible to oxidative stress. Also, ICM cells were not themselves directly counted therefore the ICM numbers derived could be due to errors in counting the TE or total cell numbers accurately. Interestingly, when sperm were exposed to H_2O_2, the resulting embryos also had reduced blastocyst total and ICM cells, similar to the phenotype observed in this study (Lane *et al.* 2014). In the current study, it is unknown whether BGP-15 had any impact on the sperm, which then manifested in improved embryo development. An effect on sperm is unlikely however, in light of the findings in Chapter 3 and in (Wu *et al.* 2015), where BGP-15 was shown to work more effectively in *in vivo* environment and in addition has no discernible effects in the absence of cellular stress (Chung *et al.* 2008; Nagy *et al.* 2010; Crul *et al.* 2013). Studies have shown that decreased ICM cell number may be a causative factor for fetal growth retardation (Lea *et al.* 1996; Kwong *et al.* 2000). A reduction in the number of ICM cells in the blastocyst is also reflective of reduced embryo viability post-transfer (Lane and Gardner 1997; Lane *et al.* 2014). Additionally, in rodents, blastocyst cell numbers as well as the proportion of cells within the late blastocysts have also been shown to positively correlate with implantation and pregnancy rates (Lane and Gardner 1997). This suggests that the presence of BGP-15 in IVF medium could potentially improve implantation and live birth rates, by restoring cell allocation in the blastocyst. It is also important to note that there was an apparent difference of approximately 20 cells in the blastocyst total cell count between Figure 5 (differential staining) and Figure 6 (Oct4/ Cdx2 immunohistochemistry). This was due to different culture

condition (single drop versus group culture) and also different stages of development of the embryos (blastocyst (Fig 5) versus expanded blastocyst (Fig 6)).

In conclusion, the present study shows that the presence of oxidative stress increases ROS, alters mitochondrial activity and disturbs normal mtDNA replication processes. In support of this, mtDNA content does not change markedly in embryos from oxidative-stressed oocytes during embryogenesis; remaining static at an average of 6.5×10^5 copies in oocytes to 6.3×10^5 copies in blastocysts. By contrast, embryos from the control oocytes increased mtDNA from 6.3×10^5 copies to 12.0×10^5 copies, as is known to occur in preparation for implantation and consistent with the results in Chapter 4 Fig. 1A. Our results also demonstrate that COCs are acutely responsive to oxidative stress which impairs cleavage rates as well as embryo differentiation potentially via effects on mitochondria. Importantly, BGP-15, a drug currently in human clinical trials, is able to alleviate the effects of oxidative stress in oocytes and improve embryo development. These provide us with a better understanding of oocyte and embryo capability to counteract stress with BGP-15 and also demonstrate the possibility that BGP-15 (or a similar) supplement in media may result in improved embryo development.

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CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

6.1 Summary and Future Directions

It is now well-established that environmental and metabolic insults occurring early in development can have long-term health implications (Perera and Herbstman 2011). In particular, maternal obesity during the peri-conceptual period negatively affects oocyte developmental competence (Robker 2008; Lane *et al.* 2015). The need to further study this effect is driven by the increasing prevalence of metabolic conditions such as obesity and diabetes. Nevertheless, although there are some known consequences of maternal obesity on oocytes such as altered mitochondrial activity and compromised embryo development (Igosheva *et al.* 2010; Wu *et al.* 2011; Luzzo *et al.* 2012; Wu *et al.* 2015; Hou *et al.* 2016), the mechanisms remain largely unknown. However, before we can hope to reverse any adverse outcomes arising from peri-conception maternal obesity, we must quantify the sensitivity of oocytes to different environmental and metabolic insults and understand the causal mechanisms involved.

The research in this thesis was aimed at explaining how a perturbed metabolic environment acts during the periconception period to causes long term developmental outcomes. Cumulatively, these studies show that 1) cholesterol in mouse oocytes is required for embryogenesis and formation of membrane functional domains in blastocysts, 2) maternal high-fat diet alters oocyte lipid raft distribution and 3) high lipid conditions or oxidative stress in oocytes impacts mitochondrial DNA (mtDNA) copy numbers in embryos. I also provide evidence in multiple species and physiological contexts that BGP-15 counteracts stress via its effect on mitochondria.

It is well-established that the cumulus-oocyte complex (COC) relies on follicular fluid for nutrients, cytokine/growth factors and other peptide hormones, steroids and energy substrates (Dumesic *et al.* 2015). The composition of follicular fluid is generally reflective of venous blood (Revelli *et al.* 2009); hence, poor maternal health, resulting in abnormal metabolic profiles systemically, is reflected within the follicular environment. One major metabolite in the follicular fluid is cholesterol, as well as triglycerides and free fatty acids. These are known to be altered in obesity creating an ‘obesogenic environment’ for the oocyte that can be mimicked and studied *in vitro* using high palmitic acid lipid or a blend of high non-esterified fatty acids (NEFA) (Leroy *et al.* 2005; Wu *et al.* 2012). Oxidative stress

markers are also increased in follicular fluid in numerous contexts, for instance it has been localised in the follicular fluid in the patients undergoing *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) (Oyawoye *et al.* 2003; Pasqualotto *et al.* 2004) and in this thesis this 'maternal environment' was emulated using simple hydrogen peroxide. These approaches enabled me to explore the effects of a variety of environmental conditions which the oocyte might experience, on its subsequent development and differentiation.

One remarkable insight from this thesis has been the importance of cholesterol in membrane raft formation that confers developmental competence. The compromised embryo development outcome and changes in cell lineage numbers that result from altering oocyte cholesterol levels support the concept that disrupted rafts may also be one of the underlying issues with obesity-mediated effects on conception rates. Although much is known about the role of cholesterol in cardiovascular disease, its relationship with the female reproductive system is only beginning to be understood. My observations have confirmed the need to better understand the roles of cholesterol metabolism within follicular fluid on oocyte quality and developmental competence. Future studies to elucidate the mechanisms that regulate intrafollicular cholesterol transport during folliculogenesis and the role of cholesterol in membrane trafficking and signaling in oocytes are needed. Based on the findings of raft distribution patterns reported in this study, it will be worth identifying the proteins that are located in the embryonic lipid rafts and to understand their role in membrane organisation and cell signaling events in the context of their location within lipid raft structures. Given that BGP-15 treatment resulted in only partial restoration of lipid rafts in the blastocysts from female mice fed a high-fat diet (HFD), this suggests that there remains some residual impact of the HFD or increased maternal adiposity. I would predict that full restoration could be achieved from a longer duration of BGP-15 interventions, i.e. through to the blastocyst stage rather than preconception only, but this remains to be determined.

I propose that mitochondrial dysfunction and oxidative stress may act as the initiators for a cascade of events that create the HFD oocyte phenotype. Operational mitochondria are crucial to normal oocyte function, with these organelles representing the primary source of adenosine triphosphate (ATP) production within both oocytes and early embryos (Babayev and Seli 2015). Diminished mitochondrial integrity in oocytes from HFD-fed mice and from

oxidative stress exposure has been demonstrated by a loss of mitochondrial activity, followed by a reduction in mtDNA in embryos. Therefore, it is likely that loss of mitochondrial integrity results in aberrant protein synthesis, particularly of the respiratory chain complexes, and inactivation or loss of mtDNA.

I also hypothesise that oxidative stress may act as the 'trigger' for a cascade of other factors that orchestrate mitochondrial dysfunction, as well as directly affecting multiple aspects of oocyte biochemistry and functionality. Oxidative stress has the potential to directly affect mitochondrial function and I have shown that oxidative stress is linked with changes in mitochondrial activity and mtDNA levels. The DNA, proteins and lipids within the mitochondria are particularly susceptible to oxidative attack, not only because of their close proximity to the source of ROS production, but in the case of mtDNA, because of the absence of protective histones and repair mechanisms (reviewed by (Alexeyev 2009)). In mammals, mtDNA is maternally inherited and transmitted for many generations (Sato and Sato 2013). Therefore, changes in mtDNA copy number and/or mutation load are carried into the next generation and in some circumstances may influence offspring metabolism, fertility and/or aging. However, not all mutations are carried through, and there are commonly thresholds above which mutation load must exist for a phenotypic effect.

The findings that environmental insults (maternal HFD, high lipid conditions and oxidative stress) negatively impacts mitochondrial activity and mtDNA provides mechanistic insight into how perturbed oocyte biology could contribute to long-term health of the embryos. In Chapter 4, I showed that mtDNA levels varied between species and acted differently in response to BGP-15. Differences in mtDNA replication observed may be due to difference in oocyte lipid profiles in the different species. Intriguingly, in invertebrate species lipid droplets seem to regulate the function of proteins related to embryo genome activation. Thus, lipid stores in oocytes may function not only as a source of energy reserve, but also as a potential reservoir for signaling factors that play a role in cellular fate for mammalian early embryo development (Kim *et al.* 2012). Importantly, I document a relationship between mtDNA quantity and improved embryo development and embryo health. This was shown in the mouse and bovine studies in Chapter 4 and oxidative stress model in Chapter 5. Notably, damage to mtDNA in the oocyte could potentially be the basis for the abnormalities

associated with the embryos; for instance impairments in the transcription of essential mitochondrial genes. Taken together, the studies in Chapters 3, 4 and 5 provide evidence that the perturbations in oocytes, embryo development and embryo health are not entirely permanent and can be at least in part reversed by BGP-15 intervention. Although the precise molecular mode of action of BGP-15 is not yet completely clear, our results implicate its effectiveness in the presence of cellular stress, consistent with previous reports (Chung *et al.* 2008; Nagy *et al.* 2010; Crul *et al.* 2013). Based on the findings in this thesis, it is clear that BGP-15 acts on the mitochondria irrespective of species. Recent studies also reported that BGP-15 has a protective role by preserving mitochondrial integrity and reducing mitochondrial ROS production (Sumegi *et al.* 2017) and improved mitochondrial function by increasing mitochondrial content (Salah *et al.* 2016). So, future studies are needed to fully delineate the molecular mechanisms by which BGP-15, directly and/or indirectly, acts on oocyte/embryo mitochondria.

Supplementation of BGP-15 in *in vitro* maturation (IVM) and *in vitro* fertilisation (IVF) provides a means to increase mtDNA in both the oocytes and embryos under stress environments. The increase in mtDNA following BGP-15 treatment suggests that there may be an increased stimulation of mitochondria biogenic pathways or mtDNA replication to compensate for impaired mitochondrial functions. However, it is yet unclear whether this increase in mtDNA is the result of a general increase in transcription of nuclear genes, or whether mitochondria have a specific role to play in this process such as a shift in mitochondrial metabolism; therefore these possibilities need to be further evaluated. Future studies could be directed towards elucidating these by using knockout mouse models for nuclear-encoded mitochondrial genes and examining whether mtDNA levels are still regulated by BGP-15. Regardless of whether mtDNA replication occurs when the embryonic genome is activated, mtDNA copy number was not increased until at least the blastocyst stage in the mouse and macaque. Future studies such as embryo transfer studies are also required to investigate the relationship of increased/decreased mtDNA in implantation, pregnancy and live birth rates in order to evaluate the potential clinical relevance of BGP-15.

Although promising, there are some limitations associated with the present studies. One of the constraints is that the experiments were *in vitro* and such experimental design may increase the risk of artefactual response as *in vitro* environments do not entirely mimic *in vivo* conditions and therefore may cause a decline in the developmental competence of IVM oocytes. An *in vitro* maturation environment would impair not only spindle assembly and cytoplasm maturation but also DNA epigenetic modifications and subsequent RNA transcription and protein expression (Anckaert *et al.* 2013; El Hajj and Haaf 2013). Another confounder is variations among species. The use of abattoir material prevents the ability to identify whether oocytes were from obese or lean females, so variation would be expected in the ability of BGP-15 to modulate oocyte or embryo characteristics. Equally, the high lipids added in the studies are tailored for each species, therefore the current findings may be due to these biological differences in lipid profiles in these species.

In conclusion, my research extends our understanding of the mechanisms by which the preimplantation period is a critical stage in which the environment impinges on development. The data generated in this thesis provide information on the interplay between membrane rafts, mitochondrial activity, mtDNA copy number and cell response upon alteration of environmental cues. These provide new insights into fundamental mechanisms that may determine human oocyte quality and subsequent healthy development, which may lead to new measurements or therapies of human fertility. In particular, this study highlights the importance of mitochondria as a determinant of developmental competence, indicating that it will be of value to increase our understanding of the biochemical processes by which mitochondria impinge on continued developmental progression and embryo metabolism. The direct mechanism by which BGP-15 exerts its effect on mtDNA levels and alleviates stress responses on subsequent embryo health is clearly multifactorial. Importantly, BGP-15 provides a means to increase mtDNA levels and improve embryo development, providing new leads for future therapeutics aimed at combatting the impact of metabolic syndrome on oocyte quality and embryo, which holds promise for improving the health of future generations.

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APPENDIX

MEDIA, SOLUTIONS AND TABLES

Table 1 High-fat diet (HFD) ingredients (made in-house using the same recipe as D12492; Research Diets, Brunswick, NJ)

Components	Weight (g)
Casein	258.44
Maltodextrin	161.5
Sucrose	88.88
Cellulose	64.6
Potassium Citrate	21.318
Dicalcium Phosphate	16.796
Mineral Mix	12.92
Vitamin Mix	12.92
Calcium Carbonate	7.106
L-Cystine	3.876
Choline Bitartrate	2.589
Soy Bean Oil	32.3
Lard	316.54

Table 2 Nutrient compositions present in the high-fat diet (HFD)

	Standard chow (SF-105)		HFD (D12492)	
Nutrient	**kcal%**	**g%**	**kcal%**	**g%**
Protein	16	19.6	20	26.2
Carbohydrate	64	48.3	20	26.3
Fat	20	10	60	34.9
Energy	3.57 kcal/g		5.24 kcal/g	

Table 3 Summary of Lineage-Specific Protein Detection in Preimplantation Embryos

Transcription factor	Expression	Function	Reference
Octamer binding transcription factor 3/4 (Oct4 / Pou5f1)	In all cells of the early embryo from 8- to 32-cell stage. By the mature blastocyst stage, its distribution is restricted to the ICM	• Essential for establishing and maintaining pluripotency of the ICM • Essential to prevent ICM from diverting towards the TE lineage • Oct4 null embryos die around the time of implantation and fail to outgrow in culture	(Nichols *et al.* 1998; Avilion *et al.* 2003; Niwa *et al.* 2005; Strumpf *et al.* 2005; Ralston and Rossant 2008; Guo *et al.* 2010)
SRY-related HMG-box 2 (Sox2)	Levels of Sox2 decrease in the embryo until it reaches its lowest levels at 8-cell stage/morula. By the mature blastocyst stage, its expression is restricted to the ICM	• Pluripotency in blastomere • May act to maintain or preserve developmental potential • Its down-regulation correlate with a commitment to differentiate, such that it is no longer expressed in cell types with restricted developmental potential	(Avilion *et al.* 2003; Niwa *et al.* 2005; Strumpf *et al.* 2005; Guo *et al.* 2010)
Homeobox Transcription Factor Nanog (Nanog)	In all cells of the early embryo from 8- to 32-cell stage. By 64-cell stage, it is exclusively expressed within the ICM. By the mature blastocyst stage, its distribution is restricted to the ICM	• Maintenance of pluripotency in ICM • Oct4 and Sox2 together promotes the expression of Nanog	(Avilion *et al.* 2003; Niwa *et al.* 2005; Guo *et al.* 2010)

Caudal-type homeobox transcription factor-2 (Cdx2)	Ubiquitous before 32-cell stage. Found only in the TE	• Key role in trophectoderm specification • Expression regulated by the transcriptional regulator Tead4 • Important for down-regulating the expression of Oct4 and Nanog • Influences cell polarity by up-regulating polarity genes such as aPKC • In the absence of Cdx2, TE cell identity cannot be maintained in the blastocyst stage	(Strumpf *et al.* 2005; Dietrich and Hiiragi 2007; Jedrusik *et al.* 2008; Ralston and Rossant 2008)
TEA Domain Transcription Factor 4 (Tead4)	Ubiquitously expressed from the 2-cell stage onwards without an apparent localisation to ICM or TE	• Act upstream of Cdx2 to regulate TE formation • Crucial for the initiation of TE formation • Tead4-null embryo exhibits defects specifically in TE	(Yagi *et al.* 2007; Nishioka *et al.* 2008; Marikawa and Alarcon 2009)
Yes-associated protein 1(Yap1) (Wwtr1 is similar to Yap)	mRNA and nuclear protein detectable from 4-cell stage onwards	• Dominant-negative Yap expression leads to reduced Cdx2 expression • Overexpression in inner cell mass of blastocyst induces Cdx2 expression	(Stephenson *et al.* 2012)
Eomesodermin (Eomes)	Protein undetectable until blastocyst stage, TE-restricted	• Encodes a T-box transcription factor	(Ciruna and Rossant 1999; Russ *et al.* 2000; Home *et al.* 2009)

	expression by the blastocyst stage	• Knockout of Eomes leads to failure to TE proliferation downstream from Cdx2 • Control the establishment of embryonic and extra-embryonic lineages	
GATA Binding Protein 3 (Gata3)	Detected at late 8-cell stage, TE restricted expression by the blastocyst stage	• Involved in the transcription factor networks that drive trophoblast cell lineage development • Expression dependent on Tead4, suggesting that Gata3 and Cdx2 act in parallel pathways downstream of Tead4 and function to activate target genes within the TE lineage	(Home *et al.* 2009; Ralston *et al.* 2010)
Kruppel-Like Factor 5 (Klf5)	Ubiquitously early, strong in TE, weak in ICM of blastocyst	• Required for the formation of the TE and ICM, and for repressing primitive endoderm development	(Lin *et al.* 2010; Stephenson *et al.* 2012)

Table 4 Techniques used to identify membrane lipid rafts. Adapted from (Simons and Toomre 2000)

Approach	Purpose	Live cells	Comments
Flotation of detergent-resistant membranes (DRMs)	Identifies putative raft association Identifies possible raft proteins	No	• Easy to do • Most common approach for identifying putative proteins involved in signaling • Weak associations with rafts are difficult to detect
Antibody patching and immunofluorescence microscopy	Identifies putative raft association	No	• Easy to do • Common approach • Better than floatation for detecting weak raft associations • Cell-cell variability makes quantification difficult
Immunoelectron microscopy	Determines location of raft components	No	• Promising results • Requires technical expertise
Chemical crosslinking	Identifies native raft protein complexes	Yes	• Straightforward • Choice of appropriate conditions and reagents is semi-empirical
Single fluorophore tracking microscopy	Monitors the diffusion and dynamics of individual raft proteins or lipids	Yes	• Requires highly specialised equipment and expertise
Photonic force microscopy	Determined the diffusion constant, size and dynamics or individual rafts	Yes	• Very informative technique • Requires highly specialised equipment and technical expertise

			• Time-consuming acquisition and analysis
Fluorescence resonance energy transfer (FRET)	Detects whether two raft components are spatially close (for example, <10nm)	Yes	• Powerful approach • Choice of appropriate donor and acceptor probes is important

* The disruption of rafts by cholesterol depletion or sequestration is especially useful as a control for each of these approaches.

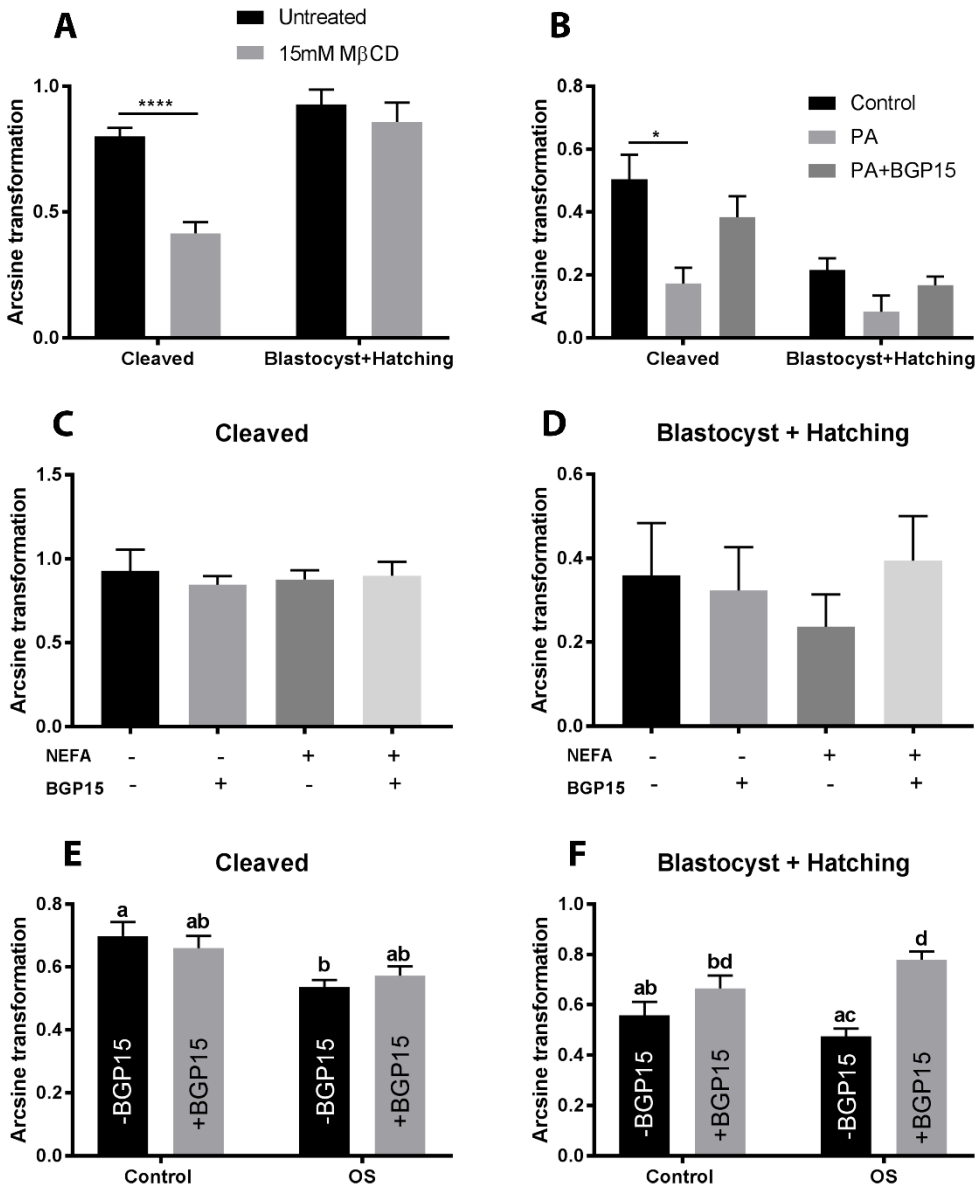

Figure 1 Arcsine Transformation on embryo developmental rates.

To validate the statistical analysis on embryo development, data was arcsine transformed. Arcsine transformed data showed no difference to the original data in the thesis. (A) Developmental data from Chapter 2 (Figure 3A). (B) Mouse developmental data from Chapter 4 (Figure 3B and 3C). (C-D) Cattle developmental data from Chapter 4 (Figure 6A and 6B). (E-F) Mouse developmental data from Chapter 5 (Figure 3B and 3C). Groups with different superscripts differ significantly by one-way ANOVA with Tukey's post hoc test. * $P \leq 0.05$, **** $P \leq 0.001$. MβCD- methyl-beta cyclodextrin, PA- palmitic acid, NEFA- non-esterified fatty acids, OS- oxidative stress.

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