Mitochondrial Sirtuin 3 and Sirtuin 5 in granulosa and cumulus cells and their contribution to the altered follicular environment in women with either reduced ovarian reserve or advanced maternal age.

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Abstract

Women with reduced ovarian reserve or advanced maternal age are known to have poorer IVF outcomes compared to younger women with normal ovarian reserve. While previous studies in humans have correlated metabolite concentrations in follicular fluid to IVF outcome, the impact of maternal age and reduced ovarian reserve have yet to be determined. Oocytes are dependent on mitochondrial metabolism for viability and disruptions to mitochondrial activity can reduce oocyte viability. It has been suggested that oocytes from women with either reduced ovarian reserve or advanced maternal age have a reduction in metabolic function, however, the exact mechanisms behind this reduction remain largely unknown. Interestingly, a family of proteins, the Sirtuins, are able to sense cellular metabolic state and post-translationally alter protein function, thus implicating these proteins in metabolic function. Sirtuin 3 (SIRT3) and Sirtuin 5 (SIRT5) are two proteins that are specifically located to mitochondria, thus may be important in understanding metabolic control in the pre-ovulatory follicle. Thus the aim of this thesis was to determine if a difference exists in the follicular environment in women with reduced ovarian reserve or advanced maternal age and if so does SIRT3 or SIRT5 play role in ovarian follicular cells.

Women (n=111) undergoing routine IVF treatment were recruited to participate in this study. They were allocated to one of three cohorts based on maternal age (young maternal age [≤35]; advanced maternal age ≥40) and ovarian reserve for age, as measured by serum anti-mullerian hormone (AMH) levels. Surplus follicular fluid, granulosa and cumulus cells were collected, de-identified and randomly allocated to experimental protocols. Follicular fluid concentrations of carbohydrates (glucose, lactate and pyruvate), hormones FSH, LH, progesterone, estrogen and AMH), and selected ions were determined. Metabolic analysis of granulosa and cumulus cells was performed. Granulosa and cumulus gene expression of phosphofructokinase platelet (*PFKP*)

and lactate dehydrogenase A (*LDHA*) was determined. *SIRT3* and *SIRT5* gene expression and protein activity was confirmed in granulosa and cumulus cells via qPCR, immunohistochemistry, western blotting and deacetylation/desuccinylation activity. Granulosa and cumulus cell carbamoyl phosphate synthase I (CPS1) protein, a SIRT5 target, was confirmed using immunohistochemistry. Follicular fluid ammonium concentration and granulosa and cumulus cell glutamate dehydrogenase (GDH) activity, a SIRT3 target, were assessed using microfluorometry. Granulosa and cumulus cell acetylated mitochondrial proteins were separated by immunoprecipitation and acetylation of GDH was assessed via western blotting. Data from young women with normal ovarian reserve were compared with those from young women with reduced ovarian reserve and those of advanced maternal age.

Young women with normal ovarian reserve had significantly lower starting FSH doses and fewer previous cycles compared to the remaining two groups. Young women with normal ovarian reserve had significantly more oocytes collected compared to young women with reduced ovarian reserve and the advanced maternal age women. Fertilisation rate was significantly higher in the young women with normal ovarian reserve compared with the advanced maternal age group. Women of young maternal age with normal ovarian reserve had significantly less embryos transferred compared to the advanced maternal age group. The clinical pregnancy rate in young women with normal ovarian reserve was significantly increased compared to both the reduced ovarian reserve and advanced maternal age groups. No differences were found in clinical pregnancy rate between the reduced ovarian reserve and advanced maternal age groups.

Follicular fluid glucose concentrations were significantly decreased, whereas lactate and progesterone concentrations, granulosa and cumulus cell glucose uptake, lactate production, and phosphofructokinase platelet gene expression were significantly increased in women with

reduced ovarian reserve and in women of advanced maternal age. *SIRT3* and *SIRT5* mRNA and active protein were present in granulosa and cumulus cells and co-localized to the mitochondria. Women with reduced ovarian reserve or advanced maternal age had decreased granulosa and cumulus cell *SIRT5* mRNA, protein, desuccinylation activity and an accumulation of follicular-fluid ammonium. CPS1 protein was present in granulosa and cumulus cells. Compared to young women with normal ovarian reserve granulosa cell *SIRT3* mRNA was decreased in young women with reduced ovarian reserve and advanced maternal age whereas cumulus cell *SIRT3* mRNA was decreased in women of advanced maternal age only. Granulosa cell GDH activity was decreased in young women with reduced ovarian reserve and in women of advanced maternal age, whereas cumulus cell GDH activity was reduced in the advanced maternal age group only. Granulosa and cumulus cell acetylated mitochondrial GDH was increased in women of advanced maternal age while young women with reduced ovarian reserve had increased granulosa cell GDH acetylation only.

The data presented within this thesis suggest that in women with either reduced ovarian reserve or advanced maternal age both SIRT3 and SIRT5 may regulate granulosa and cumulus cell GDH and CPS1 activity, therefore altering the microenvironment surrounding the oocyte, as reflected by the altered follicular environment. This perturbed microenvironment may be responsible for impaired oocyte developmental competence, subsequent embryo development and reduced clinical pregnancy rates, also reported in this study. Considering the association between the decline in pregnancy rates in women with reduced ovarian reserve and in women of advanced maternal age and the knowledge of perturbed granulosa and cumulus cell SIRT3 and SIRT5 function this may lead to novel therapies to improve mitochondrial metabolism in the oocyte and follicular cells in women undergoing IVF treatment.

Declaration

The content presented within this thesis contains no material that has previously been accepted for the award of any other degree or diploma in any university or other tertiary institution. To the best of my knowledge and belief this thesis contains no material previously published or written by any other person, except where reference is made in the text. I certify that no part of this work will in future be used in a submission 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. All experiments outlined in this thesis were performed myself and any contributions and assistance received from others is acknowledged.

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Leanne Pacella-Ince

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"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

-Marie Curie-

Glossary/Abbreviations

ACECS2	Acetyl-CoA Sythetase 2
Acetyl CoA	Acetyl Coenzyme A
aCGH	Array Comparative Genomic Hybridisation
AMH	Anti-Mullerian Hormone
AQP	Aquaporin
ART	Assisted Reproductive Technologies
ATP	Adenosine Triphosphate
BCL-2	β-Cell Lymphoma 2
BMP-15	Bone Morphogenetic Protein 15
cAMP	Cyclic Adenosine Monoposphate
CDK1	Cumulus Expansion Enabling Factor
CEEF	Cyclin-Dependent Kinase 1
COC	Cumulus Oocyte Factor
CPS1	Carbamoyl Phosphate Synthase 1
CytC	Cytochrome C
DNA	Deoxyribonucleic Acid
ETC	Electron Transport Chain
FAD	Flavin Adenine Dinucleotide
FADH2	Reduced Flavin Dinucleotide

FSH	Follicle Stimulating Hormone
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GDF-9	Growth Differentiation Factor 9
GDH	Glutamate Dehydrogenase
GLUT	Glucose Transporter
GV	Germinal Vesicle
GVBD	Germinal Vesicle Breakdown
hCG	Human Chorionic Gonadotropin
HAT	Histone Acetylase
HDAC	Histone Deacetylase
ICDH	Isocitrate Dehydrogenase
ICSI	Intracytoplasmic Sperm Injection
IMM	Inner Mitochondrial Membrane
IP_3	Inositol Trisphosphate
IVF	In Vitro Fertilisation
LDHA	Lactate Dehydrogenase A
LH	Luteinising Hormone
MAPK	Mitogen-Activated Protein Kinase
MPF	Maturation Promoting Factor
mRNA	Messenger RNA

mtDNA	Mitochondrial DNA
NAD	Nicotinamide Adenine Dinucleotide
NAD^+	Oxidised Nicotinamide Adenine Dinucleotide
NADH	Reduced Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
OMM	Outer Mitochondrial Membrane
OSF	Oocyte Secreted Factors
PCOS	Polycystic Ovarian Syndrome
PFKP	Phosphokinase Platelet
PGER	Progesterone Receptor
PKA	Protein Kinase A
PTGER2	Prostaglandin E Receptor 2
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SIR2	Silent Information Regulator 2
SIRT	Sirtuin
TCA	Tricarboxcylic Acid Cycle
TGF-β	Transforming Growth Factor-β
UPC	Uncoupling Protein
VEGF	Vascular Endothelial Growth Factor

Publications, Conferences, Scholarship and Awards

Scientific Publications

1. **Pacella, L.** Zander-Fox, D.L., Armstrong, D.T. and Lane, M. (2012). Women with reduced ovarian reserve or advanced maternal age have an altered follicular environment. *Fertility and Sterility*, 98; 986-994.

2. **Pacella-Ince, L.**, Zander-Fox, D.L. and Lane, M. (2013). Mitochondrial SIRT5 is present in follicular cells and is altered by reduced ovarian reserve and advanced maternal age. *Reproduction, Fertility and Development*. Published online 27 August 2013.

3. **Pacella-Ince, L.**, Zander-Fox, D.L. and Lane, M. (2014) Mitochondrial SIRT3 and its target glutamate dehydrogenase are altered in follicular cells of women with reduced ovarian reserve or advanced maternal age. *Human Reproduction*, 29 (7), 1490-1499.

Conferences

1. **Pacella, L.**, Zander-Fox D.L., Hussein T., Fullston, T., and Lane M. (2010). SIRT3 in ovarian cells is altered by maternal age and ovarian reserve. Society for reproductive Biology Conference, Sydney, Australia (Oral Presentation)

2. **Pacella, L.**, Zander-Fox D.L., and Lane M. (2011). Follicular fluid glucose and lactate levels are altered by maternal age and ovarian reserve. The World Congress on Reproductive Biology and Society for Reproductive Biology Conferences, Cairns, Australia (Poster Presentation)

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Scholarship and Awards

2011 – 2014 *Faculty of Health Science Postgraduate Award (Scholarship)*

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CHAPTER ONE

Literature Review

1.1 Introduction

In today's society, couples are choosing to have children later in life, with the number of women trying to conceive between the ages of 35 and 44 increasing significantly in the last decade [1]. This is of particular concern given the well-documented relationship between increasing maternal age and reduced reproductive success (Figure 1.1) [2-6]. Over the last ten years in Australia and New Zealand there has been a 42% increase in the number of women seeking assisted reproductive technologies (ART) for conception and in 2009 62% of women seeking ART were over the age of 35 [1]. However, ART is not the "cure" for women with reduced fertility, as ART pregnancy rates for women with advancing maternal age reflect normal conception, with a dramatic reduction in success rates in women over 40 years of age (34.0% live birth rate per embryo transferred in women less than 30 years compared to 9.4% in women 40-44 years and 0.8% for women greater than 45 years) [1].

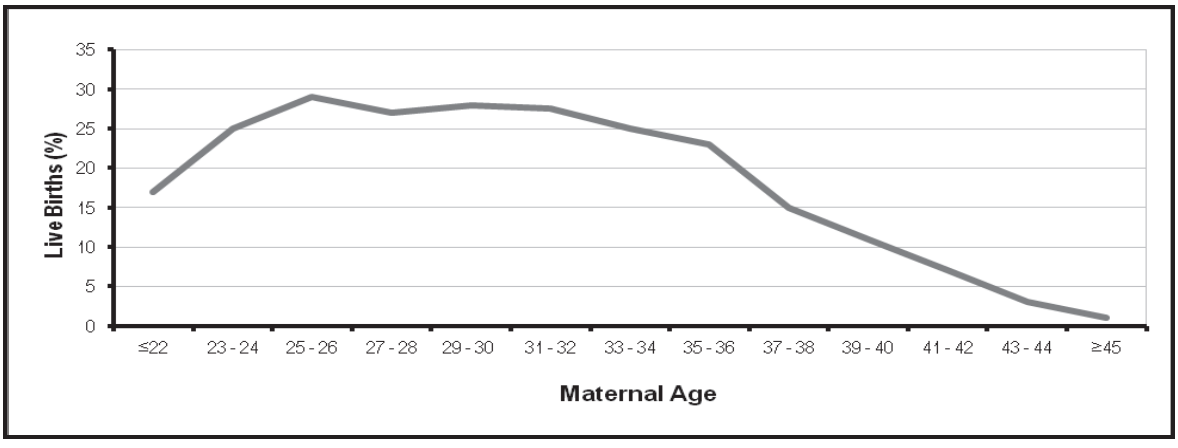

Figure 1.1: Percentage of live births using autologous oocytes in a fresh IVF/ICSI cycle in Australia and New Zealand in 2009.
With increasing maternal age there is a decline in live birth outcome. The highest live delivery rate is in women less than 32 years and this rate steadily declines with increasing maternal age with a rate of 0.8% in women who are ≥45 years [1].

This decline in fertility with increasing maternal age is predominantly a result of a decrease in oocyte quality [3-7]. This relationship is best demonstrated by studies using donor oocyte cycles,

where women undergoing ART over the age of 42 years have pregnancy rates similar to that of the age of their donor (<35years), thus suggesting an age-dependent decline in oocyte quality is a more probable cause, rather than an increase in implantation disorders (Figure 1.2) [2, 5].

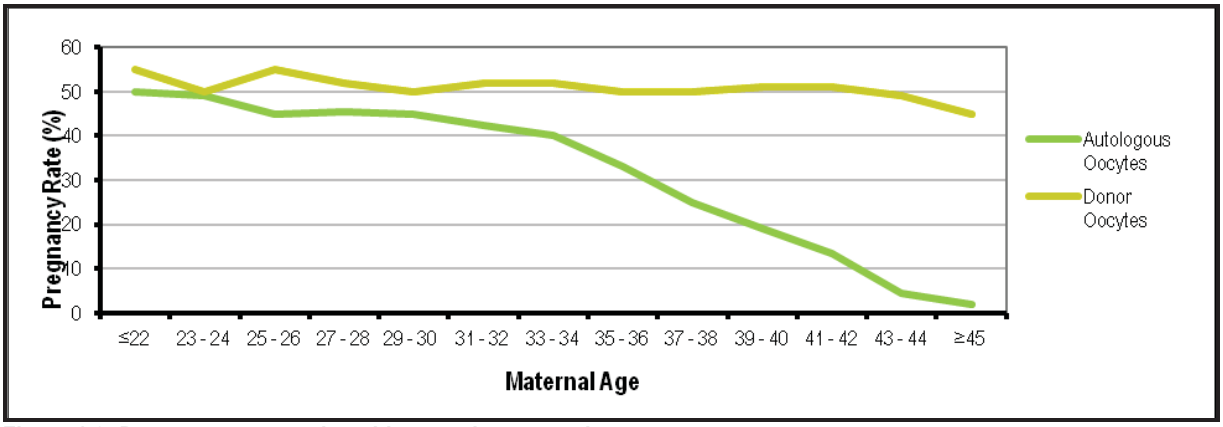

Figure 1.2: Pregnancy rates using either autologous or donor oocytes.
With increasing maternal age there is a decrease in pregnancy rates with a rapid decline occurring after 35 years when using autologous oocytes. However, pregnancies occurring with donor oocytes remain relatively steady irrespective of how old the recipient is [8].

Interestingly, women undergoing ART treatment, with reduced ovarian reserve (a reduction in the number of oocytes remaining in the resting ovarian pool) irrespective of age also exhibit reduced pregnancy rates and reduced cumulative pregnancy rates [9-16]. Currently, apart from oocyte donation there is no known intervention to improve the pregnancy rates of these women.

The follicular environment to which the oocyte is exposed plays an important role in the development of a viable oocyte and it is well-established that bi-directional communication between the oocyte and its surrounding ovarian follicular cells is essential for this development. Oocyte developmental competence is dependent on oocyte growth and energy, which is a driving force behind the production of good quality oocytes, that are able to be fertilised and then go on to form good quality embryos and ultimately a viable pregnancy [5, 6, 17]. To date the ovarian follicular environment of women that are known to have reduced pregnancy potential, specifically

women with reduced ovarian reserve or of advanced maternal age, remain largely uninvestigated. It has been suggested that oocytes from these women have a reduction in metabolic function and an increase in aneuploidy [3, 6, 18, 19]. However, aneuploidy is not the only mechanism behind the decline in pregnancy rates in women of advanced maternal age as transfer of a euploid embryo does not improve pregnancy rates to the same level as young women, thus suggesting there are other mechanisms involved in the decline in oocyte quality and thus pregnancy rates. If this reduction in energy is significant, many important cellular processes could be affected and this may impact on oocyte fertilisation and embryo development [3, 19]. Therefore, this thesis will examine the follicular environment in women with reduced ovarian reserve and in women with advanced maternal age compared to young women (≤35 years) with normal ovarian reserve as well as assessment of a potential mechanism which may contribute to the decline in oocyte quality.

1.2 The Ovarian Follicle

Precise regulation of the ovarian follicular environment is essential for the development of a competent oocyte. The following sections will review the development of the ovarian follicle, with specific focus on the oocyte, granulosa and cumulus cells.

1.2.1 Folliculogenesis

Folliculogenesis is the process in which the follicles within the ovary mature and in humans it has been estimated that this process takes >150 days (Figure 1.3) [20]. During follicular development the oocyte develops and matures, a process referred to as oogenesis. Both folliculogenesis and oogenesis follow a co-ordinated sequence of events that starts within the fetal ovary at 18-22 weeks after conception and ends with ovulation of a mature oocyte. Maturation of the oocyte is

reliant on the synchronised proliferation and differentiation of follicular cell layers, the granulosa cells and cumulus cells [21]. Once folliculogenesis is initiated it is a continuous event, however, less than one percent of the primordial follicles (explained below) will actually continue their development to ovulation. The follicles that don't make it to ovulation degenerate by atresia [20, 22]. It is important to note that the factors and timing involved in the process of folliculogenesis are species-specific [23].

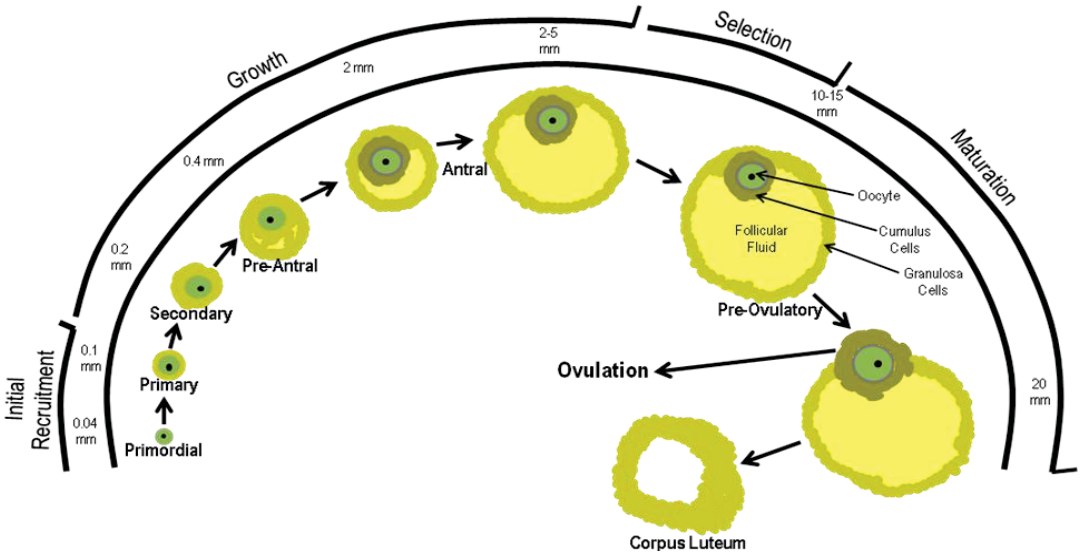

Figure 1.3: The growth of the follicle in the female adult human ovary.
Within the adult ovary follicles can be found at various sizes (as outlined in the figure), representative of the stage of folliculogenesis and in humans this takes approximately 150 days. A specific series of events must occur at each stage of folliculogenesis in order for the ovulation of a mature oocyte to occur. The follicle undergoes an initial recruitment phase, where follicles are recruited into the growing pool, then a growth phase occurs followed by selection and maturation of the one follicle that continues to grow and mature until ovulation (adapted from Gougeon, 1986) [20].

1.2.1.1 Primordial Follicle

Within the human ovary the resting pool of primordial follicles has been established at 16–22 weeks post conception and contains its peak number of 6–7 million non-growing primordial follicles measuring about 0.03–0.05mm in diameter [24]. This supplies the female with a finite pool of developing follicles and oocytes until the end of her reproductive life [25, 26]. However, from this point onwards the size of the ovarian reserve, or the number of follicles in the ovary, decreases dramatically. The human female ovary at birth contains about 2 million primordial

follicles and this number continues to decline such that by the time puberty is reached there are between 250,000 and 500,000 resting follicles in each ovary and of these only about 300 – 400 will mature into an ovulatory follicle [27]. Within these primordial follicles are primordial germ cells (30µm in size), destined to become the oocyte, which are in a quiescent state undergoing very little to no metabolic activity [28]. These primordial germ cells are enclosed by a single squamous layer of somatic cells resting on a basement membrane, which are thought to be the precursors to granulosa cells (Figure 1.4) [26].

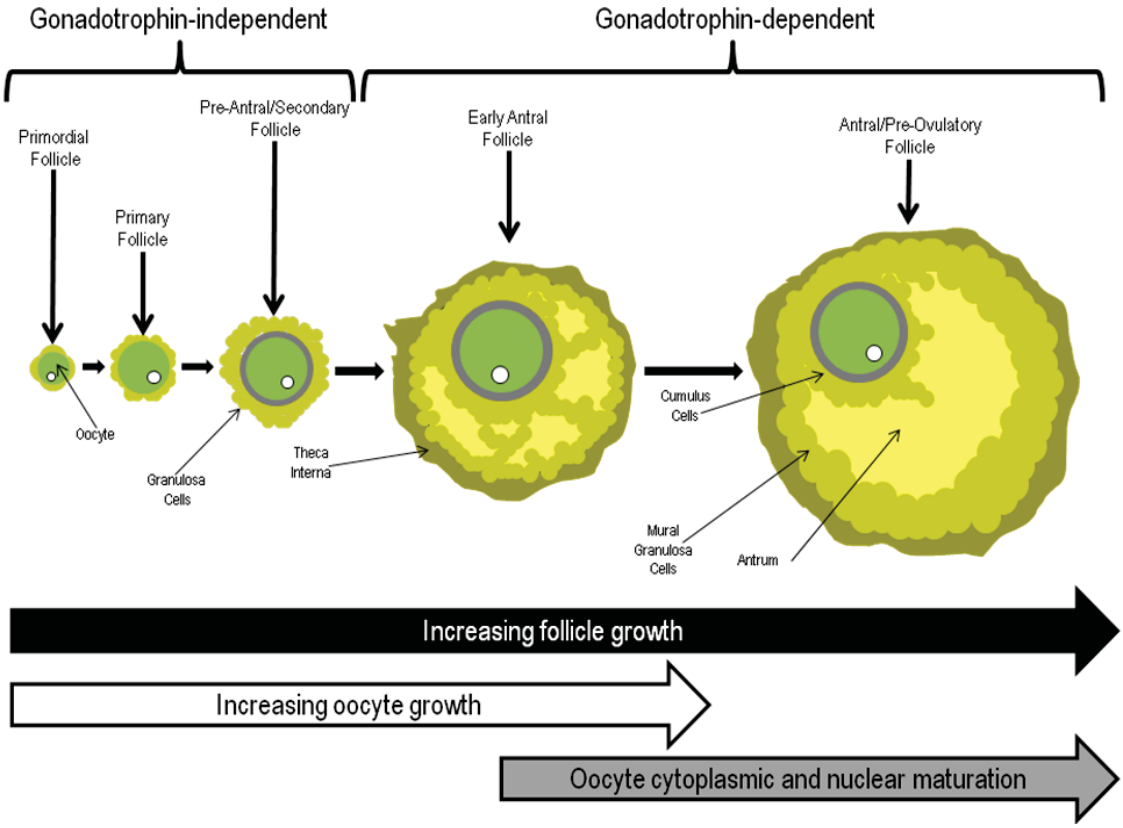

Figure 1.4: Diagram of folliculogenesis and the periods during the process when follicle growth, oocyte growth and cytoplasmic and nuclear maturation occur.
The different stages of folliculogenesis are characterised by increasing follicle growth and by proliferation and differentiation of the granulosa cells. Throughout folliculogenesis from the primordial stage to the antral stages the follicles increase in size as opposed to oocytes whose growth occurs from the primordial stage up until the pre-antral stage when the oocyte is fully grown. From the pre-antral stage up until the antral stage the oocyte undergoes maturation, which is essential to support embryonic development. From the primordial stage up until the late secondary stage growth of the follicle occurs without gonadotrophins and after this stage continued follicle growth is highly dependent on the gonadotrophins.

1.2.1.2 Primary Follicle

The transition from primordial follicle to the primary follicle, which is around 0.1mm in diameter, is characterised by granulosa cell proliferation, oocyte growth and development of the theca (Figure 1.4) [20, 28]. The single layer of squamous somatic cells surrounding the primordial germ cells proliferate by mitosis and become granulosa cells, which are cuboidal in shape and form a single layer surrounding the oocyte [26, 29, 30]. In addition to the proliferation of granulosa cells, the primordial germ cells emerge from their quiescent state and start to undergo oogenesis. These germ cells initiate their own growth phase and increase in diameter and volume by approximately 11% [28]. During this period of oogenesis the primordial germ cells transition into dividing oogonia that give rise to primary oocytes that enter the first meiotic division and arrest at metaphase I [26, 29, 30].

In conjunction with this growth phase the zona pellucida starts to develop around the oocyte. Three glycoproteins are secreted that make up the zona pellucida, which ultimately results in the separation of the oocyte from the granulosa cells [31, 32]. During the formation of the zona pellucida the granulosa cells form intercellular tight junctions as well as gap junctions into the oocyte membrane, the oolemma, via processes that pass through the developing zona pellucida [31, 33]. These gap junctions allow for bi-directional communication and allow nutrients, metabolites and growth factors into and out of the oocyte. Thus the oocyte is able to influence granulosa cell growth and differentiation and likewise the granulosa cells are able to influence growth of the oocyte, therefore perturbations to either can cause adverse effects in follicle development [31]. The zona pellucida is essential for normal follicle and oocyte development and it has previously been shown that mice treated with anti-zona pellucida antibodies result in poor gap junction formation between the oocyte and the granulosa cells impairing the cross talk with the oocyte, resulting in poor follicle and oocyte development [34]. The zona pellucida is also

important for fertilisation as it contains proteins that enable sperm binding to allow for penetration and thus fertilisation [31].

The granulosa cells of the primary follicles also begin to express receptors to the gonadotrophin, follicle-stimulating hormone (FSH), however, their growth remains gonadotrophin independent until the later stages of folliculogenesis. The exact signal for the initial recruitment of the primordial follicle into the primary follicle transition is yet to be determined, however the factors involved are thought to be intra-ovarian and produced by both the oocyte and granulosa cells. In vitro culture of cortical ovarian tissue results in activation of the primordial follicles, thus suggesting that within the ovary these follicles are under inhibitory control which allows them to be maintained in a dormant state [22, 32].

Anti-mullerian hormone (AMH) is one factor that has been demonstrated to inhibit but not completely block recruitment of follicles into the growing pool. AMH is a member of the transforming growth factor- β (TGF-β) super family and it is produced by granulosa cells at the primary follicle stage, with expression increasing as the follicle matures up until the pre-antral stage [25]. It is thought granulosa cell expression of AMH is required to maintain a balance between the number of primordial follicles to be activated and number that that remain in the resting pool [35-37]. A study in mice established that in the presence of AMH, FSH decreases thus suggesting AMH inhibits the initial recruitment of follicles by inhibiting FSH [38]. In studies performed in AMH-deficient mice it was found that the ovaries of 4 month old mice had a threefold increase in small non-atretic growing follicles and a decline in the number of primordial follicles when compared to their wild-type counterparts [36]. Interestingly, this increase began before the onset of puberty, since a higher number of growing follicles were already present at

day 25. Thus, suggesting that in the absence of AMH primordial follicles are recruited faster into the growing pool and as a result of this, the ovarian pool is prematurely depleted in the AMH-deficient mice [36]. Similarly, in the human, there has been a positive effect on the initiation of primordial follicle growth after treating with AMH, as culturing human ovarian tissue with recombinant AMH resulted in a reduction of the initiation of follicle recruitment, thus suggesting that AMH plays a regulatory role in the recruitment of follicles into the growing pool [25, 35, 37].

1.2.1.3 Secondary/Pre-Antral Follicle

Continued proliferation of primary follicle granulosa cells results in the oocyte being surrounded by several layers of cells, now known as a pre-antral or secondary follicle which has increased to around 0.2mm in diameter (Figure 1.4) [26]. The initiating factor for the development of the follicle from the primary to the pre-antral stage is not well established and is speculated to be gonadotrophin independent and controlled by oocyte paracrine and autocrine factors [39]. One such possible oocyte paracrine factor is a member of the TGF-β signalling family, growth differentiation factor-9 (GDF-9). Gene knockout of GDF-9 in mice resulted in failure of pre-antral follicle development, thus demonstrating that GDF-9 plays an essential role in development of the follicle to the pre-antral stage [39].

Even though pre-antral follicle development is gonadotrophin independent, these follicles do respond to luteinising hormone (LH) and FSH, thus suggesting that for optimal development to the pre-antral stage these hormones are required, as FSH receptors are present in the granulosa cells of rats [40], cows [41] and sheep [42]. Furthermore, in vitro it has been established that FSH is required for granulosa cell proliferation and differentiation and for the reduction in the number of atretic follicles in several species such as rodents, cattle and humans [22]. In rats, LH

receptors have been detected on the developing thecal layer of the pre-antral follicle and it is hypothesised that androgens are produced by the theca cells which are able to further stimulate formation of the FSH receptors on the granulosa cells thus further suggesting that FSH and LH are required for optimal follicle growth and development [30, 43]. Therefore, even though gonadotrophins may not be essential for follicle development at this stage they may play a role in supporting follicular development and preventing follicular degeneration [44].

Oocyte growth forms a significant part of pre-antral follicle development. It is during this growth period that the oocyte acquires the ability to support subsequent preimplantation embryo development. The growing oocyte is a metabolically active cell, it synthesises mRNAs and proteins that are important not only for oocyte growth but also support development through to the stages of the early preimplantation embryo [45]. During this phase of oocyte growth there is a 60-fold increase in oocyte volume resulting in an oocyte of 120μm in diameter compared to an immature oocyte within the primordial follicle which is around 30μm in diameter [20, 28]. The cytoplasm within the oocyte becomes highly organised by the re-organisation and modification of its organelles. Due to the increase in RNA and protein synthesis, the number of ribosomes and mitochondria increases in the growing oocyte.

1.2.1.4 Antral Follicle

Antral follicles are characterised by the presence of a fluid-filled cavity, called an antrum, which forms within the granulosa cells layers and is filled with follicular fluid. The exact initiating factor for the development of the follicle from the pre-antral to antral stage is not well established but this process occurs once the oocyte reaches a certain species-specific size and the cavities that form gradually becomes confluent to form one large antrum. In conjunction with antrum formation

the oocyte is capable of resuming meiosis [46]. As a result of antrum formation the granulosa cells surrounding the oocyte differentiate into a specialised cell type called cumulus cells, the granulosa cells are located around the periphery of the follicle, while the cumulus cells surround the oocyte (Figure 1.4). Together with the oocyte, the cumulus cells form a unit called the cumulus-oocyte complex (COC). Each follicle is enveloped by a basal lamina, a specialised type of extra-cellular matrix and separates the follicle from the theca cells. In humans, development of the follicle from the pre-antral stage to the early antral and preovulatory stages takes approximately 85 days, with the antral growth stage lasting until the follicles are approximately 2-4mm in diameter and preovulatory follicles increasing in diameter from 5mm to more than 20mm at ovulation [20].

Antral follicle development is dependent on the presence of the gonadotrophins, FSH and LH and occurs in waves of growth and regression [26]. FSH supports the growth of a group of antral follicles until the largest dominant follicle, the preovulatory follicle (also known as Graafian follicles) begins to produce estradiol and inhibin, which acts as an FSH suppressor and this follicle becomes dependent on LH. As the levels of FSH decline the remaining antral follicles regress via atresia and as a result the dominant follicle is the only follicle capable of ovulation. Ovulation occurs mid-cycle in response to the LH surge and the follicular material, primarily the granulosa cells, that remain behind transform into the corpus luteum, which predominantly produces progesterone, as opposed to estrogen. In humans, usually only one dominant preovulatory follicle develops during each menstrual cycle.

1.2.1.4.1 Follicular Fluid

The granulosa cells at all stages of folliculogenesis are avascular and do not possess tight junctions between the cells. Instead the granulosa cells are connected via gap junctions and this connection is essential as in mice, deletions of connexin-43 prevents secondary follicles from forming antra [47]. It is thought that the fluid within the antrum is a serum transudate as previous studies have shown that the electrolyte composition of the follicular fluid is similar to blood serum [48]. The antrum of the antral follicle forms when fluid, derived from serum accumulates in between the granulosa cells. This occurs due to the presence of an osmotic gradient between the follicle and the vasculature of the theca [49]. The movement of the fluid occurs via passive diffusion however, aquaporins found on the granulosa cells are thought to facilitate the movement of the fluid into the follicle. In vitro mouse follicle culture has demonstrated expression of *Aqp7* and *Aqp8* on granulosa cells [50]. Furthermore, in rats inhibition of aquaporins on granulosa cells resulted in reduced fluid diffusion [50].

The follicular fluid that surrounds the COC is an important microenvironment for the supply of nutrients to the granulosa and cumulus cells as well as the oocyte. In addition, the substances produced by the oocyte and its surrounding follicular cells are secreted into the follicular fluid and thus alter the composition further. Apart from being comprised of hormones, metabolites and secreted factors that are produced and secreted by the oocyte, granulosa and cumulus cells, it is also comprised of ions obtained from the blood serum. Thus, the components of follicular fluid are reflective of the activity of the follicular cells as well as being influenced by the systemic environment.

Follicular fluid concentrations of gonadotrophins FSH, LH and human chorionic gonadotropin (hCG) have been positively correlated with oocyte maturation and have been associated with increased fertilisation, thus demonstrating increased oocyte developmental competence [51]. A human in vitro study established that oocytes that were mature and able to be fertilised had an increased number of granulosa cells with LH receptors and thus were more responsive to these gonadotrophins. Another human study established that higher LH concentrations in the follicular fluid resulted in embryos that lead to a successful IVF outcome compared to those follicles with lower concentrations of LH [52]. The concentration of FSH in follicular fluid has also been examined in humans, with higher concentrations positively correlating with a mature oocyte that was able to be fertilised [53]. Steroid hormones have also been associated with fertilisation, with studies establishing a positive relationship between follicular fluid estradiol concentrations and fertilisation outcome [54, 55]. However, elevated follicular fluid concentrations of progesterone have been associated with abnormal fertilisation of oocytes [56]. These studies suggest that there is an intimate relationship that exists between the hormonal profile of the follicular fluid and the resulting oocyte developmental competence.

Recently, it has also been suggested that differences in the concentration of follicular fluid metabolites correlate with oocyte developmental competence. In humans it was established that elevated concentrations of glucose and decreased concentrations of lactate in the follicular fluid resulted in the failure of fertilised oocytes to cleave [57]. Another study examined the metabolic profile of human follicular fluid via Nuclear Magnetic Resonance and found that there was a negative correlation between the concentration of glucose with lactate and pyruvate and a positive correlation between the concentrations of lactate and pyruvate. However, this study only measured the metabolic profile of the follicular fluid and did not correlate its findings to embryo development or pregnancy outcome [57]. Due to the intimate relationship between these

metabolites the follicular fluid has a role in providing an energy source for the COC [58]. This study suggested that there may not be one individual metabolite concentration that will be able to provide a marker for the developmental potential of oocytes, but rather that the control of metabolism could be related to fertilisation outcome and ultimately pregnancy [58]. These studies in humans on the hormone and metabolite composition of follicular fluid have usually focussed on the identification of a marker for selection of a developmentally competent oocyte. Currently, little remains known about the follicular fluid environment in women that are known to have a poor IVF outcome, such as women of advanced maternal age and women that have reduced ovarian reserve.

1.2.2 The Pre-Ovulatory Follicle

As outlined above follicular maturation from the primordial follicle stage to the preovulatory stage is the culmination of a lengthy process in which a series of specific and timed events must occur to produce a follicle that contains a developmentally competent oocyte. As previously mentioned, both FSH and LH are essential for the development of the follicle from the early antral to the pre-ovulatory stage. In order for the selection and growth of one pre-antral follicle to continue a 10–30% increase in FSH levels are required and this is termed an FSH threshold. If this FSH threshold it is not reached initiation of pre-ovulatory follicle development does not occur and the follicles undergo atresia [59]. The mechanism behind how this selection process occurs without stimulating the remaining pre-antral follicles remains largely unknown. However, one theory suggests that once the pre-ovulatory follicle has been selected and has responded to the increase in FSH, the requirement of FSH to maintain pre-ovulatory follicle development is decreased. This hypothesis was tested in primates where gonadotrophin secretion was blocked and exogenous FSH was administered to initiate pre-ovulatory follicle development. After pre-ovulatory follicle development was evident, the concentration of exogenous FSH was decreased

back to baseline and pre-ovulatory follicle function was still evident. Thus, suggesting that once the pre-ovulatory follicle has developed it does not required the increased FSH levels to maintain its development and this may be due to the follicle increasing its sensitivity to FSH such that it is still able to function and develop. Therefore the decrease in FSH concentration results in the inability of the remaining pre-antral follicles to develop and instead they degenerate by atresia [60].

Granulosa cells initially only contain FSH receptors and during pre-ovulatory follicle development, in response to FSH, estradiol is able to be synthesised and secreted by the granulosa cells [61]. This rise in estradiol causes follicular growth through granulosa cell proliferation and as a consequence the number of FSH receptors increases, which enhances the responsiveness of granulosa cells to FSH [61]. FSH and estradiol also induce the development of LH receptors on granulosa cells, which is important for the continual development of the follicle and especially for the initiation of ovulation [61]. LH acts on both the thecal cells and the granulosa cells to produce estradiol and as a result of increasing estradiol concentration, FSH levels are suppressed via negative feedback. Thus, the follicle gradually becomes dependent on LH for continual growth and development [61].

Once the threshold concentration of LH has been reached, the LH surge occurs, which triggers ovulation of the COC. This surge in LH is responsible for the changes that occur in the follicle including the suppression of granulosa cell proliferation and the induction of gene expression in the granulosa cells that are required for luteinisation and the production of progesterone. Such genes include those that are pro-inflammatory factors (such as interleukin-1), angiogenic factors

(such as VEGF), proteases that are required for tissue remodelling and factors required for progesterone production (such as progesterone receptor [PGER]) [62].

The LH surge stimulates the production of prostaglandins by the granulosa cells via the Prostaglandin E Receptor 2 (PTGER2). Prostaglandins are essential during the process of ovulation as treating humans with a PTGER2 inhibitor resulted in disrupted ovulation [63]. In addition, *Ptger2* null mice are unable to undergo ovulation or produce prostaglandins, however after administration of exogenous prostaglandins ovulation was able to progress [64]. Progesterone is also important for ovulation as it has been found *Pger* knockout mice were unable to ovulate [65].

1.2.2.1 The Cumulus Oocyte Complex

Starting from the early stages of folliculogenesis the interactions between the oocyte and granulosa cells, essential for controlling the rate of follicle growth and differentiation, are mediated by either gap junctions or paracrine signalling. As mentioned previously the COC forms during the antral stage of folliculogenesis and the granulosa cells around the oocyte differentiate into cumulus cells. After differentiation has occurred the communication between the oocyte and cumulus cells is important for oocyte nuclear and cytoplasmic maturation, which determines the developmental competency of the oocyte. The positive effect of cumulus cells on oocyte development and consequently on early embryo development has previously been reported in many mammals including murine, ovine, bovine and humans [66]. These interactions between the oocyte and its surrounding follicular cells are necessary both before and after the LH surge [26, 67].

Before the LH surge, the germinal vesicle oocyte of the antral follicle has been shown to be a powerful regulator of cumulus cell function, as these immature oocytes were more effective in suppressing LH receptor mRNA expression in mouse granulosa cells compared to mature oocytes (oocytes with a polar body present) [67]. Germinal vesicle oocytes are also able to promote the expression of cumulus cell transcripts, whereas in granulosa cells they suppress transcription. Apart from influencing granulosa cell proliferation and differentiation the germinal vesicle oocyte also regulates the metabolic activity (e.g. glycolysis) of the cumulus cells within the COC [68]. After the LH surge the oocyte regulates the expression of cumulus cell genes responsible for the mucification and expansion process, which are essential for ovulation [69].

1.2.2.1.1 Bi-Directional Communication

At the pre-antral stage of follicle development, oocyte growth is nearly complete and as discussed above the follicles are now reliant on gonadotrophins for the selection and growth of one follicle to continue its development [21]. However, the development of the follicle from the pre-antral to the pre-ovulatory stage is a complex and crucial event, which is dependent on the co-ordination of specific signals. This transition from the pre-antral to pre-ovulatory stage is especially important for the production of RNA and proteins required for maturation of the oocyte and subsequent pre-implantation development [21, 22, 70]. It is not until these final stages of folliculogenesis, that the oocyte is able to resume the first meiotic division and progress to metaphase II. However, for the oocyte to accomplish this, communication between the different cell types within the follicle is important. The communication between cumulus cells and the oocyte occurs in a bi-directional manner and is essential for cumulus cell development and for maintaining oocyte health (Figure 1.5) [21, 26].

As mentioned above there are two modes of communication that occur between the oocyte and the cumulus cells and vice versa. The cumulus cells form gap junctions with the oocyte by penetrating the zona pellucida to connect with the oocyte membrane by trans-zonal cytoplasmic projections (Figure 1.5). These gap junctions, made up of units called connexins, allow for the passage of low molecular weight (<1000M_r) molecules [31], such as ions, cAMP [70], amino acids [29], metabolites, such as pyruvate [29], nucleotides [70] and small regulatory molecules [70], all of which are required for oocyte nuclear and cytoplasmic maturation and therefore the development of a viable oocyte [26, 29, 67, 70]. Larger molecules pass into the oocyte via receptor mediated endocytosis. Throughout the stages of folliculogenesis and oogenesis there is continuous communication via these gap junctions between the oocyte and its follicular cells and they have been detected from the primordial stage of folliculogenesis in mice and in the secondary follicle in cattle thus the stage of folliculogenesis when gap junctions are expressed is species specific [22]. Furthermore, this mode of transport is especially important during the growth period of the oocyte, during the primordial to pre-ovulatory follicle stages, when it increases in size due to the accumulation of water, ions, carbohydrates and lipids [22]. Homozygous null mutations to connexin-43, a member of the connexin family, in the ovaries of mice have demonstrated that folliculogenesis is unable to proceed past the primary follicle stage [71]. Furthermore, mice deficient in connexin-37, another member of the connexin family and an essential component for the gap junctions, display a lack of pre-ovulatory follicles and meiotic competence is unable to be achieved in the oocytes [72]. Taken together, it is evident the there is an essential role for gap junctions during folliculogenesis and oogenesis.

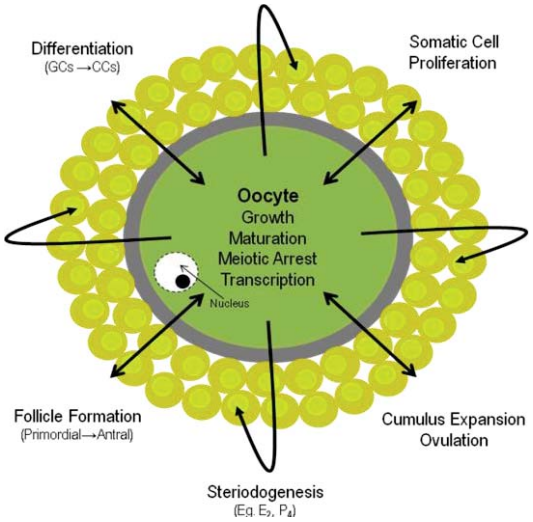

Figure 1.5: Schematic representation of the bi-directional communication that occurs between the cumulus cells and the oocytes.
This bi-directional communication is essential for many process needed for the development of a viable oocyte and for the development of cumulus cells. The main two types of communication that occurs between cumulus cells and the oocyte are paracrine signalling (curved arrows) and via gap junctions (straight arrows) (adapted from Eppig, 2001) [26].

This gap-junctional communication is of particular importance for metabolism, particularly for the generation of adenosine triphosphste (ATP). The oocyte requires energy in the form of ATP but it lacks the ability to utilise glucose as its energy source, compared to cumulus cells which are able to utilise glucose and this has previously been demonstrated by measuring the glycolytic activity of cultured COCs compared to denuded oocytes (oocytes which have had their cumulus cells removed) [68]. This study in mice demonstrated that denuded oocytes cultured in media containing glucose without cumulus cells were unable to mature to metaphase II, however if either pyruvate or oxaloacetate were in the culture media oocytes were able to mature to the metaphase II stage. However, when cumulus cells were present progression of the oocyte to metaphase II occurred in the presence of glucose [73]. Thus, oocytes require the metabolite pyruvate produced from the metabolism of glucose by cumulus cells as a source of energy to support growth and maturation. Therefore, cumulus cells are required to metabolise glucose to provide pyruvate needed for the oocyte, which is transported into the oocyte via gap junctions and the oocyte is then able to generate ATP [70]. However, it has been documented that the

glycolytic activity of cumulus cells is regulated by the oocyte and this has previously been researched by examining cumulus cell transcripts involved in the process of glycolysis [68]. Gene expression in mice of glycolytic enzymes such as Phosphofructokinase Platelet (*Pfkp*) and Lactate Dehydrogensae A (*Ldha*) in cumulus cells were found to be down-regulated in cultured cumulus cells compared to COCs. However, if cumulus cells were cultured with oocytes normal glycolytic enzyme expression was restored [68]. This demonstrates that cooperation between the oocyte and the cumulus cells occurs and the metabolic activity of the cumulus cells is regulated by the oocyte [68, 69].

The second mode of communication between the oocytes and the cumulus cells is paracrine signalling (Figure 1.5). This is of particular importance as it has been well-established that folliculogenesis is unsuccessful without oocyte paracrine signalling [67]. When removed from the antral follicular environment, oocytes undergo spontaneous meiotic resumption in culture. Subsequent studies have demonstrated that ovarian follicular cells, particularly cumulus cells, are essential for growth and for the timing of both nuclear and cytoplasmic maturation of the oocyte. The oocyte is able to regulate differentiation, proliferation and functions of cumulus cells by the secretion of OSFs, such as GDF-9 and bone morphogenic protein-15 (BMP-15). These oocyte secreted factors play an important role in the differentiation of granulosa cells into cumulus cells [69]. OSFs have the ability to regulate the function of the surrounding cumulus cells by promoting cumulus cell growth, proliferation and expansion and they are also able to prevent luteinisation of cumulus cells by regulating steroidogenesis [26]. These findings support the concept that the oocyte is able to promote the maintenance of the cumulus cell phenotype and thus maintain a specialised environment via the production and secretion of paracrine factors.

BMP-15 and GDF-9, are members of the TGF-β super family and in humans both can be detected in the primary follicles of the ovary with continued expression and changing roles throughout the subsequent stages of folliculogenesis [74]. However the expression of GDF-9 within the follicle is species-specific, for example GDF9 mRNA and protein are expressed in the oocytes of the primary follicles in mice, rats and humans whereas in the bovine and ovine it is expressed in the primordial follicles [75]. In addition, it was originally thought that expression of GDF-9 was exclusive to the oocyte but it has now been detected in human cumulus cells [75]. *Gdf9* female knockout mice have been demonstrated to be infertile due to follicles being arrested at the primary stage of folliculogenesis, thus indicating GDF-9 is essential for follicle development [39]. In contrast, in *Bmp15* knockout mice there was no effect on follicle development but the mice had reduced ovulation and fertilisation rates [76]. However, the follicles in sheep homozygous for BMP15 were arrested at the primary stages of folliculogenesis and as a result were infertile [77]. These studies demonstrate that both GDF-9 and BMP-15 are essential for folliculogenesis and their actions are species specific.

Another example of the importance of these paracrine factors is in response to FSH, mouse oocytes secrete paracrine signals that allow cumulus cells to undergo expansion, an essential process that occurs during ovulation. However, as important as the oocyte is in producing paracrine factors, follicular cells are also able to produce paracrine factors that affect the oocyte. An example is porcine granulosa and cumulus cells which are able to produce cumulus expansion-enabling factor (CEEF), which is involved in cumulus cell expansion, which is different to mouse, as mentioned above it is the oocyte that produces the paracrine factors involved in cumulus cell expansion [78]. This indicates that the production and regulation of paracrine factors by the oocyte, cumulus cells and granulosa cells is also species specific.

Therefore, the development of the oocyte, ovarian follicular cells and differentiation of granulosa cells into cumulus cells are intimately associated, and any perturbations to either the oocyte or ovarian follicular cells are a reflection of the follicular environment. Due to the existence of this unique relationship between the oocyte and the follicular cells, granulosa cells and cumulus cells can provide us an indication of the developmental competence of the oocyte.

1.2.2.2 Oogenesis

1.2.2.2.1 Oocyte Maturation

Oocyte developmental competence is gradually obtained throughout a series of structural and functional changes, that occur during folliculogenesis with one of the final steps in the development of an oocyte being oocyte maturation [70, 79, 80]. The process of maturation in the oocyte is commonly designated into two aspects, nuclear maturation and cytoplasmic maturation, however even though these are two separate functions they are intimately related. It is during these processes that the oocyte gains the ability to undergo fertilisation and subsequently sustain embryonic development [70]. The completion of nuclear maturation does not mean that the oocyte is able to be fertilised and support early embryonic development, as there are still many processes that need to happen, independent to nuclear maturation, within the cytoplasm for this to occur [81].

1.2.2.2.1.1 Nuclear Maturation

Oocytes acquire meiotic competence (the ability of the oocyte to resume meiosis and become nuclearly mature) during the time of antrum formation of the follicle during folliculogenesis and at this stage human oocytes are approximately 80% of their final size [81]. The changes that occur

during nuclear maturation include; transcription and translation of RNA, DNA replication and repair, chromosome condensation, spindle formation and preparation for fertilisation [21].

Oocytes undergo a process of meiosis to produce a haploid gamete (an oocyte with 23 chromosomes), which is achieved by completing one round of DNA replication and two cell divisions. Oocyte maturation is initiated by a preovulatory surge of gonadotrophins, in particular by the LH surge. Up until the LH surge the oocyte is arrested at the germinal vesicle (GV) stage of the first meiotic prophase, also known as the diplotene stage [82]. Meiotic resumption is characterised by the breakdown of the nuclear membrane within the oocyte, also called germinal vesicle breakdown (GVBD). The first division (meiosis I) results in the 23 homologous (pairs) chromosomes or 46 chromosomes (2N) separating just prior to ovulation. This is characterised by the formation of the first polar body (which contains half of the chromosomes), the other 23 chromosomes (N) remain within the oocyte which arrests at metaphase II [66, 82]. The second division (meiosis II) involves the segregation of sister chromatids and this happens during fertilisation. If either of these divisions do not occur correctly this could result in abnormal separation of the chromosomes leading to aneuploidy and 80% of errors during meiosis are thought to occur during meiosis I [125]. Aneuploidy can result in poor embryo development [83] and affects pregnancy outcome as it can cause implantation failure [18], miscarriage [84] and birth defects [85]. During the process of meiosis the oocyte requires a significant amount of energy, which is met by the transport of energy substrates via the gap junctions from the cumulus cells into the oocyte and by cumulus cell oxidative phosphorylation of glucose to provide pyruvate to the oocyte [18, 29].

The resumption of meiosis is thought to occur due to the actions of gonadotrophins on the surrounding oocyte follicular cells, rather than direct actions on the oocyte, due to the lack of gonadotrophin receptors on the oocyte and that removal of the oocyte from the follicle results in spontaneous resumption of meiosis [21, 66, 80, 82]. The molecular mechanisms behind meiotic resumption may require the removal of the meiosis inhibiting factors and/or the acquisition of the oocyte maturation signals [82]. As mentioned previously, gap junctions between the cumulus cells and the oocyte allows for the transport of regulatory substances important for oocyte nuclear maturation, such as steroids, calcium, inositol trisphosphate (IP_3), cyclic adenosine monophosphate (cAMP) and purines, between them (Figure 1.6) [66]. Thus, communication with the cumulus cells is essential for correct timing of nuclear maturation.

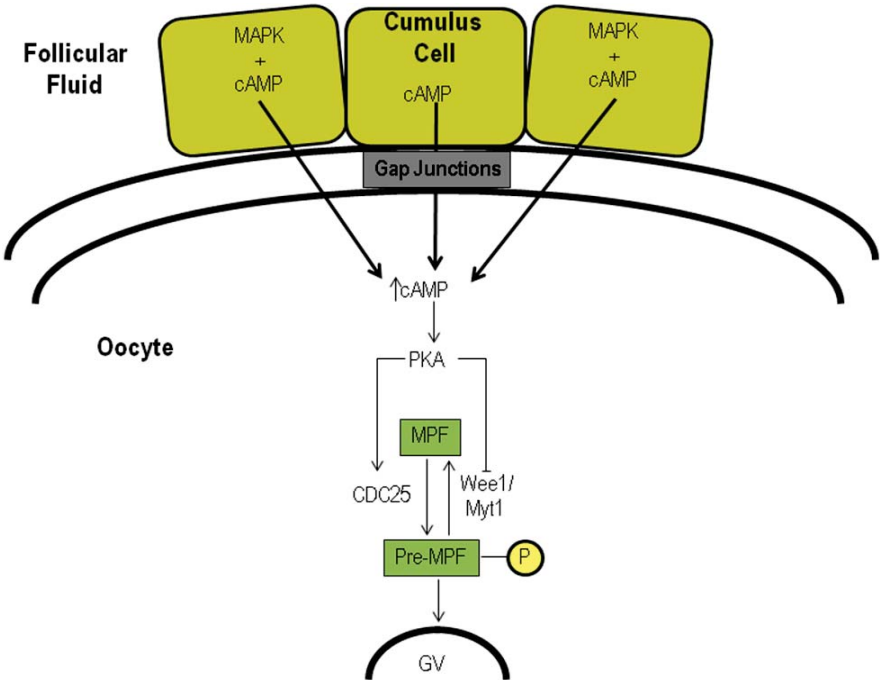

Figure 1.6: Schematic showing the process of oocyte maturation within a cumulus-oocyte complex.
Cyclic adenosine monophosphate from the cumulus cells could be entering the oocyte via gap junctions and thus keeping maturation promoting factor phosphorylated and hence deactivated. Once cAMP level from within the oocyte decreases (i.e. a loss of the gap junctional communication between the two types of cells e.g. during cumulus expansion) MPF is activated (dephosphorylated) and thus causes germinal vesicle breakdown and the resumption of meiosis.

Once cAMP enters the oocyte it is thought that it acts as a second messenger that triggers the cAMP-dependent protein kinase A (PKA) to phosphorylate protein maturation promoting factor (MPF), a complex made up of the sub-units CDK1 and cyclin B, via the CDC25 phosphatase, keeping it in its inactive state (pre-MPF), or dephosphorylate it via kinase WEE1/MYT1, thus activating it (Figure 1.6) [82]. Therefore, high levels of cAMP results in PKA acting upon CDC25, thus phosphorylating MPF and keeping it in it pre-MPF state and maintaining meiotic arrest [6, 80, 82]. Hence, a decrease in cAMP levels, possibly induced by the loss of gap junctions between the oocyte and its surrounding cumulus cells, causes the opposite effect with meiosis resuming. This occurs by PKA acting upon WEE1/MYT1, which allows for the dephosphorylation of MPF and allowing germinal vesicle breakdown (GVBD) to occur and meiosis to resume [82]. In addition, activation of mitogen-activated protein kinase (MAPK) in the surrounding cumulus cells may be having an effect. However, the exact mechanisms on how this occurs or the interactions between cAMP and MAPK still remain unknown [82].

1.2.2.2.1.2 Cytoplasmic Maturation

Developmental competence (the ability of the oocyte to develop into a healthy embryo that is capable of forming a healthy pregnancy) is related to oocyte cytoplasmic maturity and this maturation process occurs concurrently with nuclear maturation. Unfortunately, currently the mechanisms behind cytoplasmic maturation still remain unclear and there is no method to measure its completion, apart from successful fertilisation, embryonic development and fetal development [70].

At different time points during the maturation process the oocyte displays polarity, spatial patterning and several proteins display a polarised cortical arrangement [66]. The cytoplasmic

organelles the mitochondria, endoplasmic reticulum and Golgi apparatus and the Balbiani body (a concentration of material that contains RNA and proteins) change their location to surround the germinal vesicle. In addition, the mitochondria number increases to approximately 200,000 in a fully grown human oocyte thus mitochondrial activity also increases and there are modifications to the Golgi apparatus and ribosomes also accumulate [81, 86]. Once germinal vesicle breakdown has occurred the mitochondria disperse during extrusion of the first polar body. The cytoplasmic changes that occur during this process are mRNA transcription, protein translation, post-translational changes in proteins and ultra-structural changes, such as epigenetic/chromatin remodelling [86].

If cytoplasmic maturation does not occur successfully the oocyte may be unable to be fertilised or it will be unable to develop into a viable embryo and studies in mice have established that blastocyst formation is dependent on the transcripts generated during oocyte maturation and mRNAs and proteins involved in fertilisation and regulation of embryo development are also accumulated at this stage [87]. In addition, inappropriate cytoplasmic maturation of the oocyte results in the failure of successful male pronuclear formation, consequently causing an increase in abnormalities after fertilisation [66].

1.3 Cumulus Oocyte Complex Metabolism

As mentioned above the human oocyte exists in a complex with the cumulus cells, whereby the cumulus cells can utilise glucose as an energy source releasing pyruvate, via the Embden-Meyerhof pathway, which can subsequently be transported into and utilised by the oocyte (Figure 1.7). Thus, glycolysis accounts for a large proportion of glucose metabolism by the COC and therefore has an important role in providing the majority of energy for the oocyte with a metabolic

co-operativity occuring between the oocyte and its cumulus cells. Once pyruvate has entered the oocyte it is able to be metabolised via the tracarboxylic acid cyle (TCA) and then by oxidative phosphorylation. The pentose phosphate pathway is also an important glucose metabolic pathway during maturation of the oocyte however, in the COC only a small proportion of glucose is metabolised this way due to the majority of glucose metabolism being metabolised via glycolysis within the cumulus cells (Figure 1.7) [88].

1.3.1 Cumulus Cell Metabolism

Cumulus cell glycolysis is the predominant pathway for glucose metabolism within the COC. The ability for cumulus cells to metabolise glucose is due to the presence of glucose transporters and the high activity of glycolytic enzymes such as *PFKP* and *LDHA* [68]. The high rate of glycolytic activity of cumulus cells is required to generate ATP and produce pyruvate, lactate, malate and oxalacetate, which are utilised as oxidative substances by the oocyte [89]. During the process of glucose metabolism the reducing agent nicotinamide adenine dinucleotide (NADH) is produced. The metabolite NADH is primarily synthesised by the cumulus cells during glycolysis by the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [89]. NADH also has an important role in both cytosolic and mitochondrial redox potential [90]. The redox state describes the sum of interactions between oxidised and reduced forms of a variety of molecules including the ratio between NAD(P):NAD(P)H, FAD:FADH2, reduced glutathione:oxidised glutathione [90].

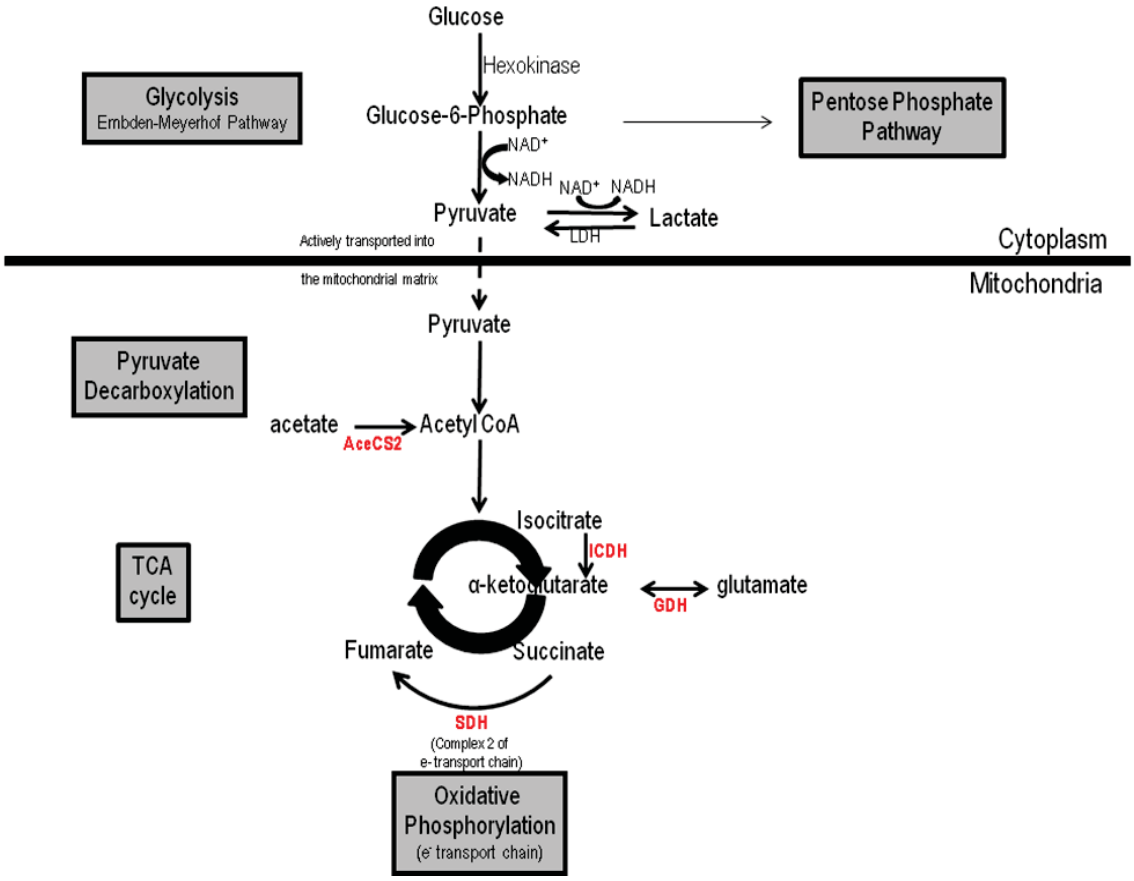

Figure 1.7: Diagram showing the possible different energy metabolism pathways in which cumulus-oocyte complexes utilise.
There are three possible pathways; glycolysis, the pentose phosphate pathway (PPP) and the tricarboxcylic acid (TCA) cycle.

1.3.2 Oocyte Metabolism

As mentioned previously, in contrast to cumulus cells, the oocyte lacks the capacity to undergo glucose metabolism, to the level required to maintain viability or meiotic maturation, thus it this heavily reliant upon the cumulus cells to produce oxidative substances (pyruvate, lactate, malate and oxalacetate) which are required for its own metabolism. In the absence of cumulus cells, oocytes in mice and cows exhibit very low glucose uptake, glycolytic activity and glucose oxidation [91, 92]. Furthermore, the human oocyte expresses only one of the facilitative glucose transporter isoforms (GLUT1) compared to cumulus cells that express four isoforms [93]. The oocyte requires pyruvate as its main energy substrate and it is able to metabolise pyruvate through the TCA cycle and through oxidative metabolism (both explained in more detail below),

which produces the majority of ATP (Figure 1.8) [94, 95]. Targeted deletion of pyruvate dehydrogenase, an enzyme required for metabolism, resulted in defects to both nuclear and cytoplasmic maturation [96]. Lactate is known to be abundant in follicular fluid and in the reproductive tract and it is able to be oxidised to pyruvate by lactate dehydrogenase, however, in denuded mouse oocytes the generation of pyruvate from lactate does not fuel mitochondrial ATP production and it is instead important for the regulation of cytosolic redox state [90]. NADH is also able to be utilised by the oocyte via a reaction catalysed by α-ketoglutarate dehydrogenase and malate dehydrogenase of the TCA cycle. Thus, within the oocyte the majority of ATP generated is via mitochondrial respiration through the metabolism of pyruvate with the levels of glucose uptake, glycolytic activity and glucose oxidation being comparatively low [17, 97-99].

1.3.3 Mitochondria

Mitochondria are double-membrane bound organelles found within the cytoplasm. Compared to other cell types, which contain between 1 and 10^3, the oocyte has the largest number of mitochondria with approximately 10^5 mitochondria located within a mature human oocyte [100] and they each contain their own specific mitochondrial DNA (mtDNA), which is distinct from nuclear DNA [17, 101-103]. There has been evidence that suggests abnormal mitochondrial function in somatic cells is linked with pathologies such as diabetes, ageing and some neurodegenerative disorders, cancer and infertility [104, 105].

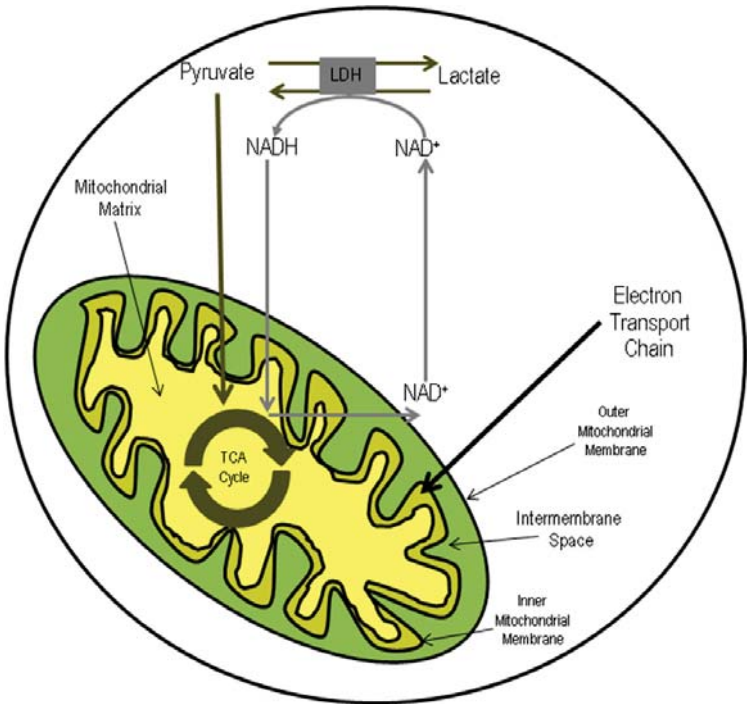

Figure 1.8: Mitochondrion within a cell and the processes by which ATP is produced.
Pyruvate is the metabolite required for the oocyte to produce ATP. Once pyruvate enters the oocyte it is able to enter the mitochondria where it is utilised by the TCA cycle to produce ATP (adapted from Wilding et al., 2001 [94]).

Mitochondrial number increases significantly in conjunction with the increase in cytoplasmic volume during oogenesis. During this period of oocyte growth, the mitochondria within the oocyte also change ultrastructure, with primary oocytes containing mitochondria with numerous transversely orientated cristae, however immature oocytes have round and oval shaped mitochondria with columnar-shaped cristae and at ovulation the mitochondria have a spherical immature structure, are highly vacuolated and have a dense matrix with only a few cristae [106]. As mentioned previously, during oocyte maturation the mitochondria are re-located to different areas within the oocyte presumably due to differences in energy requirements [94, 101, 106].

In addition to energy production, mitochondria within the oocyte also have an important role in the regulation of sperm-triggered calcium oscillations, which are important for oocyte activation, which occurs during fertilisation [106, 107]. Oocyte activation is induced by an increase in the concentration of intracellular calcium and the mitochondria within the oocyte may be acting as a

calcium store and may have a role in the oscillations, as well as in the maintenance of calcium clearance. These calcium oscillations trigger the mitochondria to produce a burst of ATP at fertilisation, via the up-regulation of oxidative phosphorylation, which is also important for the maintenance of these oscillations [106]. Therefore, oxidative phosphorylation only increases when ATP is needed and this is triggered by calcium oscillations. This is beneficial as the production of reactive oxygen species (ROS) by the electron transport chain (ETC) is reduced, which limits the mitochondria to low amounts of oxidative stress [106].

Mitochondria are also involved in the process of apoptosis, therefore down regulation of survival-promoting signals result in the apoptotic pathway being activated. A key regulator of apoptosis is the β-cell Lymphoma 2 (BCL2) family of proteins, which are both anti-apoptotic and pro-apoptotic. Their main site of target is the mitochondrial membrane and they are able to regulate the release of cytochrome C (CytC), a key component in the cascade of event that results in apoptosis [108]. Members of the BCL2 family have important roles in follicular growth and atresia by regulating oocyte, cumulus and granulosa cell apoptosis. Studies in mice deficient for BCL2 have demonstrated a decrease in the number of oocytes and primordial follicles, conversely over expression of BCL2 resulted in a decrease in granulosa cell apoptosis of antral follicles [108]. BAX, a member of the BCL2 family, has been demonstrated to be expressed in both oocytes and granulosa cells, with strong expression in the granulosa cells of atretic follicles compared to healthy follicles in the human ovary [108]. Considering this family of proteins initiate the release of cytochrome C it has been suggested the mitochondria are involved in the apoptotic process that occurs in granulosa cells. Thus the mitochondria may be essential in determining whether follicles undergo atresia or continue to develop [108, 109].

1.3.1.1 TCA Cycle

Within the oocyte ATP can be produced via aerobic respiration [98]. Aerobic respiration utilises oxygen and in this situation pyruvate in the cytoplasm is able to enter the mitochondria. Once in the mitochondria, it is broken down via a series of chemical reactions and the energy produced is utilised in a cyclical reaction, called the TCA cycle, from this 12 ATP is produced via the ETC (Figure 1.9) [98].

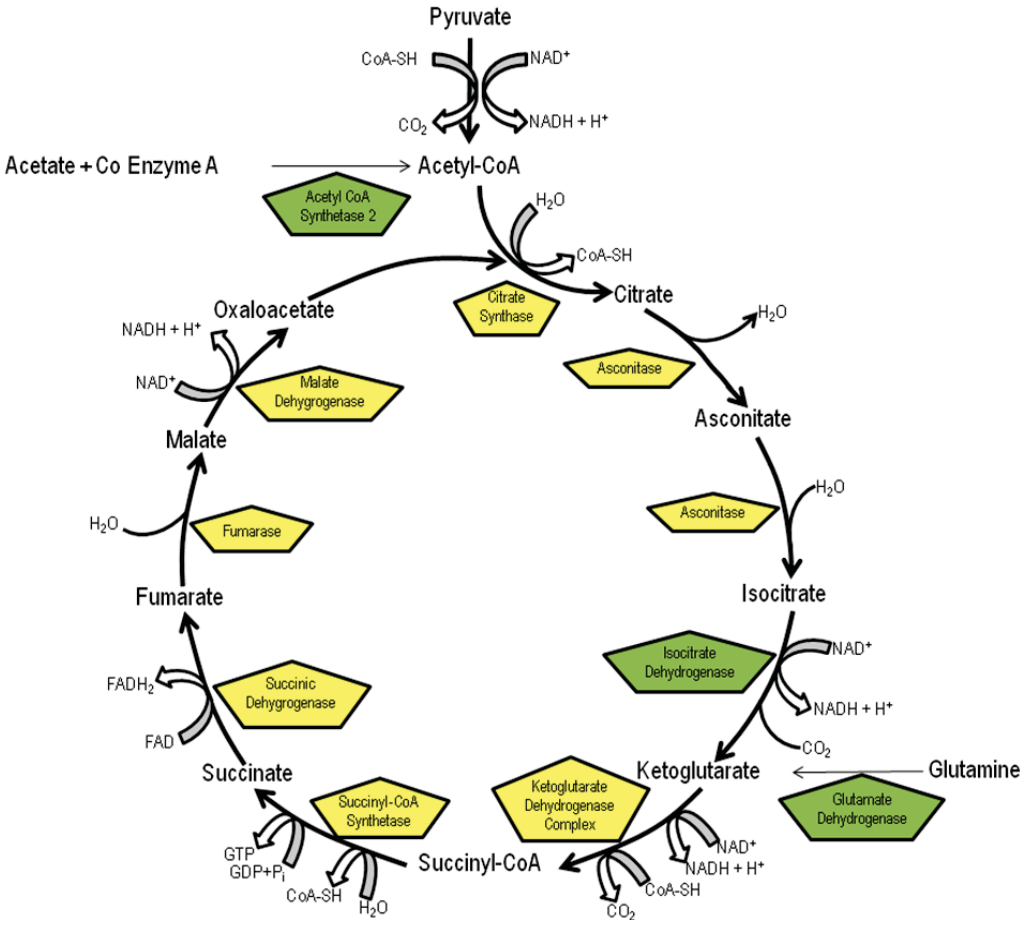

Figure 1.9: Schematic of the tricarboxcylic acid (TCA) cycle.
Within the mitochondria, the energy produced from the breakdown of pyruvate is able to be used in the various reactions that occur within the TCA cycle and these reactions have the ability to form adenosine triphosphate (ATP). In addition the reaction that occurs between succinate and fumarate is important at complex II of the electron transport chain.

In addition to pyruvate, lactate and glucose, COCs may also have the ability to utilise amino acids as an energy supply. In response to the LH surge cumulus cells are able to convert glutamine to α-ketoglutarate via the enzyme glutamate dehydrogenase. α-ketoglutarate can then be used in

the TCA cycle to produce ATP. Production of energy in this way is important for oocyte maturation in various species [110].

1.3.1.2 Electron Transport Chain

Energy for the oocyte is produced by mitochondria in the form of ATP through the process of oxidative phosphorylation. This process involves electron transport chain (also known as the mitochondrial respiratory chain) and is made up of four complexes (I–IV). Mitochondria are bound by two membranes; the outer mitochondrial membrane (OMM), which is permeable and allows the passage of molecules up to 10kDa in size and the inner mitochondrial membrane (IMM), which is impermeable and contains the enzymes (complexes) involved in the ETC [98]. The ETC utilises electron donors, produced by the TCA cycle, to produce ATP via a sequence of redox reactions. Electrons are led through the complexes of the ETC to the final acceptor, molecular oxygen. Proton pumping, due to electron transfer, into the intermembrane space, occurs at complexes I, III and IV and creates a chemical and electric gradient, which is used to control the production of ATP by complex V (ATP synthase). [106]. Complexes I and II oxidise products that are produced during the TCA cycle and the electrons produced from this process are transferred to ubiquinone (also known as co-enzyme Q10) and then to complex III. This complex reduces cytochrome C, also a member of the electron transport chain, and transfers the electrons to complex IV. Thus, energy is produced from transferring electrons along the transport chain and this energy is used to release protons into the mitochondrial intermembrane space creating a gradient that is made up of both charge and pH. ATP sythase, another component of the electron transport chain allows the protons back into the mitochondrial matrix and this process supplies the energy needed to phosphorylate ADP into ATP and therefore the energy supply [98]. However, it is thought that ROS is continuously produced in the mitochondria due to the ‘leakage’ of high energy electrons along this chain [111, 112].

To supply energy the mitochondria utilise approximately 85-90% of a cell's oxygen supply and as a consequence harmful reactive oxygen species (ROS) are also produced [106]. Therefore, it is not surprising that mitochondria are the major producers of ROS. At complexes along the ETC, electrons are able to react with oxygen and other electron acceptors to produce free radicals, which can potentially have adverse effects upon the mitochondria and ultimately the cell. Thus, mitochondria require protection and this is achieved by the presence of a range of anti-oxidants and by several enzymatic defence systems within the cytoplasm [106]. Uncoupling proteins (UCPs), also located on the IMM, are essential for controlling mitochondrial ROS production. They are able to do this by allowing protons back into the mitochondrial matrix and thus decreasing the total movement of protons thereby protecting the cell from the generation of ROS as the protons are isolated to the mitochondrial matrix and are unable to react to produce ROS [106].

1.3.1.3 Mitochondrial DNA

Mitochondrial DNA (mtDNA) is a 16.6kb double stranded circular genome that encodes 13 of the proteins that are involved in the electron transport chain [105]. mtDNA is always maternally inherited and even though sperm carries paternal mitochondria into the oocyte it is thought that they are usually destroyed immediately after penetration into the oocyte or during the early embryonic stages of development [17, 18].

Longitudinal developmental studies on mice have demonstrated that mtDNA replication begins during primordial germ cell formation and at this stage it is thought that there are approximately 200 mitochondrial genomes enclosed with the immature mitochondria. As a result of the number of mitochondria increasing during the process of oogenesis the number of mtDNA copies also

increases so that by the time the oocyte is fully developed each oocyte contains about 10,000 mtDNA copies, organized at 1-2 copies per organelle. This exponential increase in copy number ceases after fertilisation and does not start to increase again until after implantation occurs [114]. Studies have shown that there is large variation in mtDNA copy number between oocytes, the exact significance of this still remains unknown, however there have been studies that have shown an association with decreased mitochondrial copy number and poorer fertilisation outcome and also with ovarian insufficiency [115, 116].

Mitochondrial DNA lacks protective histones and the DNA repair capabilities that exist in the nucleus, and together with the location of the mtDNA near the inner mitochondrial membrane they can be a target of oxidative attack [90]. This may cause a cascade of events that ultimately causes mitochondrial dysfunction, which in turn affects ATP production and other cellular processes that require energy [98]. High levels of ROS are known to have detrimental effects to oocyte maturation and embryo development and it can cause mitochondria-dependent apoptosis of the oocyte and early embryo. As mammalian oocytes contain both anti-apoptotic and pro-apoptotic factors the balance between these influences oocyte survival. It has been established that mitochondria are involved in the apoptotic cascade of events [106]. ROS produced by the mitochondria, are known to induce cytochrome C leakage [17, 106].

1.3.1.4 Regulation of Mitochondrial Activity

Protein post-translational modifications are one of the most efficient processes for regulating cellular physiology [117]. Lysine residues in proteins can be subjected to various post-translational modifications including methylation, acetylation, biotinylation, ubiquination, succinylation and malonylation [117-119]. Post-translational acetylation of proteins is the covalent

addition of an acetyl group to the ε-amino group of lysine residues. Mitochondrial protein acetylation is fast becoming recognised as a fundamental mechanism for regulating the activity of mitochondrial proteins and overall mitochondrial function [120]. This simple modification can influence many protein functions including transcriptional activity, celluar localisation, protein stability and protein activity. The acetylation state of proteins results from the actions of histone acetytransferases (HATs) and histone deacetylases (HDACs), these enzymes catalyse the addition or removal of an acetyl group from the lysine residue. A proteomic study of cellular proteins revealed that a large number of mitochondrial proteins are targets of reversible lysine acetylation and that this type of acetylation was a post-translational modification and could occur in the mitochondria [121]. A study has shown that about 20% of all mitochondrial proteins are acetylated [121] which suggests that enzymes that are involved in glycolysis and the TCA cycle may actually be acetylated.

It has been established that mitochondrial protein acetylation is sensitive to metabolic perturbations as in the liver of mice mitochondrial protein acetylation increased during fasting with 14% of acetylated mitochondrial proteins unique to fed mice and 24% unique to fasted mice [121]. Interestingly, the acetylation of mitochondrial proteins is also increased in mice during long-term high fat diet feeding [122]. Therefore, an altered metabolic state such as nutrient deficiency or nutrient excess is able to increase the amount of mitochondrial proteins that are acetylated [120].

1.4 Ageing and Ovarian Reserve

1.4.1 Ageing

Reproductive ageing results in a decline in fecundity and this can be attributed to changes that occur in ovarian function, the loss of follicles from within the ovary and a reduction in oocyte quality that occurs with age. The decline in oocyte viability with increasing maternal age suggests that there is a factor within these oocytes that degenerates with age and such factors could be a decrease in protein synthesis, errors in DNA synthesis or the inability to form an appropriate spindle which is important for normal chromosome segregation and for all of these processes to occur there is a need for energy, which implicates the mitochondria [4]. However, other reasons for the decline in oocyte quality could be attributed to exposure to environmental factors, such as radiation, xeno-estrogens and heavy metals, which may cause damage to the oocyte during a woman's life or ovulation of the most viable oocytes first thus leaving the less viable oocytes behind and hence a gradual decrease in oocyte quality occurs [123].

It is thought the number of follicles declines dramatically once numbers fall below a critical level of 25,000 and this usually occurs at approximately 37.5 years of age. However, the process in which follicle numbers decline varies among women, where some women will not enter menopause until their fifties while others start to undergo early menopause in their thirties. Women of advanced maternal age (>40 years) are generally known to have elevated serum FSH concentrations. One theory behind this increase can be attributed to the decline in follicle number and therefore FSH is unable to bind to its receptors to induce estrogen production, which is important for upregulating the number of FSH sites, and as a consequence FSH concentrations remain elevated. A consequence of the elevated FSH levels is inappropriate maturation of the granulosa cells, which may cause asynchronous maturation of the follicle and the oocyte which is

amplified as FSH continues to rise [123]. Therefore, female reproductive ageing may be related to changes that occur in ovarian function and the decrease in follicle number is responsible for the gradual changes that occur in the menstrual cycle and in fecundity [123].

Oocytes are the largest cells within the body and so it is essential that they obtain enough energy to support the transcription and translation that occurs during folliculogenesis, consequently, even minor deficiencies to energy availability could result in a decline in oocyte quality. If not enough energy is available abnormal chromosome segregation and spindle formation could occur leading to increased meiotic nondisjunction resulting in aneuploidy, which is commonly seen in the embryos of women with advanced maternal age contributing to the increased number of miscarriages and chromosomal abnormalities seen in these women. As mentioned previously, the mitochondria are responsible for the production of energy within the oocyte and even though immature human oocytes are not very metabolically active the mitochondria are important for activation of the oocyte and for its survival. Thus, a decrease in the quality of mitochondria may be responsible for the decline in oocyte quality that is seen in women of advanced maternal age [124].

Studies have suggested that there is a relationship between increasing maternal age and decreased oocyte mitochondrial activity and it is thought that this may be due to the increase in mitochondrial mtDNA deletions and mutations thus leading to abnormal mitochondrial function and a reduction in energy. This reduction in energy could be a contributing factor to the increase in aneuploidy that is seen in women of advanced maternal age. Interestingly, studies have demonstrated that transfer of a chromosomally normally embryo does not necessarily guarantee implantation and thus a successful pregnancy. Furthermore, transfer of the cytoplasm from

known developmentally competent oocyte into an oocyte of poor developmental competency has also been successful in achieving pregnancy and it has been speculated that the reasoning behind this could be the normally functioning mitochondria and thus sufficient energy supply for subsequent embryo development [4, 18]. Thus, demonstrating that a euploid oocyte is not the only factor that affects implantation and pregnancy and that mitochondrial function is also an important factor.

Cumulus cells are important to the oocyte for the supply of pyruvate, thus if these cells are defective this could lead to abnormal oocyte development, interestingly is has been demonstrated the there is a decline in the number of normal mitochondria in these cells from women over 38 years compared to younger women with mitochondria from older women having an abnormal morphology and disrupted cristae [103]. A study that examined the impact of maternal age on the molecular signature of human cumulus cells established that gene expression of 110 cumulus cell proteins is altered compared to younger women with some of the genes detected relating specifically to oxidative phosphorylation and to mitochondrial function [126]. Specifically decreased expression of two factors involved in oxidative phosphorylation at the gene and protein level were *ATP5I*, a protein that is part of the ATPase complex and *NDUFA1*, which is part of Complex I. In addition, increased gene expression of *ATP6V1A*, another component of ATPase and COX10, a component of cytochrome C of the electron transport chain was detected. Furthermore, another study established that granulosa cells from women over 38 years had a decrease in number of normally functioning mitochondria and this was due to an increase in mtDNA mutations (as measured by a 4977-bp deletion) [109]. Taken together these studies suggest that mitochondria within these cells are compromised which could potentially have an effect on their functionality and ultimately affect oocyte viability.

1.4.2 Diminished Ovarian Reserve

Although ovarian reserve declines with age, the rate of follicular decline varies between women and there is a subset of women where this decline happens more rapidly. Epidemiological studies have suggested that approximately 10% of women in their early 30s could be nearing their perimenopausal phase [127]. The mechanisms on how this decline occurs remains to be elucidated however a theory is an acceleration of the process of follicular recruitment [123]. Interestingly, it has been established that younger women with reduced ovarian reserve exhibit similar outcomes to women of advanced maternal age, with a poor response to ovarian stimulation, a decrease in the number of oocytes collected during an IVF cycle, a decline in pregnancy rates after ART treatment, an increase in miscarriage and elevated aneuploidy rates [10]. Currently, apart from ART there is no treatment available for women affected with reduced ovarian reserve.

Young women with reduced ovarian reserve have differences in their hormonal profile compared to women of the same age with a normal ovarian reserve. Interestingly, the granulosa cells in these women are thought to have a decline in proliferation and an increase in apoptosis [128]. This decrease in both granulosa cell and cumulus cell viability has been observed in women with elevated FSH levels and occurs independent to a woman's age [128]. Furthermore, premature luteinisation of the granulosa cells (differentiation of granulosa cells into cells of the corpus luteum) has been associated in women with reduced ovarian reserve and occurs due to a premature increase in progesterone levels. Women with premature luteinisation have elevated FSH levels and require a higher dosage of ovarian stimulation. These women are also thought to be associated with decreased pregnancy rates and cumulative pregnancy rates [129].

1.4.2.1 Markers of Ovarian Reserve

To date, there are several methods to assess a woman's ovarian reserve, the main measure's being serum FSH levels, antral follicle count, AMH or a combination of these.

1.4.2.1.1 Follicle-Stimulating Hormone

FSH is a glycoprotein that is synthesised and secreted by the anterior pituitary gland. It acts on FSH receptors on the granulosa cells during the late secondary follicle stage to stimulate the continuing growth of the follicle and it stimulates the secretion of estradiol from the granulosa cells of the antral follicle. As mentioned above, once ovarian follicles become depleted there is an increase in FSH concentration, possibly due to the decrease in the number of follicles and therefore granulosa cells resulting in less receptors available to respond to FSH. Therefore, a combination of serum FSH and maternal age has been suggested to be better than age alone in predicting IVF outcome in women. However, the limitation of using serum FSH is the monthly variation in basal FSH and FSH concentrations appear to be a late indication of ovarian ageing [123].

1.4.2.1.2 Antral Follicle Count

There are approximately 20 to 150 early follicles (0.05 – 2.0mm in diameter) present at any one time in the ovaries of women between the ages of 25 to 40 years. These growing follicles are too small to be identified using conventional scanning techniques. The follicles that are recruited to develop and grow (2 – 10mm in diameter) can be detected via a transvaginal scan. Histological analysis of human ovaries has proven the number of primordial follicles can be correlated to the number of antral follicles detected by a scan and therefore provides an indication of ovarian reserve [130]. Additionally, antral follicle count has been associated with the occurrence of the

transition to menopause. Low numbers of antral follicles are a sign of ovarian ageing and are seen earlier compared to the rise in serum FSH levels. The limitations of antral follicle count are differences in count between cycles and intra-observer differences. However, it has been suggested that antral follicle count is a superior indicator compared to maternal age, however, the quality of the follicles detected in the scan cannot be assessed [123]. The responsiveness to ovarian stimulation is an indicator used to determine the number of oocytes as generally the most responsive follicles contain an oocyte [130].

1.4.2.1.3 Anti-Mullerian Hormone

AMH is a member of the TGF-β super family and is produced by the granulosa cells of the primary, secondary and small antral follicles. In humans, serum AMH levels are at its highest once puberty has been reached and after this point serum AMH concentrations start to decline. Once the follicle has developed from the primordial stage AMH is produced and continues until the follicles have reached the pre-antral stages and it can be measured in blood serum. Expression of AMH is at its highest in the pre-antral (≤4mm) and early antral follicle and it gradually declines in the subsequent stages of follicular development, with no expression of AMH during final stages of FSH-dependent growth or when the follicle becomes atretic [25, 127]. This profile of AMH production lead to the suggestion that serum AMH levels during the early follicular phase maybe representative of the size of the growing cohort of follicles within the ovary [127].

Considering serum AMH levels are a reflection of the growing pool of follicles this hormone has been utilised as a biochemical marker for the detection of decreased ovarian reserve, independent of maternal age and independent to the phase of the menstrual cycle [131]. Furthermore, Lekamge *et al*., (2007) found that oocytes from women of low AMH gave poor

fertilisation rates and embryos had reduced pregnancy rates, compared to women with high AMH [10]. These women with high AMH had more oocytes collected, higher fertilisation rates and thus better oocyte quality, more embryos produced, a lower occurrence of miscarriage after a fresh embryo transfer and a doubling in pregnancy rates per IVF cycle [10]. This suggests that low AMH is a predictor of poorer oocyte quality and reproductive ageing is related not only to maternal age but also to a decrease in ovarian reserve, however, the causes and mechanisms behind the development of these poor oocytes in women of low AMH remain unclear.

Table 1.1: Values indicating serum AMH levels for ovarian reserve status.

Age	Low AMH Values	Normal AMH Values	High AMH Values
26-29	<2.0	2.0-5.9	>5.9
30-35	<1.4	1.4-4.2	>4.2
36-39	<0.8	0.8-2.8	>2.8
40-44	<0.4	0.4-1.4	>1.4

Serum AMH for the chronological age of women, thus demonstrating that serum AMH for age is essential in determining the ovarian reserve status of a woman (adapted from [132, 133]).

There have been multiple studies that have correlated serum AMH with antral follicle count and FSH as a predictor of ovarian reserve [130, 132-135]. Serum AMH was established to be superior to FSH in determining ovarian reserve, as a decline in ovarian reserve was detected earlier using AMH compared to FSH. In addition, serum AMH was found to be comparable with antral follicle count in determining ovarian reserve. Thus serum AMH alone can be utilised as an accurate marker of ovarian reserve. Furthermore, the studies by Tremellen and Kolo (2010) and Wiweko *et al.,* 2013 developed percentile charts of serum AMH for the reproductive ages of women and determined that a woman's ovarian reserve status is relative to her age related peers. Thus, a single cut-off AMH concentration for determining the ovarian reserve status for women of all ages cannot be utilised as an accurate marker of ovarian reserve and a women's

chronological age must be taken into account to determine if she has reduced or normal ovarian reserve relative to her maternal age (Table 1.1).

1.5 Sirtuin Proteins

1.5.1 Overview of the Sirtuins

It has been established that a family of proteins, the Sirtuins, have the ability to post-translationally influence and regulate many cellular functions. These proteins are nutrient sensing Class III histone (NAD^+-dependent) deacetylases. This family of proteins all contain a NAD^+ binding domain, which is conserved across mammals and a unique N-terminal and/or C-terminal sequence, which varies in length, in addition four of the proteins contain a catalytic core domain of about 250 amino acids (Figure 1.10) [136]. This family of proteins were first discovered in yeast where it was found that the Silent Information Regulator 2 (Sir2p), prolonged lifespan in low glucose conditions [137]. In addition, Sir2p has been found to be involved in the repair of chromosomal double-strand breaks, cell cycle progression and chromosomal stability [138].

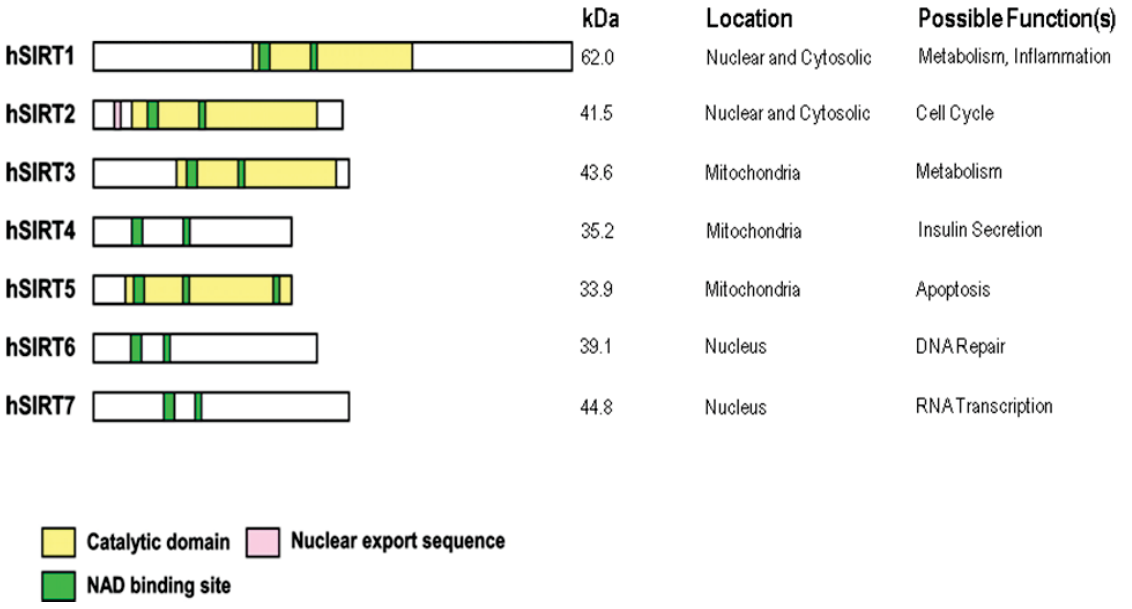

Figure 1.10: Diagrammatic representation of SIRTs 1 – 7.
The mammalian Sirtuin family of proteins all have a conserved catalytic domain and NAD^+ binding domain. In humans, the Sirtuin family of proteins, their size, location and possible function [136].

In mammals, there are 7 homologs that make up the Sirtuin family, SIRT1-7 and all share the same catalytic domain of Sir2p. In mammals, Sirtuins are thought to be involved in the regulation of metabolism and are important factors in slowing the ageing process. In lower organisms, it has been found that caloric restriction, a diet in which 20% fewer calories are consumed, extends lifespan [104]. Animals on a restricted diet are found to have a later onset of age related diseases such as cancers and they are able to delay the onset of characteristics usually associated with ageing, such as the decrease collagen elasticity, the increase in insulin resistance, the decline in immunity and the attainment of neuro-behavioural impairments. Sirtuins are thought to have key roles in the positive outcome seen in these animals, in which lifespan was increased with caloric restriction. Thus, it is logical to think that this family of proteins are regulated by diet, which is able to regulate many physiological processes that ultimately results in extending lifespan [104, 139, 140] (Figure 1.11).

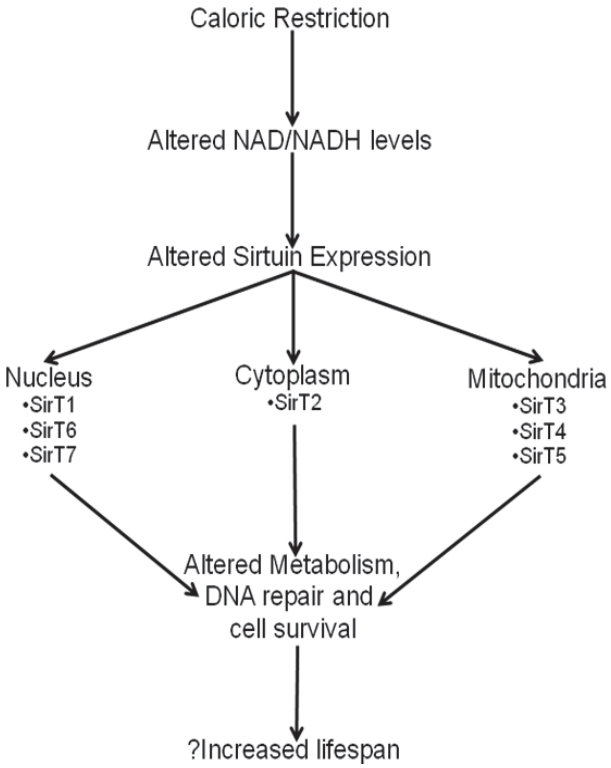

Figure 1.11: Flowchart depicting the possible action of caloric restriction and the way in which it can increase mammalian lifespan (adapted from Haigis and Guarente, 2006) [140].

Of the 7 SIRTs, SIRT1, 2, 6 and 7 are located in the nucleus or cytoplasm or both and SIRT3, 4, and 5 are mitochondrially located [137, 141]. To date, SIRT1 is the most studied and has been

found to be both nuclear and cytosolic. The localisation of this protein appears to differ between tissue types and it has been suggested that it may control many physiological processes, related to cell survival and metabolism, which are known to be affected by ageing. It has been found to have many targets and protects against cellular oxidative stress and DNA damage. In the pancreas, SIRT1 up-regulates the secretion of insulin in response to glucose, in the liver it increases gluconeogenesis and suppresses glycolysis and in adipose tissue it inhibits fat storage and increases lipolysis [136, 139, 140].

SIRT2 is located within the nucleus and cytoplasm and specifically co-localises with tublin. Studies on the mammalian cell cycle have found that SIRT2 is important in its regulation. It has been found that the levels of SIRT2 increase during the mitotic phase and over expression of this protein results in a delay in mitosis. SIRT2 may also have a role in other phases of the cell cycle, as it was found that in mouse embryonic fibroblast cells that were null for *SIRT2*, the G_1 phase was lengthened and the S phase was shortened [140].

SIRT4, localised to the mitochondria, is a protein that is able to regulate energy consumption, however it does not possess deacetylation activity, but instead has ADP-ribosyltransferase activity. In pancreatic cells, SIRT4 has an essential role in the control of amino acid-stimulated insulin secretion by inhibiting glutamate dehydrogenase (GDH), a mitochondrial metabolic regulator. GDH converts glutamate to α-ketoglutarate, which can then be utilised in the TCA cycle [140].

In the mouse, the nuclear protein SIRT6 is expressed in many tissues. It has been shown to have weak deacetylation activity but it also has ADP-ribosyltransferase activity. *Sirt6* knockout mice

show signs of premature ageing, including loss of subcutaneous fat, decreased bone density and die within four weeks after birth [140]. These mice have been found to have a deficiency in a specific form of DNA repair. Mouse embryonic fibroblasts that are null for *Sirt6*, have impaired proliferation and are more sensitive to DNA damage. Chromosomal translocations and detached centrosomes were found in *Sirt6* knockout mouse embryonic fibroblasts, however they still had normal cell cycle checkpoints and double-strand break DNA repair [140].

SIRT7 is another nuclear protein but it is specifically localised to the nucleolus. Its expression is related to growth and is found to be highly expressed in tissues that have high proliferation, such as the liver, spleen and testes. Thus, it is not surprising to find that in tissues that are non-proliferating, such as the heart, brain and muscle, SIRT7 is either non-existent or is very low. SIRT7 may have actions in regulating cellular growth and metabolism and in the nucleolus it may be working with rDNA and RNA polymerase I. In response to an alteration in the metabolic environment, this Sirtuin appears to regulate cell growth and cellular metabolism by inducing ribosome synthesis in proliferating cells [140].

1.5.2 Sirtuin Proteins and the Mitochondria

As mentioned previously, acetylation of mitochondrial proteins is becoming increasingly recognised as a regulator of mitochondrial protein activity [142]. Therefore, proteins such as SIRT3 and SIRT5, which are located within the mitochondria and are able to sense metabolic state and post-translationally alter mitochondrial proteins, may be important in understanding metabolic control.

1.5.2.1 Sirtuin 3

SIRT3 is a protein whose localisation and role is thought to be species-specific, for example in mice it is located in the mitochondrial inner membrane and in humans it is located in the mitochondrial matrix [137]. Using the 293F TREX cell line, Scher *et al*., (2007), demonstrated that the long isoform of *SIRT3* is located in the nucleus and that it can localise to the mitochondria. They also demonstrated that this translocation happens when the cell is stressed, either by the environment or even by a significant over expression of *SIRT3* [143]. In mice it was initially thought that SIRT3 was exclusively localised to the mitochondria where it acts as a NAD^+-dependent deacetylase (Figure 1.12) [104, 136, 139]. This was disputed by Nakamura *et al*., (2008), who suggested that the localisation of SIRT3 in mice changes from the mitochondria to the nucleus when it is co-expressed with SIRT5 [137]. However, further studies have established that there are actually two isoforms of SIRT3, the short isoform (257 amino acids) and a long isoform (399 amino acids) [144]. Onyango *et al*., 2002 cloned a portion of SIRT3 that did not have the first 142 amino acids, as it is this portion that is different to the other Sirtuins, they discovered that the full-length SIRT3 localised to the mitochondria, while the cloned version was found throughout the cytoplasm. Thus, these results suggest that the first 142 amino acids are required for targeting SIRT3 into the mitochondria. However, another study deleted the first 25 amino acids and they found that there was no significant localisation to the nucleus compared to the full length SIRT3. Thus, it is only the first 25 amino acids that are required for SIRT3 to localise to the mitochondria.

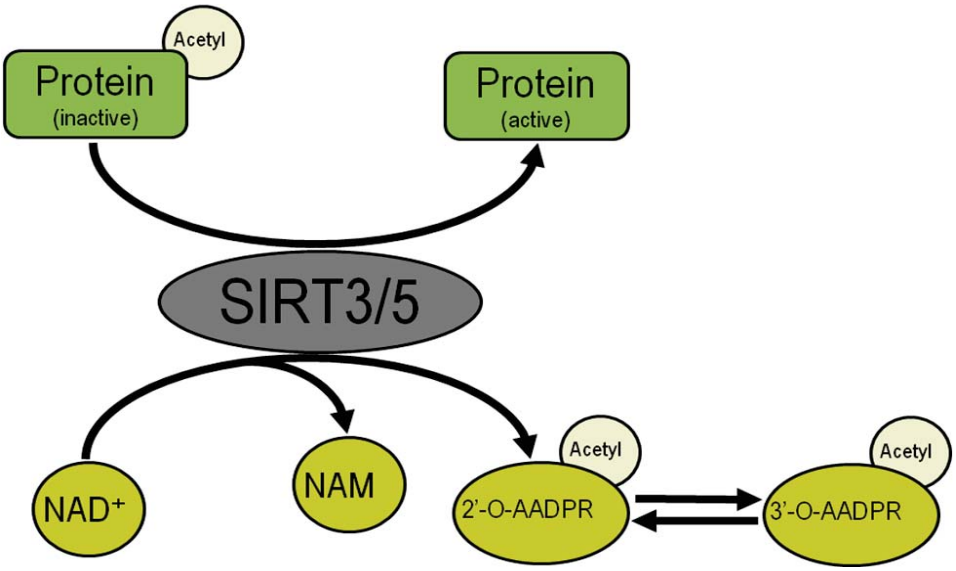

Figure 1.12: Schematic depicting the action of SIRT3 and SIRT5 as deacetylases.
SIRT3 and SIRT5 utilise NAD^+ and together they are able to deacetylase a protein as well as produce the substrates; nicotinamide [145], 2'-O-acetyl-ADP ribose (2'-O-AADPR) and 3'-O-acetyl-ADP ribose (3'-O-AADPR), which are formed by the transfer of the acetyl group to the ADP-ribose part of NAD^+ (adapted from Yamamoto et al., 2007 and Michan and Sinclair, 2007) [104, 136].

In order for a protein to move into the mitochondria it needs a targeting signal which is usually an amphipathic α-helix which is made up of positively charged hydrophobic and hydroxylated amino acids. SIRT3 structure predictions reveal that an N-terminal peptide corresponding to resides 1-25 has a high probability of containing an amphipathic α-helix. If these amino acids are arranged into a helical wheel they show positively charged amino acid arginine residues on one side of the helix opposed by hydrophobic amino acids and this is a typical feature of mitochondrial pre-sequences [146]. A study has shown the helical structure and its positive charge is important for the import of human SIRT3 (hSIRT3) into the mitochondria and alterations to this structure reduced mitochondrial import. The majority of hSIRT3 is located in the mitochondria as a 28kDa protein as opposed to a 43kDa protein. The mitochondrial processing peptidase is able to process hSIRT3 resulting in activation of hSIRT3 activity. This suggests that hSIRT3 is synthesised in an inactive form which allows transportation through the cytoplasm to the mitochondrial matrix where it is processed to become an active protein. This processing could

allow for the safe transfer of the enzyme to the mitochondria and also for selective activation when required [146].

SIRT3 may have a direct relationship to extended life span and play a role in the regulation of ageing in humans [136]. A study has found that SIRT3 is important in controlling basal ATP levels, by deacetylation of the mammalian enzymes Acetyl-CoA Synthetase 2 (ACECS2), Glutamate Dehydrogenase (GDH) and Isocitrate Dehydrogenase 2 (ICDH2), three key metabolic regulators in the mitochondrial matrix and therefore, energy homeostasis [143, 147, 148]. In mammals, ACECS2 converts acetate, obtained from the diet or from cellular reactions, into acetyl-CoA, which is important in the production of fatty acids, amino acids, ketone bodies and ATP (via the TCA cycle), which is important in the mitochondria of the cumulus oocyte complex for energy production [104]. SIRT3 has been shown to be stimulated during periods of caloric restriction, thus it has been proposed that during these times, when food is restricted, SIRT3 has the ability to control both the production of fatty acids and the amount of carbon that is transported into the TCA cycle [104, 147].

A study conducted in mice, found that SIRT3 has high expression levels in brown adipose tissue and low expression levels in white adipose tissue. However, under calorie restriction, expression levels of SIRT3 are up-regulated in both white and brown adipose tissue [149]. Interestingly, it was also discovered that SIRT3 expression increases during cold temperatures and was lower at room temperatures, the reason behind this was that SIRT3 increased mitochondrial respiration. In addition, it was discovered that SIRT3 had the ability to make changes to mitochondrial function by decreasing both mitochondrial membrane potential and ROS and by increasing oxygen consumption [149]. In a study performed using *SIRT3* null mice, it was found that ATP

levels in tissues that usually have a high level of ATP, such as the liver, heart and kidney were significantly decreased and this is due to the deficiency of SIRT3 deacetylase activity. Further, SIRT3 controls acetylation and activity of Complex I of the ETC, thus playing a role in the regulation and maintenance of ATP levels. However, interestingly *Sirt3* null mice had no obvious phenotypic adverse effects [150]. This finding is consistent with another study, in which *Sirt3* null mice were found to be healthy and have normal metabolism, even under mild stress conditions, such as fasting for short periods of time or cold exposure [147]. Such studies indicate that there are some redundancies in the function of the Sirtuins.

1.5.2.1.1 Sirtuin 3 and Glutamate Dehydrogenase

Glutamate dehydrogenase (GDH) is an enzyme that catalyses the reaction between L-glutamate and α-ketoglutarate using pyridine nucleotides (NAD(P)H) as coenzymes. Glutamate is a multi-functional amino acid that acts as a neurotransmitter, has a role in ammonia metabolism and is associated with mitochondrial metabolism as it is a precursor to the TCA cycle and α-ketoglutarate is directly involve in the TCA cycle and thus in the production of energy [151]. GDH is found in all mammals and is a homopolymer that is made up of two to six subunits with a molecular weight that ranges from 40 to 60kDa and is located in the mitochondrial matrix.

GDH is a central regulator of mitochondrial metabolism and studies have shown that SIRT3 can directly deacetylate GDH, which controls glutamate oxidative stress and generates NADPH. Glutamate is then able to convert to α-ketoglutarate, which is an enzyme that is part of the TCA cycle [147]. Incubation of SIRT3 with NAD^+ increased deacetylation of GDH and therefore its activity increased, this activity increased even more in the presence of more SIRT3 [148]. GDH is co-localised with SIRT3 in the mitochondrial matrix and it has been documented that in *Sirt3*

knockout mice GDH was hyperacetylated compared to wild-type mice [147]. Thus, SIRT3 is able to deacetylate GDH and as a result of the deacetylation, GDH is activated. Activation of GDH is thought to promote the synthesis of ATP and glucose by allowing amino acids to be utilised as fuel for the TCA cycle [148].

1.5.2.1.2 Sirtuin 3 and Isocitrate Dehydrogenase

Mammalian isocitrate dehydrogenase 2 (ICDH2) is a mitochondrial TCA cycle enzyme that catalyses the reaction of isocitrate to α-ketoglutarate, while reducing NAD^+ to NADH. It is a heterotetramer that is made up of three subunits each with a molecular mass of between 37 and 39kDa. The amino acid sequence of each of the subunits is approximately 25-34% identical to E.coli and about 12-18% identical to the pig compared to the human, thus demonstrating that there is low conservation between species [152].

ICDH2 has the ability to generate NADPH from oxidative decarboxylation of isocitrate to α-ketoglutarate, NADPH is required for the generation of reduced glutathione which is the major antioxidant responsible for the prevention of ROS. A previous study in mouse fibroblasts demonstrated ICDH2 is upregulated in response to ROS, and decreased levels of ICDH2 resulted in increased levels of ROS resulting in an accumulation of oxidative damage to DNA, interestingly SIRT3 was implicated in its regulation [153]. Thus in oocytes from women with advanced maternal age where it has been shown that mitochondrial function is impaired, SIRT3 may be implicated as it plays an important role in the deacetylation of mitochondrial proteins that are involved in energy metabolism. It has also been demonstrated that SIRT3 is able to deacetylate ICDH2 similar to GDH, incubation of SIRT3 and NAD^+ with ICDH increased its

activity and this activity increased in the presence of increasing amount of SIRT3, thus SIRT3 is able to active ICDH by deacetylation [148].

SIRT3 is able to deacetylate and therefore activate ICDH2, which is a key regulation point for flux throughout the TCA cycle. ICDH2 is the isoform that is targeted by SIRT3 and this isoform is able to produce NADPH instead of NADH, which is used for ATP synthesis. The activity of NADPH is thought to be necessary for the NADPH-dependent regeneration of antioxidants and therefore stimulation of SIRT3 is theorised to slow oxidative damage and the cellular ageing process [148].

1.5.2.2 Sirtuin 5

SIRT5 is soluble and located within the intermembrane space of the mitochondria [137]. Initially SIRT5 was thought to possess only deacetylation activity however, recently it was discovered to also possess desuccinylation and demalonylation activities. Thus, SIRT5 is the only Sirtuin to possess desuccinylation, demalonylation and deacetylation activities.

1.5.2.2.1 Sirtuin 5 and Deacetylation

SIRT5 was initially described as having weak deacetylase activity and *Sirt5* knockout mice were demonstrated not to have any changes in deacetylation [147]. One of the first substrates established to have SIRT5 dependent deacetylation was Carbamoyl Phosphate Sythetase (CPS1). CPS1 has an important role in the urea cycle, which is important in the removal of ammonia and *Sirt5* knockout mice were less able to clear ammonia via the urea cycle due to the decreased activity of CPS1 [154]. However, it has also been established that SIRT5 has the ability to deacetylate the IMS protein, cytochrome C. Cytochrome C has important functions in

oxidative metabolism and apoptosis, therefore implicating SIRT5 in the regulation of these functions [148].

1.5.2.2.2 Sirtuin 5 and Desuccinylation

Until recently SIRT5 has only been known as a weak deacetylase but it seems that this protein is more active as a desuccinylase [119]. *Sirt5* knockout mice displayed global protein hypersuccinylation in the liver whereas no difference in global protein deacetylation were detected [119]. This suggests that SIRT5 desuccinylase activity is much higher compared to its deacetylation activity. SIRT5 has been demonstrated to have the unique ability to remove succinyl groups from lysine residues [119]. It has been suggested that Succinyl-CoA is the cofactor of enzyme-mediated lysine succinylation. Succinyl-CoA is an important metabolic intermediate in various metabolic pathways including the TCA cycle and porphyrin synthesis [117]. These desuccinylation modifications have been detected in a variety of organisms raging from yeast to human and have been detected in metabolic enzymes such as ICDH2, malate dehydrogenase (MDH), citrate sythase and CPS1 [117, 118, 155]. The exact biological significance of this type of post-translational modification and how it plays a role in the regulation of metabolic enzymatic activity has yet to be determined. As mentioned above SIRT5 is also able to desuccinylate CPS1 and *Sirt5* knockout mice had a 15% reduction in CPS1 activity compared to wild-type mice [119]. It has also been shown to activate ICDH2.

1.5.2.2.3 Sirtuin 5 and Demalonylation

As well as desuccinylation activity SIRT5 has also been demonstrated to possess demalonylation activity [118]. *Sirt5* knockout mice have been demonstrated to have global hypermalonylation in the liver [118]. Malonyl-CoA is thought to be the cofactor of enzyme-mediated lysine malonylation

and is an important intermediate in the pathways of cellular metabolism [118]. Malonyl-CoA has also been demonstrated to also be an important regulatory molecule as interruption to the regulation of malonyl-CoA is able to negatively affect cellular physiology [118]. Similar to the succinyl lysine modifications, lysine malonyl modifications have also been detected in a variety of organisms [118, 155]. The exact biological significance of protein demalonylation and how it plays a role in the regulation of metabolic enzymatic activity has yet to be determined. However, glutamate dehydrogenase and malate dehydrogenase have been demonstrated to have malonyl lysine residues, thus suggesting that these metabolic proteins may be a target for protein demalonylation [119].

Table 1.2: SIRT3 and SIRT5 locations within the mitochondria, their functions and the tissues that have been studied to date.

	Mammalian Tissue Types	Location in the Mitochondria	Functions
SIRT3	• Kidney, heart and liver • Embryonic fibroblasts • Adipose tissue (brown and white)	• Mitochondrial Matrix (human) • Mitochondrial Inner Membrane (mice) • ?Nucleus	• Activate GDH • Activate ICDH • Activate ACECS2 • Reduce membrane potential • Reduce ROS • Increase oxygen consumption • Regulate activity of Complex I
SIRT5	• Lymphoblasts • Heart muscle cells • Liver	• Mitochondrial Intermembrane Space • Mitochondrial Matrix	• Activate Cytochrome C • Activate CPS1 • Activate ICDH2 • Activate MDH • Activate Citrate Synthase

Interestingly, the targets of SIRT5 protein desuccinylation and demalonylation are similar to those of SIRT3 deacetylation, thus suggesting that all of these post-translational modifications are

important in regulating mitochondrial metabolism. Considering that mitochondrial metabolism is essential in the supply of energy to the ovarian follicle it is logical to think that SIRT3 and SIRT5 may be playing an essential role in this regulation. However, currently the localisation and function of the Sirtuins in ovarian tissue remains largely unknown and no information about SIRT3 or SIRT5 is known within the oocyte, granulosa or cumulus cells (Table 1.2).

1.6 Conclusion

Currently, there is little information available about the follicular environment in women with reduced ovarian reserve or advanced maternal age and how this compares to young women with normal ovarian reserve. In different tissues types, SIRT3 has been found to be located within the mitochondria and can regulate basal ATP levels via the TCA cycle and ETC. SIRT5, also located in within the mitochondria, has been implicated in activating cytochrome C, CPS1 and ICDH2. Previous research has also found that a relationship may exist between the expression levels of SIRT3 and SIRT5, thus these proteins may have the potential to affect mitochondrial activity within the ovarian follicle [104, 137, 143, 147, 148]. The production of ATP, by the mitochondria, is important in maintaining many cellular activities, including spindle formation, which may lead to a decline in oocyte viability [17, 97, 98]. However, a relationship between the mitochondrial proteins, SIRT3 and SIRT5 has yet to be determined in the oocyte and the ovarian follicular cells. Thus, the aim of this study was to determine if a difference exists in the follicular environment in women with advanced maternal age and in women with reduced ovarian reserve compared to young women with normal ovarian reserve. Furthermore, to determine if SIRT3 and SIRT5 are located in granulosa and cumulus cells and if they are altered in these groups of women. This is of particular importance as in today's society women are having children later on in life and it has been suggested that with increasing maternal age, there may be an increase in mitochondrial

dysfunction. In addition, it has been found that in women with low AMH, oocytes have a decreased developmental potential [10]. In 2004, in Australia, the average age of women who gave birth for the first time was 28.0 years, an increase from 26.5 years in 1995. In 2006, the average age of women who had fertility treatment was 35.6 years, which was higher than in 2002, which demonstrates that women are leaving it later in life to have children. [60]. This may have implications on the health system, due to the increase in number of older women seeking age-related fertility treatment [4].

It is well-known that the success rate for both natural conception and assisted reproduction in women who are over 38 years of age is significantly lower compared to younger women [4]. Women of advanced maternal age also have an increased incidence of having children with a genetic disorder thus implying that there is a relationship between maternal age and oocyte quality. Furthermore, these women are known to have high infant mortality rates and increased risk of low birth weight, regardless of whether they had conceived naturally or had assisted reproduction [4]. New evidence has revealed that women with reduced ovarian reserve are also likely to have poorer IVF outcomes, however the obstetric and neonatal outcomes still remain to be reported. Taken together, this suggests that women with either a reduced ovarian reserve or advanced maternal age not only have a decreased chance of a successful pregnancy but also of having a baby that is of poor health [4]. Therefore, if the mechanisms behind maternal oocyte “ageing” can be understood, it can lead to steps to improve oocyte quality in women seeking IVF treatment and specifically in women with reduced ovarian reserve and in women of advanced maternal age. Ultimately, the success rate of a viable pregnancy can be increased and improve the health of the baby.

1.7 References

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Hypothesis and Aims

Hypothesis

Women with reduced ovarian reserve or of advanced maternal age have an altered follicular environment and altered mitochondrial Sirtuin 3 and Sirtuin 5 in granulosa and cumulus cells therefore having an effect on its targets glutamate dehydrogenase and carbamoyl phosphate synthase I and thus contributing to this altered follicular environment.

Experimental Aims

To create a patient cohort and allocate patients to one of three cohorts based on their age and AMH levels and establish the relationship to IVF outcome.

To determine the metabolic profile of granulosa and cumulus cells and the follicular fluid content of carbohydrates, selected ions and hormones and the effects of advanced maternal age or reduced ovarian reserve.

To determine the presence and location of SIRT5 and examine the presence and function of CPS1 (a SIRT5 target) in granulosa and cumulus cells in women with reduced ovarian reserve and in women of advanced maternal age.

To determine the presence and location of SIRT3 and examine its target glutamate dehydrogenase in granulosa and cumulus cells in women with reduced ovarian reserve or of advanced maternal age.

CHAPTER TWO

Patient Demographics

2.1 Introduction

In recent years there has been a trend towards delaying childbirth. In Australia in 2009 13.7% of first time mothers were over 35 years of age compared to 10.3% in 2000 [1, 2]. Furthermore, during this same time period there has been a 1.6% increase in women over the age of 40 giving birth [2]. It has been well-documented that pregnancy success rates decline with increasing maternal age and it is thought that this is due to a decrease in oocyte number and an increase in oocyte aneuploidy rates [3-5]. In addition, increasing maternal age is an important risk factor of adverse obstetric (placental praevia, intra uterine growth restriction and gestational diabetes) and perinatal (pre-term birth, small for gestational age infant and perinatal death) outcomes [6].

This delay in childbearing has resulted in an increase in the number of women aged over 35 years seeking ART treatment. Unfortunately, the outcomes of ART are also negatively affected by maternal age. Women over the age of 40 have a reduction in ovarian response to ovarian stimulation, decreased fertilisation rates and pregnancy outcome compared women under the age of 40 [7]. However, even though chronological age is the most important predictor of IVF outcome, the rate of reproductive aging varies between women, thus biological age is not necessarily a reflection of reproductive age. A natural decline in ovarian reserve occurs in women of advanced maternal age however, some young women may exhibit an accelerated decline in ovarian reserve as well. The measurement of serum AMH concentration is increasingly being utilised as a measure of a woman's ovarian reserve independent to her age and can thus serve as a reflection of reproductive age. Interestingly, previous research has determined that women with diminished ovarian reserve are also more likely to have poorer IVF outcomes including a poor response to ovarian stimulation, a decrease in the number of oocytes collected during an

IVF cycle, a decline in pregnancy rates after ART treatment, an increase in miscarriage and elevated aneuploidy rates [13].

Therefore, this study determined the IVF outcomes of a cohort of women with either reduced ovarian reserve or advanced maternal age.

2.2 Materials and Methods

2.2.1 Patient Recruitment and Cohorts

Informed consent (Appendix 1) was obtained from women (n=111) undergoing IVF treatment at Repromed (Dulwich, South Australia, Australia) and they were recruited for participation in this study (exclusion criteria were donor/recipient cycles, pre-implantation genetic screening cycles and PCOS patients). Each woman was only represented once within the study period. Samples were de-identified and randomly allocated to experimental protocols (Appendix 2), based on age (young maternal age: ≤35 and advanced maternal age: ≥40) or ovarian reserve (Table 2.1).

Table 2.1: Allocation of patients to each cohort.

Age/AMH	Normal Ovarian Reserve (NOR)	Reduced Ovarian Reserve (ROR)
Young Maternal Age ≤35 **(YMA)**	YMA & NOR	YMA & ROR
Advanced Maternal Age ≥40 **(AMA)**	AMA & NOR	AMA & ROR[#]

[#] This cohort was initially included during the initial recruitment period. However, due to cycle cancellations (as a result of poor stimulation) or unavailability of sufficient material it was decided not to include this cohort for the remainder of the study.

Serum AMH was utilised as the measure of ovarian reserve. The AMH range for young women (≤35) with normal ovarian reserve was 10-42pmol/L and the young women (≤35) with reduced ovarian reserve had serum AMH concentrations of <10pmol/L. AMH values for women of advanced maternal age (≥40) were 3-10pmol/L. These values represent 25-75% quartiles for the age groups and thus these values are considered to be normal for maternal age (Table 2.2) [8]. Ethical approval was obtained from the Women's and Children's Hospital Research Ethics Committee (North Adelaide, South Australia, Australia) and from the Repromed Scientific

Advisory Committee (Dulwich, South Australia, Australia) and written informed consent was obtained for use of surplus follicular fluid, granulosa and cumulus cells (Appendix 1). Initially this study also included an advanced maternal age (≥40) with reduced ovarian reserve (AMH <3pmol/L) group however, due to the low patient numbers and the difficulty in collecting enough sample material this group was omitted.

Table 2.2: Patients were allocated to each cohort based on serum AMH concentrations based on maternal age [8][#].

Age	Low AMH (pmol/L)	Normal AMH (pmol/L)	High AMH (pmol/L)
26-29	<14	14-42	>42
30-35	<10	10-30	>30
36-39	<6	6-20	>20
40-44	<3	3-10	>10

[#]The reference ranges used within this study are taken from Tremellen and Kolo (2010), which use the same method and reference ranges as samples included in this study [8].

2.2.2 Ovarian Stimulation and Clinical Outcomes

All women underwent antagonist cycles using gonadotropin stimulation with recombinant FSH (*Puregon (100-375U), Schering-Plough, New South Wales, Australia or Gonal F (150-350U), Merck Serono, New South Wales, Australia)*, administered from menstrual cycle day 2/3. On day 6/7 a GnRH antagonist *(Orgalutran (250mcgms), Schering-Plough)* was administered. Follicle growth was monitored via ultrasound and when 2 or more follicles of ≥17mm in diameter were present, a hCG trigger (*Ovidrel, (250mcgms, Merck Serono)* was administered and oocytes were collected 36hrs later. Data on the number of previous IVF cycles, days of FSH administration and infertility diagnoses were obtained by retrospective review of patient clinical records. Clinical pregnancy rate is defined as per the Australian and New Zealand Assisted Reproduction Database (ANZARD) [1]. Implantation rate is defined as the number of embryos successfully

implanted (as measured by the presence of a fetal heart at the 8 week ultrasound scan) compared to the number of embryos that were transferred. Utilisation of oocytes is defined as the number of embryos that were either frozen or transferred compared to the number of oocytes that were either injected or inseminated.

2.2.3 Embryo Culture and Grading

All embryos were cultured using the sequential culture system using G1/G2 media (Vitrolife, Göteborg, Sweden) and were cultured at 37°C in 6% CO_2/ 5% O_2. Embryos were assessed on day 3 and graded 1-4 based on cell number and percentage fragmentation (grade 1 being the best quality). All embryos underwent extended culture and were transferred on either day 4 or day 5 of culture. Embryo freezing occurred on either day 4 or day 5. Day 4 embryo assessment is based on degree of compaction, cell number and fragmentation [14]. Day 5 embryo assessment is based on the Gardner's scale which assesses degree of expansion, inner cell mass and trophectoderm formation [15].

2.2.4 Statistics

All statistical analyses were performed using GraphPad Prism Software Version 6 (Graphpad Software, San Diego, CA, USA). Data are expressed as mean $\pm$ standard error of the mean (SEM). To determine significance between patient groups a Student's T-test was used and a Chi Square Test was used for pregnancy rates and infertility diagnoses. For embryo development, a one way analysis of variance (ANOVA) with Tukey's post-hoc test was used. A P-value <0.05 was considered statistically significant.

2.3 Results

2.3.1 Patient Demographics

Mean maternal age, serum AMH level and infertility diagnosis for each cohort are indicated in Table 2.3. As expected, mean maternal age was significantly higher in the advanced maternal age groups compared to the young maternal age groups ($p<0.05$; Table 2.3). Serum AMH concentration was elevated in the young maternal age with normal ovarian reserve group compared to the remaining two cohorts as indicated in Table 2.3 ($p<0.05$). There was no difference in serum AMH (based on maternal age) between the young maternal age with reduced ovarian reserve and the advanced maternal age with normal ovarian reserve group. No significant differences were detected between the cohorts in body mass index (BMI) or the number of days of FSH administration required (Table 2.3). Young women with normal ovarian reserve had significantly lower starting FSH doses and fewer previous cycles compared to the remaining two groups ($p<0.05$; Table 2.3). No significant differences were detected in infertility diagnoses aside from age related diminishing fertility (a natural decline in oocyte number/viability) [8] which was significantly higher in the advanced maternal age group compared to the young maternal age groups ($p<0.05$; Table 2.3). (Note that Table 2.3 assesses all patients included in this study, for the following chapters only patients allocated to the experimental protocol are assessed in the patient demographics table specific for that chapter.)

Table 2.3: Patient demographics, IVF cycle details and patient infertility diagnoses.

	Young Maternal Age with Normal Ovarian Reserve (n = 45)	Young Maternal Age with Reduced Ovarian Reserve (n = 28)	Advanced Maternal Age with Normal Ovarian Reserve (n = 38)
Age (range)	30.9±0.6[a] (23 – 35)	32.4±0.5[a] (26 – 35)	41.8±0.4[b] (40 – 47)
Serum AMH (pmol/L) (range)	24.3±1.4[a] (11.1 – 39.8)	7.7±0.8[b] (<3.0 – 11.7)	8.4±0.6[b] (4.3 – 10.0)
BMI (range)	24.6±0.9[a] (17.9 – 30.0)	24.3±0.9[a] (18.8 – 31.2)	28.6±1.5[a] (18.3 – 34.1)
Starting FSH Dose (IU) (range)	186.0±9.2[a] (100 – 300)	292.8±6.9[b] (225 – 350)	302.6±5.2[b] (225 – 350)
# previous fresh cycles (IVF/ICSI)	1.0±0.2[a]	2.5±0.5[b]	2.3±0.4[b]
Days of FSH (range)	13.6±0.2[a] (11 – 17)	14.0±0.4[a] (11 – 21)	13.6±0.3[a] (11 – 18)
Infertility Diagnosis (%)[#]			
Ovulatory	12.2%[a]	3.8%[a]	0.0%[a]
Tubal	14.6%[a]	7.7%[a]	6.3%[a]
Endometriosis	4.9%[a]	7.7%[a]	3.1%[a]
Unexplained/Other	29.3%[a]	19.2%[a]	12.5%[a]
Diminished Fertility [9]	0.0%[a]	0.0%[a]	31.3%[b]
Male Factor	53.7%[a]	69.2%[a]	50.0%[a]

[#] Some patients may have more than one infertility diagnosis.

2.3.2 Cycle Outcomes

Young women with normal ovarian reserve had significantly more oocytes collected compared to young women with reduced ovarian reserve and the advanced maternal age women ($p<0.05$; Table 2.4). Fertilisation rate was significantly higher in the young women with normal ovarian reserve compared with the advanced maternal age group ($p<0.05$, Table 2.4). There was no significant differences in insemination method (ICSI rate), fertilisation rate between the young women with normal ovarian reserve and the reduced ovarian reserve group and between the reduced ovarian reserve and advanced maternal age group (Table 2.4).

Women of young maternal age with normal ovarian reserve had significantly less embryos transferred compared to the advanced maternal age group ($p<0.05$, Table 2.4) but no difference was found compared to the reduced ovarian reserve group (Table 2.4). No difference was found in the number of embryos transferred between the reduced ovarian reserve and advanced maternal age groups (Table 2.4). Women with reduced ovarian reserve and women of advanced maternal age had significantly more embryo transfers cancelled compared to the young women with normal ovarian reserve ($p<0.05$, Table 2.4). Embryo freezing was higher in the young women with normal ovarian reserve compared to both the reduced ovarian reserve and advanced maternal age groups ($p<0.05$, Table 2,4) however, no differences were detected between the reduced ovarian reserve and advanced maternal age groups in the mean number of embryos cryopreserved (Table 2.4).

The clinical pregnancy rate in young women with normal ovarian reserve was significantly higher compared to both the reduced ovarian reserve and advanced maternal age groups ($p<0.05$, Table 2.4). No differences were found in clinical pregnancy rate between the reduced ovarian reserve and advanced maternal age groups (Table 2.4). Implantation rate was significantly higher

in young women with normal ovarian reserve compared to the remaining two cohorts ($p<0.05$, Table 2.4). In addition, implantation rate was significantly higher in the reduced ovarian reserve group compared to the advanced maternal age group ($p<0.05$, Table 2.4). Oocyte utilisation was also significantly higher in young women with normal ovarian reserve compared to both the reduced ovarian reserve and advanced maternal age groups ($p<0.05$, Table 2.4). Oocyte utilisation was also significantly higher in the reduced ovarian reserve gorup compared to the advanced maternal age group ($p<0.05$, Table 2.4). (Note that Table 2.4 assesses all patients included in this study, for the following chapters only patients allocated to the experimental protocol are assessed in the cycle outcomes table specific for that chapter).

Table 2.4: IVF outcome in young women with normal and reduced ovarian reserve and in women of advanced maternal age with normal ovarian reserve.

	Young Maternal Age with Normal Ovarian Reserve (n = 45)	Young Maternal Age with Reduced Ovarian Reserve (n = 28)	Advanced Maternal Age with Normal Ovarian Reserve (n = 38)
No. of Oocytes Collected	12.0±0.9[a]	8.2±0.7[b]	6.8±0.8[b]
ICSI Rate (%)	93.0[a]	96.2[a]	87.5[a]
Fertilisation Rate (%)	69.2±4.0[a]	64.9±3.9[ab]	53.1±5.0[b]
Mean No. Embryos Transferred	1.2±0.1[a]	1.3±0.1[ab]	1.6±0.1[bc]
No. of Embryo Transfers Cancelled (%)	4.9[a]	12.5[b]	11.5[b]
Mean No. Embryos Frozen	2.9±0.4[a]	1.1±0.2[b]	0.5±0.2[b]
Pregnancy Rate (%)	56.7[a]	34.9[b]	17.9[b]
Implantation Rate (%)	39.0[a]	19.4[b]	8.9[c]
Utilisation of Oocytes (%)	77.5±3.6[a]	55.8±5.0[b]	28.0±4.0[c]

2.3.2.*1* *Embryo Development*

No significant differences were found between the three cohorts in day 3 embryo development (Figure 2.1). There was significantly better day 4 development in young women with normal ovarian reserve and in advanced maternal age women compared to young women with normal ovarian reserve ($p<0.05$, Figure 2.1). No difference was found in day 4 embryo development between young women with normal ovarian reserve and advanced maternal age women (Figure 2.1). Day 5 embryo development was significantly better in young women with normal ovarian reserve compared to both the reduced ovarian reserve and advanced maternal age women ($p<0.05$, Figure 2.1). Day 5 embryo quality (grade 1 and 2 only) was also significantly better in women with reduced ovarian reserve compared to the advanced maternal age group ($p<0.05$, Figure 2.1)

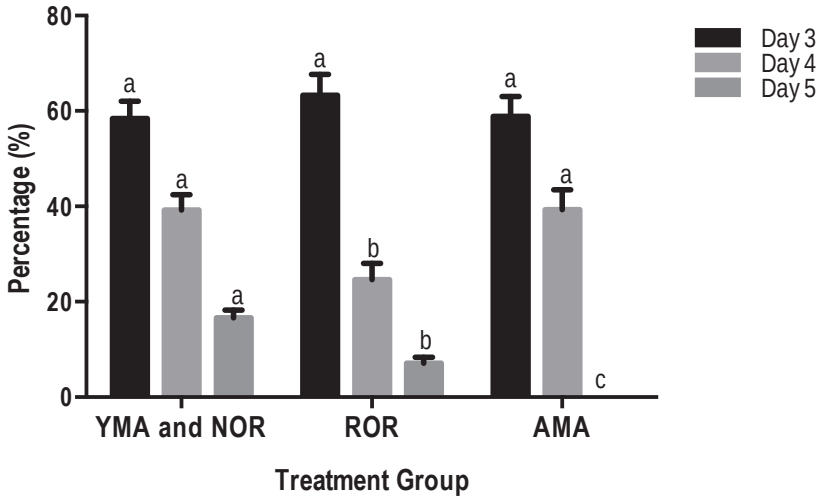

Figure 2.1: Embryo development for each cohort on day 3, 4 and 5.
Embryo development assessed as the percentage of embryos on time for embryo development (grade 1 and 2). n (YMA & NOR) = 45, n (YMA & ROR) = 28, n (AMA & NOR) = 38. a –c indicate significant differences between cohorts for day of embryo development ($p<0.05$). YMA – young maternal age, NOR – normal ovarian reserve, ROR – reduced ovarian reserve and AMA – advanced maternal age. Data expressed as mean ± SEM.

2.4 Discussion

The results from this cohort have established that young women with a normal ovarian reserve for their age have a superior IVF outcome compared to women (≤35 years) who had a low ovarian reserve (as measured by serum AMH concentration) and older women (≥40 years) with a normal ovarian reserve (for their age) when measured by clinical pregnancy rates, implantation rates, embryo quality on day 4 and day 5 and utilisation of oocytes. Clinical pregnancy rates in older women have long been known to be poorer than those women that are younger. Furthermore, women over the age of 35 on average take longer to conceive and require more cycles of IVF treatment compared to younger women and this is consistent with the findings in this study in which women of advanced maternal age had already had more IVF cycles [10]. However, the association of a poorer IVF outcome with reduced ovarian reserve has been subject to controversy.

Compared to young women (≤35 years), pregnancy rates in women of advanced maternal age are thought to be reduced due to the decline in oocyte quality and this is consistent with what was found in this study. A study of almost 3000 cycles showed the cumulative pregnancy rate in women under the age of 30 was 62% compared to 34% in women over the age of 30 after 12 IVF cycles [10]. It has been well-established that the rate of aneuploidy in oocytes increases with maternal age. A study using cytogenetic analysis on over 3000 unfertilised human oocytes established that the oocyte aneuploidy rate in women <35 is between 10 – 15% and this rate increases to about 24 – 43% between the ages of 35 – 40 and >58% at 43 years of age or older [11]. Not surprisingly, this increase in oocyte aneuploidy also correlates with an increase in chromosomally abnormal pregnancies and spontaneous abortions that occurs with increasing age [12]. It is also established that pregnancy loss in women <35 years of age with diminished

ovarian reserve (as measured by serum FSH) is very high at 57.1% [13]. In addition altered spindle formation has also been reported in younger women (<38 years) with reduced ovarian reserve [16]. Altered spindle formation is thought to be associated with mis-alignment and mis-segregation of chromosomes which can result in aneuploidy [17, 18].

Oocyte aneuploidy results predominantly from the mis-segregation of homologous chromosomes during the first meiotic division. Several molecular mechanisms have been suggested to explain the origin of aneuploidy, these include errors in recombination, improper spindle formation, a decline in interaction between the microtubules and the kinetochore and a deficiency in the spindle assembly checkpoint [5, 19]. It has been suggested that there is also a decline in chromosome cohesion in the oocytes of women of advanced maternal age [20, 21]. The cohesion proteins, important for keeping both homologous chromosomes and sister chromatids together, are thought to only be produced during fetal development thus in humans these proteins must remain functional for years to ensure correct chromosome separation occurs [20, 21]. However, studies on the association between ovarian reserve and aneuploidy have not confirmed this, suggestive of a different mechanism for the reduction in clinical pregnancy rates [22].

Another mechanism contributing to a reduction in oocyte and embryo viability may be energy production and in particular mitochondrial function. It has previously been demonstrated that the ATP content of unfertilised oocytes reflected the developmental competence of sibling oocytes [23]. Further there have been several animal models demonstrating causality of reduced mitochondrial function in oocytes and embryos and reduced viability [24-28]. However, in the human a direct link between mitochondrial energy production within ovarian cells and reduced oocyte viability remains to be established.

Another contributing factor to the decline in oocyte quality seen with increasing maternal age are changes in oocyte gene expression. A study examined the gene expression profiles of single metaphase II oocytes and established a difference in gene expression of 342 genes in women with advanced maternal age, specifically in genes involved in cell cycle regulation, chromosome alignment, chromatid separation and oxidative stress compared to younger women [29]. The transforming growth factor-β (TGF-β) signalling pathway was one such pathway that was the most significantly affected by maternal age [29]. BMP-15 and GDF-9, two members of the TGF-β family, are known to be secreted by the oocyte with these two proteins have been found previously to act upon cumulus cells and increase developmental competence of the oocyte [30, 31]. Thus, this decline in the TGF-β pathways in women with of advanced maternal age may offer one explanation as to why oocytes from these women result in poorer embryo development and the observed decline in pregnancy rates however this remains to be fully investigated.

In this present study we utilised serum AMH levels as an indicator of ovarian reserve, with this relationship having previously been established in multiple studies [8, 32-34]. Previous studies have shown a positive association between reduced ovarian reserve and a poorer IVF outcome including a lower response to ovarian stimulation [35, 36], less oocytes retrieved [34-36], a decrease in the number of normally fertilised (oocytes with 2 pronuclei) [35, 37, 38], a decline in pregnancy rates [13], an increase cycle cancellations [35] and higher miscarriage rates [13, 37, 39]. However, studies examining the possibility of using serum AMH as a predictive tool for IVF outcome remain subject to controversy with other studies showing no correlation between serum AMH concentrations and pregnancy rates but rather a predictor of ovarian response [8, 40, 41]. Consistent with these findings, in the present study, young women with reduced ovarian reserve

had less oocytes collected and a decrease in pregnancy rates compared to their age matched counterparts with normal ovarian reserve. These women also had poorer day 4 and day 5 embryo development. AMH has previously been positively correlated to blastocyst development and this is consistent with the findings of this study in which young women with a normal ovarian reserve had significantly better day 5 embryo development, an increase in the number of embryos cryopreserved and hence an increase in overall oocyte utilisation [42-45]. This is significant as this may contribute to the increase in cumulative pregnancy rates that has also been determined in women with a higher AMH concentration [45].

The findings presented in this study are consistent with many that report a relationship between reduced ovarian reserve or advanced maternal age and IVF outcome. However, what still remains to be determined is the mechanism behind this decline in IVF outcome, specifically in women with reduced ovarian reserve and to determine if these mechanisms are consistent with women of advanced maternal age. As discussed, current research has been focussed around oocyte aneuploidy as being the major factor contributing to the poor IVF outcomes seen in women of advanced maternal age. However, even though array Comparative Genomic Hybridisation (aCGH) of embryos from women of advanced maternal age increases pregnancy rates, it does not increase them to the same level seen in younger women, suggesting that aneuploidy is not the only contributing factor to the decline in pregnancy rates and an alternative mechanism may also be involved.

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CHAPTER THREE

Women with Reduced Ovarian Reserve or Advanced Maternal Age have an Altered Follicular Environment

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Women with Reduced Ovarian Reserve or Advanced Maternal Age have an Altered Follicular Environment.

Introduction

In western society the age of women trying to conceive is increasing which correlates with decreased oocyte quality and pregnancy rates and increased pregnancy complications (1-4). This age-related decline in oocyte quality results from increased aneuploidy and reduced oocyte metabolic function (5,6). Interestingly, reduced ovarian reserve (follicle number remaining in the ovarian pool); irrespective of age, is also associated with decreased pregnancy and cumulative pregnancy rates (7,8). Furthermore, women with reduced ovarian reserve have fewer oocytes collected, reduced embryo development and increased miscarriage rates (7).

The ovarian follicle provides an important environment for the support and development of the oocyte and surrounding ovarian cells. Follicular fluid is comprised of factors that maintain the oocyte in meiotic arrest and aid in ovulation and fertilisation (9). The oocyte and surrounding follicular cells play interdependent roles during follicular development, regulating growth, selection and ovulation of a competent oocyte via signaling of paracrine factors and gonadotrophins (10). These factors are synthesised by the follicular cells and oocyte and are secreted into the follicular fluid or originate from blood (9,11). The follicular fluid microenvironment is integral to the growing oocyte and changes in follicular dynamics are reflected in follicular fluid composition.

Human follicular fluid contains carbohydrates such as glucose, lactate and pyruvate, with a recent study correlating concentration to IVF outcome and therefore oocyte quality (12). Hormones are also important components of follicular fluid, which act as key regulators of follicular cell function and development. Previous studies have demonstrated that high follicular fluid concentrations of follicle-stimulating hormone (FSH) and luteinising hormone (LH) are associated with increased fertilisation, and embryos that result in successful IVF outcome are likely to have originated from follicles with high follicular fluid LH levels (11,13). The concentration of steroid hormones is also predictive of implantation and pregnancy with increased estrogen associated with improved oocyte maturation and increased progesterone associated with higher abnormal oocyte fertilisation rates (oocytes with one or >2 pronuclei), suggesting a link between hormonal regulation and oocyte competence (11).

In the antral follicle, differentiation of the somatic cells surrounding the oocyte into cumulus cells is essential for oocyte development and viability (14). Cumulus cells exhibit functional characteristics different to granulosa cells with higher rates of proliferation, low steroidogenic capabilities (especially progesterone) and low LH receptor expression (15,16). In response to the LH surge, cumulus cells undergo expansion and secrete macromolecules, whereas granulosa cells undergo luteinisation and increase progesterone production (14). These changes are dependent on oocyte secreted factors and to date few studies have addressed the effect of ovarian reserve or maternal age on cellular differentiation (17).

Thus far, little is understood as to how the follicular environment and follicular cell functions is altered as a result of reduced ovarian reserve or chronological age. This study aimed to determine the follicular fluid carbohydrate and hormone content, and the metabolic profile and

steroidogenic activity of granulosa and cumulus cells in women with reduced ovarian reserve or advanced maternal age compared to young women with normal ovarian reserve.

Materials and Methods

Patient Recruitment and Cohorts

Women (n=54) undergoing IVF treatment at Repromed *(Dulwich, South Australia (S.A.), Australia)* were recruited for participation in this study (exclusion criteria were donor/recipient cycles, preimplantation genetic screening and PCOS patients). Each woman was only represented once within the study period. Samples were de-identified and randomly allocated to experimental protocols (Supplementary Figure 3.1), based on age (young maternal age: ≤35 and advanced maternal age: ≥40) or ovarian reserve. Serum anti-mullerian hormone (AMH) was used as a measure of ovarian reserve. The AMH range for young women (≤35) with normal ovarian reserve was 1.4-4.2μg/L and the reduced ovarian reserve group had serum AMH concentrations of <1.4μg/L (these women were all ≤35 years). AMH values for women of advanced maternal age (>40) were 0.4-1.4 μg/L and these values represent 25-75% quartiles for this age group (18). Ethical approval was obtained from the Women's and Children's Hospital Research Ethics Committee *(North Adelaide, S.A.)* and written informed consent was obtained for use of surplus follicular fluid, granulosa and cumulus cells.

Ovarian Simulation and Follicular Fluid Collection

All women underwent antagonist cycles using gonadotropin stimulation with recombinant FSH (*Puregon (100-375U), Schering-Plough, New South Wales (N.S.W), Australia or Gonal F (150-350U), Merck Serono, (N.S.W.), Australia)*, administered from menstrual cycle day 2/3. On day 7 a GnRH antagonist *(Orgalutran (250mcgms), Schering-Plough)* was administered. Follicle growth was monitored via ultrasound and when ≥2 follicles ≥17mm were present, the hCG trigger (*Ovidrel (250mcgms)* was administered and oocytes collected after 36hrs. Follicular fluid was aspirated with a 17-gauge single-lumen needle, under sedation (no media flush), with the

guidance of a transvaginal ultrasound from ovarian follicles. The surplus follicular fluid, containing no or minimal blood contamination, was centrifuged for 10mins at 300g, frozen and stored at -80°C, each tube of follicular fluid contained 1-2 follicles (≥18mm) and were processed separately. Consistent with previous findings by Robker et al., 2009, there were no differences between follicular fluid concentrations of metabolites and hormones from follicles ≥18mm within patients (Appendix 3) (19).

Granulosa and Cumulus Cell Collection and Culture

Immediately following oocyte retrieval, granulosa cells were removed from the surplus follicular fluid and washed into Dulbecco's Phosphate Buffered Saline (PBS) *(Sigma-Aldrich, N.S.W., Australia)*. Cells were purified by density gradient centrifugation using 60%/40% silica solution *(Spermgrad, Vitrolife, Göteborg, Sweden)* and spun for 30mins at 470g. Cumulus cells were collected by trimming cumulus oocyte complexes (COCs) of the outer layers of cells and washed through Dulbecco's PBS immediately after oocyte collection. Cell clumps were disassociated by pipetting, granulosa and cumulus cell counts were performed using a haemocytometer, and cell suspensions of $1x10^6$cells/ml were cultured in modified G-1 media (as described in Gardner *et al*., 2004, minus lactate) at 37°C in $5\%O_2/6\%CO_2$ for 4hrs (20). A modified G-1 media only control was included.

Carbohydrate and Glycolytic Index Assessment

Follicular fluid glucose concentration was measured using the Optium Xceed diabetes monitoring system *(Abbott Diabetes Care, Victoria, Australia)*.

Follicular fluid pyruvate and lactate, and spent media glucose and lactate concentrations from cultured granulosa and cumulus were assessed using a microfluorometric assay. The concentration of each metabolite was determined using enzyme-linked reactions to pyridine nucleotides (NAD(P)H), as described previously (21). A standard curve (r^2>0.98) was run with each analysis and patient samples were assessed in triplicate and expressed as uptake or production per 1x10^6cells/hr.

Glycolysis was calculated on the basis of the breakdown of 1 molecule of glucose to 2 molecules of lactate expressed as a percentage.

Hormone Assessment

Estradiol, progesterone, LH and FSH levels were analyzed by an ADVIAR Centaur XP *(Siemens Healthcare Diagnostics Inc., Victoria, Australia),* according to manufacturer's instructions by an externally accredited laboratory. AMH concentration was determined using an Immunotech ELISA immunoassay *(Beckman Coulter, N.S.W., Australia)* according to manufacturer's instructions.

Ion, Lactate, Total Protein, Albumin and Cholesterol Assessment

Follicular fluid was analyzed for ions (sodium, potassium, chloride, bicarbonate, and calcium), lactate, total protein, albumin and cholesterol on a Siemens Advia Chemistry System *(Siemens Healthcare Diagnostics Inc.)* by an external accredited laboratory.

RNA Isolation and qPCR in Granulosa and Cumulus Cells

Granulosa and cumulus cell RNA was extracted as per manufacturer's instructions using an RNeasy Kit *(Qiagen, Victoria, Australia)*. RNA concentration was determined using the Nanodrop 1000 spectrophotometer *(Thermo Scientific, Waltham, Massachusetts)*. Complementary DNA (cDNA) was synthesized using Superscript III Reverse Transcriptase *(Invitrogen, Victoria, Australia)* as per manufacturer's instructions. qPCR primers were designed to human sequences using NCBI Blast and synthesized by Geneworks *(Hindmarsh, S.A., Australia)* (Supplementary Table 3.1). Samples were run in triplicate on the Corbett Rotor Gene 6000 *(Corbett Life Sciences, Qiagen, Victoria, Australia)*. For each reaction 2µl of cDNA was added to a master mix containing 1µl of each forward and reverse primer (diluted to 10pmol/µl), 10µl of SYBR Green *(Applied Biosystems, Victoria, Australia)* and 6µl of H_2O, giving a total volume of 20µl. PCR cycling conditions were 50°C for 2mins, 95°C for 10mins, 40 amplification cycles of 95°C for 15secs and 60°C for 1min. Analysis was performed using delta, delta ct, normalized to 18S (22).

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). To determine significance between groups a Student's T-test was used for patient demographics and a one-way ANOVA with Tukey's post-hoc test was used for the remainder of analyses. A p-value <0.05 was considered statistically significant.

Results

Table 3.1: Patient demographics and cycle outcomes.[1]

		Young Maternal Age with Normal Ovarian Reserve	Reduced Ovarian Reserve	Advanced Maternal Age
Age		30.9±0.9[a]	32.5±0.6[a]	41.5±0.5[b]
Serum AMH (µg/L)		3.1±0.3[a]	0.7±0.1[b]	1.2±0.1[c]
BMI		24.4±1.2[a]	24.5±1.6[a]	27.6±0.5[a]
Starting FSH Dose (IU)		190.5±14.0[a]	303.5±11.4[b]	307.2±8.1[b]
# previous fresh cycles (IVF/ICSI)		0.8±0.2[a]	2.6±0.7[b]	3.2±0.7[b]
# oocytes collected		11.4±0.9[a]	8.0±1.3[b]	7.8±1.3[b]
Fertilisation Rate (%)		63.5±6.3[a]	63.4±6.5[a]	50.0±6.6[a]
Mean # Embryos Transferred		1.2±0.1[a]	1.3±0.2[ab]	1.6±0.1[b]
Mean # Embryos Frozen		3.4±0.7[a]	1.2±0.4[b]	0.6±0.3[b]
Pregnancy Rate (%)		57.9[a]	50.0[a]	13.3[b]
Implantation Rate (%)		45.5[a]	36.3[a]	8.3[b]
Utilisation of Oocytes (%)[2]		45.0[a]	27.2[b]	28.0[b]
Infertility Diagnosis (%)[3]	**Ovulatory**	4.8[a]	9.1[a]	0.0[a]
	Tubal	9.5[a]	9.1[a]	5.9[a]
	Endometriosis	19.1[a]	18.2[a]	0.0[a]
	Unexplained	9.5[a]	9.1[a]	0.0[a]
	Male Factor	71.4[a]	81.8[a]	70.6[a]

[1] n(Young Maternal Age with Normal Ovarian Reserve)=21, n(Reduced Ovarian Reserve)=16, n(Advanced Maternal Age)=17, data expressed as mean±SEM, a-c indicate significant differences between cohorts, $p<0.05$. Exclusion criteria: donor/recipient cycles, preimplantation genetic screening and PCOS patients

[2] Utilization of Oocytes was calculated as follows: (frozen embryos + embryos transferred) / oocytes

[3] Infertility diagnosis: multiple diagnoses for some patients

Patient Demographics

Mean maternal age, serum AMH levels and etiology for young women with normal ovarian reserve, reduced ovarian reserve and advanced maternal age are indicated in Table 3.1. No significant differences in BMI or fertilisation rates were found between groups. Young women with normal ovarian reserve had significantly lower starting FSH doses, fewer previous cycles and more oocytes collected compared to women with reduced ovarian reserve or advanced maternal age. Young women with normal ovarian reserve had increased number of embryos frozen and higher implantation rates compared to women with reduced ovarian reserve or advanced maternal age ($p<0.05$; Table 3.1).

Carbohydrate Concentrations in Follicular Fluid

Follicular fluid glucose concentration was decreased and lactate and pyruvate concentrations increased in young women with reduced ovarian reserve and women with advanced maternal age compared to young women with normal ovarian reserve ($p<0.05$; Table 3.2). Furthermore, follicular fluid glucose concentration was decreased and lactate concentration increased in women with reduced ovarian reserve compared to women of advanced maternal age ($p<0.05$; Table 3.2).

Hormone Concentrations in Follicular Fluid

Follicular fluid estradiol concentration was higher in women with reduced ovarian reserve and lower in women with advanced maternal age, compared to young women with normal ovarian reserve ($p<0.05$; Table 3.2). Compared to young women with normal ovarian reserve, follicular fluid progesterone concentration was increased and AMH concentration was decreased in the

reduced ovarian reserve and advanced maternal age groups ($p<0.05$; Table 3.2). No significant differences were found in follicular fluid concentrations of LH or FSH between groups.

Table 3.2: Follicular fluid concentrations of carbohydrates, hormones and ions in young women with either normal ovarian reserve or reduced ovarian reserve and in women of advanced maternal age.[1]

		Young Maternal Age with Normal Ovarian Reserve	Reduced Ovarian Reserve	Advanced Maternal Age
Carbohydrates	**Glucose** (mmol/L)	3.9±0.2[a]	2.3±0.2[b]	2.9±0.2[c]
	Lactate (mmol/L)	2.6±0.1[a]	6.0±0.6[b]	3.9±0.5[c]
	Pyruvate (mmol/L)	0.6±0.1[a]	0.9±0.1[b]	0.7±0.1[b]
Hormones	**Estradiol** (pmol/L)	1634.4±149.6[a]	3971.9±106.0[b]	770.3±117.1[c]
	Progesterone (nmol/L)	2917.9±229.1[a]	3765.1±200.2[b]	4701.0±547.6[b]
	AMH (µg/L)	5.8±0.4[a]	1.4±0.1[b]	1.1±0.1[b]
	LH (IU/L)	1.2±0.2[a]	1.0±0.0[a]	1.4±0.2[a]
	FSH (IU/L)	6.2±0.7[a]	5.3±0.7[a]	6.1±0.6[a]
Ions	**Sodium** (mmol/L)	129.6±2.7[a]	124.0±3.6[a]	124.6±5.2[a]
	Potassium (mmol/L)	4.1±0.1[a]	4.1±0.2[a]	4.0±0.2[a]
	Chloride (mmol/L)	103.2±1.7[a]	99.4±2.4[a]	100.0±2.9[a]
	Bicarb (mmol/L)	19.0±0.6[a]	17.6±0.8[a]	17.4±1.7[a]
	Calcium (mmol/L)	1.76±0.1[a]	1.61±0.1[a]	1.68±0.1[a]
	Protein (g/L)	45.8±2.8[a]	36.2±7.5[a]	38.4±6.9[a]
	Albumin (g/L)	30.6±1.9[a]	24.2±4.9[a]	26.2±4.8[a]
	Cholesterol (mmol/L)	0.66±0.1[a]	0.5±0.0[a]	0.67±0.0[a]

[1] n(Young Maternal Age & Normal Ovarian Reserve)=10, n(Reduced Ovarian Reserve)=8, n(Advanced Maternal Age)=12, data expressed as mean±SEM, a-c indicates significant difference between cohorts ($p<0.05$).

Ion, Total Protein, Albumin and Cholesterol Concentrations in Follicular Fluid

Follicular fluid concentrations of sodium, potassium, chloride, bicarbonate, calcium, protein, albumin and cholesterol were not different between groups (Table 3.2).

Glucose Uptake and Lactate Production by Cultured Granulosa Cells and Cumulus Cells

To assess the follicular cell contribution to the differences in follicular fluid glucose and lactate concentration, the carbohydrate metabolism of granulosa and cumulus cells was assessed. In both cell types the rate of glucose uptake and lactate production in women with reduced ovarian reserve or advanced maternal age was increased compared to young women with normal ovarian reserve. Granulosa and cumulus cell glucose uptake and lactate production was increased in the reduced ovarian reserve compared to the advanced maternal age groups ($p<0.05$; Figure 3.1A and 3.1B).

The Glycolytic Index of the Granulosa and Cumulus Cells

To further assess the differences in glucose uptake and lactate production between granulosa and cumulus cells, the glycolytic index was determined. Compared to granulosa cells an increased glycolytic index (increase in lactate per glucose consumed) was exhibited in cumulus cells, irrespective of cohort ($p<0.05$; Figure 3.1C). No significant differences existed in the glycolytic index of granulosa cells in the reduced ovarian reserve or advanced maternal age groups compared to the young women with normal ovarian reserve group. However, an increased glycolytic index was observed in the granulosa cells from women with reduced ovarian reserve compared to the advanced maternal age group ($p<0.05$; Figure 3.1C). In cumulus cells,

young women with normal ovarian reserve had an elevated glycolytic index compared to the reduced ovarian reserve and the advanced maternal age groups ($p<0.05$; Figure 3.1C).

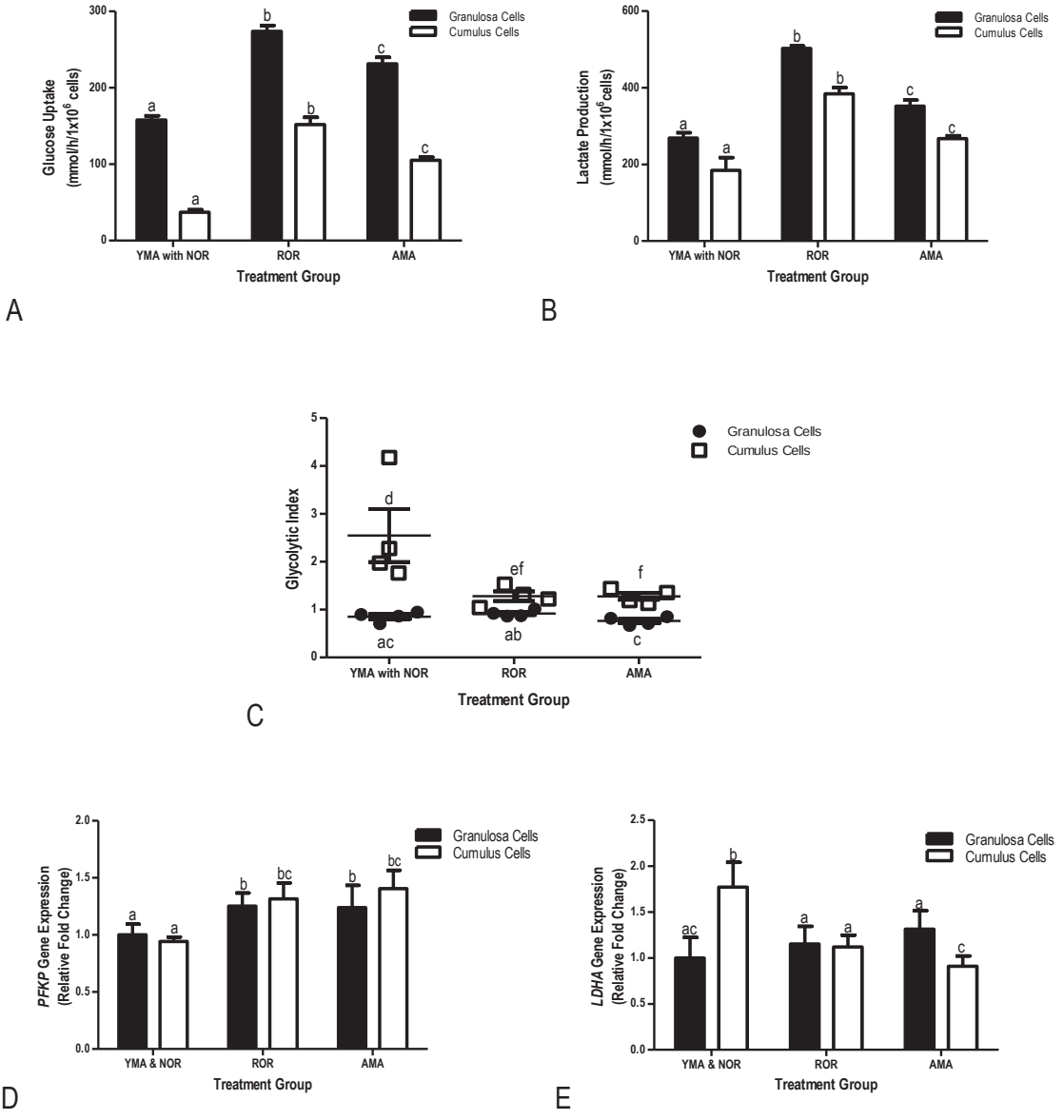

Figure 3.1: Comparison of granulosa cell and cumulus cell glucose uptake (A), lactate production (B), glycolytic index of granulosa and cumulus cells (C) and gene expression of Phosphofructokinase Platelet *(PFKP)* (D) and Lactate Dehydrogenase A *(LDHA)* (E).
Figure 3.1A – C: n=4 patients per cohort. Figure 3.1A and 3.1B: a-c – indicate significant differences between cohorts for cell type ($p<0.05$). Figure 3.1C: a-f – indicate significant differences between cohorts. Figure 3.1D and 3.1E: n=5 patients per cohort, a-c – indicate significant differences between cohorts. YMA – young maternal age, NOR – normal ovarian reserve, ROR – reduced ovarian reserve and AMA – advanced maternal age.

Glycolytic Gene Expression in the Granulosa and Cumulus Cells

To further assess the differences in glycolysis, gene expression of the glycolytic enzymes, phosphofructokinase platelet (*PFKP*) and lactate dehydrogenase A (*LDHA*) were assessed. Levels of *PFKP* gene expression were similar between cumulus and granulosa cells within treatments. *PFKP* levels were increased in granulosa and cumulus cells in the reduced ovarian reserve and advanced maternal age groups compared to young women with normal ovarian reserve concomitant with the observed increase in glucose uptake ($p<0.05$; Figure 3.1D). In contrast, *LDHA* gene expression was decreased in the cumulus cells from women with reduced ovarian reserve with a further decrease observed with advanced maternal age groups ($p<0.05$; Figure 3.1E), such that the normal increase in gene expression with differentiation of the cumulus cells was lost in the reduced ovarian reserve and advanced maternal age groups.

Progesterone Production by the Granulosa and Cumulus Cells

As there were differences in follicular fluid progesterone concentration between groups, progesterone secretion by the follicular cells was determined. Spent media concentrations of progesterone produced by the granulosa cells was significantly lower in the reduced ovarian reserve and advanced maternal age groups compared to young women with normal ovarian reserve ($p<0.05$; Figure 3.2A). Conversely, cumulus cell progesterone production was increased in the reduced ovarian reserve and advanced maternal age groups compared to young women with normal ovarian reserve. Cumulus cell progesterone production in the reduced ovarian reserve group was elevated compared to the advanced maternal age group ($p<0.05$; Figure 3.2B).

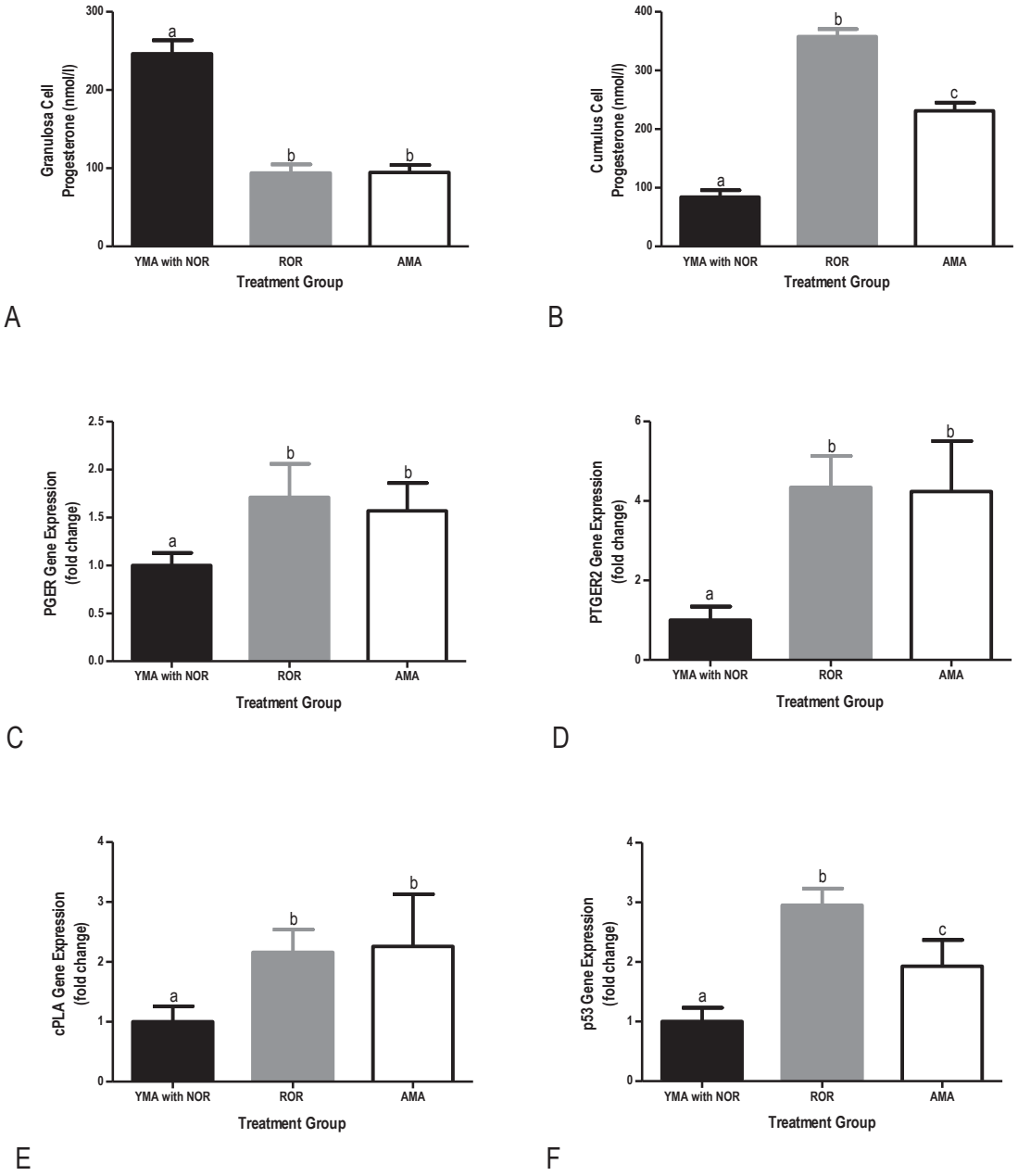

Figure 3.2: Progesterone production by cultured granulosa cells (A) and cumulus cells (B) and: gene expression of the progesterone receptor *(PGER)* (C), prostaglandin E receptor-2 *(PTGER2)* (D), cytosolic phospholipase A2 *(cPLA2)* (E) and tumour protein 53 *(p53)* (F) in cumulus cells.

Figure 3.1A and 3.1B: n=6 patients per cohort, a-c indicate significant differences between cohorts ($p<0.05$). Figure 3.1C – 3.1F n=3 patients per cohort, a-c indicate significant differences between cohorts ($p<0.05$). YMA – young maternal age, NOR – normal ovarian reserve, ROR – reduced ovarian reserve and AMA – advanced maternal age.

Progesterone Receptor, Prostaglandin and Phospholipase Activity and Apoptotic Marker Gene Expression in Cumulus Cells

To assess the molecular differences in progesterone production and apoptosis, gene expression was performed on cumulus cells for genes involved in the regulation of progesterone, prostaglandins and apoptosis. Gene expression levels for *Progesterone Receptor (PGER)*, *Prostaglandin E Receptor-2 (PTGER2)*, *Cytosolic Phospholipase A2 (cPLA2) Tumor Protein 53 (p53)* were higher in cumulus cells of women with reduced ovarian reserve or of advanced maternal age compared to young women with normal ovarian reserve ($p<0.05$; Figure 3.2C-F). However, no differences were found in gene expression of *Prostaglandin Endoperoxide Synthase 2, Secretory Phospholipase A2* or *Retinoblastoma 1*.

Discussion

The results from this study have established that carbohydrate (glucose, lactate and pyruvate) and hormone (progesterone, estrogen and AMH) levels are altered in the follicular fluid of women with reduced ovarian reserve or advanced maternal age. Cumulus cells from these women did not display the changes in both metabolic and steroidogenic activity that were observed in the young women with normal ovarian reserve, reflecting a phenotype more commonly associated with granulosa cells, suggesting altered or lack of differentiation in response to the LH surge. This data would suggest that there are metabolic and molecular changes to the follicular cells resulting in changes to the follicular fluid content which may be responsible for the reduced oocyte competence that were observed in this study. There has been some contradictory evidence in the literature regarding the ability of AMH to predict pregnancy outcomes with some studies demonstrating a relationship with pregnancy rates (7,8) and similar to our findings others demonstrating little effect (18). However, in this study, implantation rates were reduced in the advanced maternal age group, and the mean number of embryos that were able to be frozen or utilized was significantly less in both the reduced ovarian reserve and advanced maternal age group compared to the young women with normal ovarian reserve. These findings indicate that there was a decrease in the percentage of viable oocytes in both the reduced ovarian reserve and advanced maternal age groups, which is similar to the reduction in cumulative pregnancy rates previously reported (7).

Follicular fluid is not only a serum transudate but is comprised of hormones and carbohydrates that are synthesized by follicular cells (23). Ions in particular are components that are able to be filtered from blood serum. In this study the levels of ions, as well as albumin, were similar to that previously reported for serum and reflects the levels reported previously for follicular fluid, which were not altered by reduced ovarian reserve or advanced maternal age (24).

However, in direct contrast to the ionic composition, follicular fluid carbohydrate composition was altered in women with reduced ovarian reserve or advanced maternal age. The decrease in glucose and concomitant increase in follicular fluid lactate concentrations reflects the altered metabolic profile of granulosa and cumulus cells in these women. These cells take up more glucose and produce higher lactate quantities, thus increasing follicular fluid lactate levels. Follicular fluid pyruvate concentrations were also elevated in women with reduced ovarian reserve and advanced maternal age, possibly reflective of increased metabolic rate. Previous studies in granulosa cells of PCOS patients report a down-regulation in insulin-dependent glucose uptake and lactate production with no change in LH-mediated glucose metabolism (25). In contrast, we report an increase in glucose metabolism, possibly LH-mediated, resulting in increased lactate production in the reduced ovarian reserve and advanced maternal age groups. Similar to women with reduced ovarian reserve, obese women are also known to have elevated follicular fluid glucose and lactate concentrations (19,26). The consequences of this up-regulation in glycolysis are currently unknown however, a consequence of increased follicular lactate production may be a decrease in follicular fluid pH. Follicular fluid is considered to be alkaline and decreased pH is related to reduced oocyte fertilization (7,24,27).

In young women with normal ovarian reserve cumulus cell metabolism underwent an up-regulation in glycolytic index however, no such change occurred in the women with reduced ovarian reserve or advanced maternal age. This increase in glucose consumption increased the levels of the allosteric glycolytic regulator *PFKP*. Further, *LDHA* which converts pyruvate to lactate in the glycolysis pathway similarly reflected the changes in glycolysis and lactate production with increases in cumulus cells in young women with normal ovarian reserve. Similarly, cumulus cell *LDHA* was reduced in women with reduced ovarian reserve and advanced maternal age where glycolysis, specifically lactate production was also reduced. Changes in

glycolytic enzyme levels between granulosa and cumulus cells are at least in part mediated by the oocyte suggesting that oocyte signaling is impaired, possibly affecting the transition of granulosa to cumulus cell phenotype. This transition within the follicle has been shown to require endocrine signals from the follicle but is also reliant on paracrine signaling from the oocyte (28). This bi-directional communication between cumulus cells and the oocyte is essential for maintaining oocyte competence (29). It is therefore plausible that oocyte signaling from women with reduced ovarian reserve or advanced maternal age may be reduced, affecting the capacity of the cells surrounding the oocyte to differentiate into the cumulus cell phenotype. However, it is plausible impairment of the follicular cells may be affecting their ability to differentiate. In animal models reduced levels of oocyte secreted factors (OSFs), such as Growth Differentiation Factor-9 and Bone Morphogenetic Protein-15, result in decreased oocyte competence and changes in cumulus cell function, such as increased apoptotic markers (30). It has previously been demonstrated that granulosa cell viability is reduced with declining ovarian reserve and in our study cumulus cells have increased gene expression of the apoptotic marker *p53* (31). Therefore, the levels of OSFs in women with reduced ovarian reserve or advanced maternal age warrant further investigation.

Studies have demonstrated that increased follicular fluid progesterone or decreased estrogen concentrations are associated with reduced oocyte competence (11,13). Not surprisingly in older women, whose oocyte competence is reduced, there were elevated levels of progesterone and lower levels of estrogen in follicular fluid. However, in women with reduced ovarian reserve, although there was an increase in progesterone levels, estrogen was also increased. As estrogen is secreted by the theca and granulosa cells this would indicate functional differences in steroidogenic capacity in these cells (32). Furthermore, cumulus cells from women with reduced ovarian reserve and women of advanced maternal age had different steroidogenic profiles,

particularly the expression of *PGER*, *PTGER2* and *cPLA* which regulate progesterone and prostaglandin actions and production, were up-regulated compared to the young women with normal ovarian reserve. Similar to these findings a recent study by Skiadas *et al*., 2012 also reported altered gene expression in the granulosa cells of young women with reduced ovarian reserve compared to women with a higher ovarian reserve, particularly in genes that are involved with progesterone production/response (33). Therefore, similar to the metabolic changes, the hormonal profile of cumulus cells are altered in women with reduced ovarian reserve and advanced maternal age reflecting a more granulosa-like physiology further suggesting these cells may have failed to differentiate completely into a cumulus cell phenotype.

The increase in follicular fluid concentration of progesterone and cumulus cell progesterone production in the reduced ovarian reserve and advanced maternal age groups may be attributed to the increased gene expression of both PGER and PTGER2. In addition, the increase in gene expression of cPLA2 in cumulus cells in women with reduced ovarian reserve or advanced maternal age may result in greater phospholipolytic activity, presumably yielding increased levels of arachidonic acid, thereby augmenting the availability of substrate for prostaglandin E2 (PGE2) synthesis. The resulting increase in PGE2, acting through the elevated PTGER2, would increase cAMP concentration which, in turn, could augment progesterone synthesis (34). Linoleic acid, another product of the phospholipid lipolysis, may contribute directly to the reduced oocyte quality observed in women with reduced ovarian reserve or advanced maternal age. This fatty acid has recently been reported to inhibit cumulus expansion and meiotic maturation in isolated bovine COCs, resulting in impaired embryo development (35).

Interestingly, follicular fluid from women with reduced ovarian reserve and advanced maternal age contained lower AMH levels compared to the young women with normal ovarian reserve. AMH is secreted by early granulosa cells in the primary and small antral follicles (36). However, AMH expression still exists in cumulus cells of the pre-ovulatory follicle with lower expression in granulosa cells, suggesting that these cells still have the capacity to secrete AMH into the follicular fluid at these later stages of folliculogenesis (37). Previous studies have demonstrated a positive correlation between follicular fluid AMH concentration and successful oocyte fertilisation suggesting AMH production and activity is reduced in granulosa and cumulus cells from these patients (8,37,38). Andersen *et al*., 2006, reported that follicular fluid FSH can act as a negative regulator of AMH expression, however in this study follicular fluid FSH and LH concentrations were not different between the groups (39). However, AMH null mice have higher oocyte degeneration, defined as the presence of oocyte remnants within follicles, suggesting that oocytes in the follicles from women with reduced ovarian reserve may be degrading and serves as an explanation for the decline in oocyte number seen in these women (7,40).

These results demonstrate an association between perturbed ovarian follicular cell metabolism in women with reduced ovarian reserve and advanced maternal age exists. However, the mechanisms appear to differ between these two groups of women with differences observed in metabolic and steroid measures as well as in their cycle outcomes. However, although it is possible that the elevated FSH dose received by women with reduced ovarian reserve or advanced maternal age may be contributing to this altered follicular metabolism, the dose was similar in these two groups, implying that other factors are also involved. Interestingly, in this study follicular fluid FSH concentration was not different between the three cohorts, thus suggesting an FSH dose effect on the immediate environment of the follicular cells may not be occurring. In this study, the altered follicular environment was characterized by what resembled a

lack of differentiation of granulosa cells into cumulus cells which has been implicated to result from reduced oocyte paracrine factors. This bi-directional communication between the oocyte and surrounding cells is important for oocyte viability. Therefore it is possible that the decrease in overall oocyte quality, as demonstrated in the reduction of oocyte utilization, seen in women with reduced ovarian reserve or advanced maternal age may be due to the perturbed follicular cell metabolism/environment or the reverse may be true with the altered follicular cell metabolism/environment potentially causing the decrease in overall oocyte quality, therefore the exact causes resulting in these perturbations still remain to be elucidated. Knowledge of these perturbations could lead to changes in timing of oocyte retrieval or improving media requirements for these poorer prognosis women undergoing IVF treatment.

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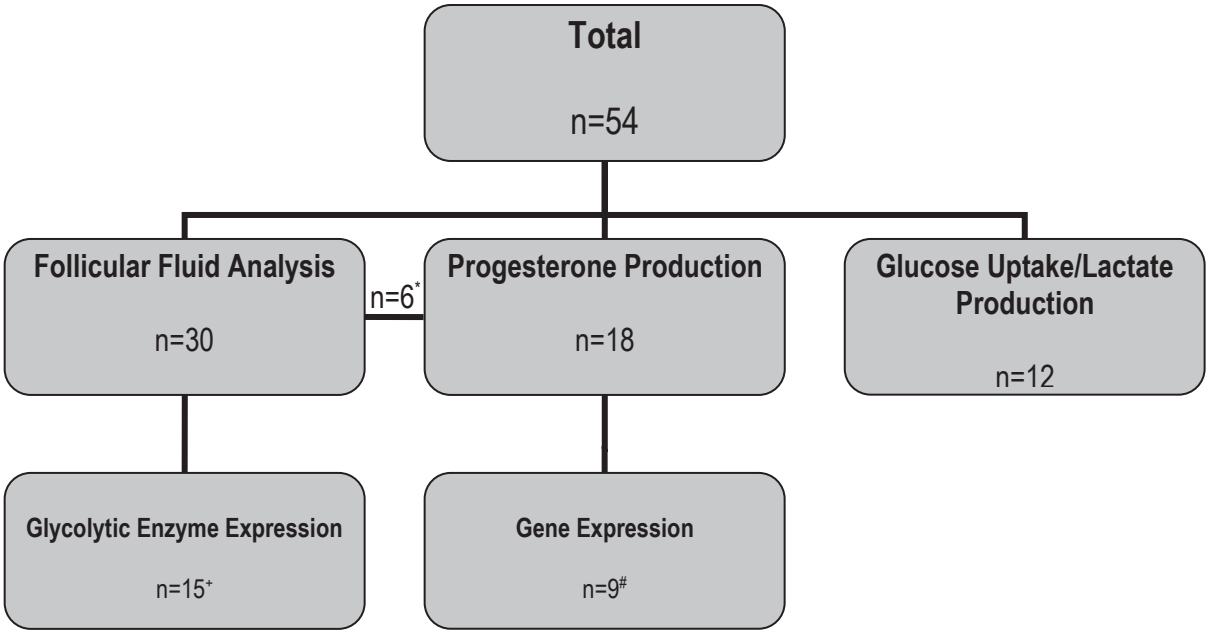
Supplementary Figure 3.1: Random allocation of patients to each experimental protocol.



*6 patients were randomly allocated to both the follicular fluid analysis and progesterone production experimental protocols, #9 patients were randomly allocated to both the progesterone production and gene expression experimental protocols and +15 patients were randomly allocated to both the follicular fluid and glycolytic enzyme expression experimental protocols.

Supplementary Table 3.1: Primer designs for qPCR

Gene	Accession No.	Sequence	Amplicon Size
Progesterone Receptor (PGER)	NM_001202474	**F:** CCAGGGACAGGACCCCTCCG **R:** GCTGCCTCCAGCACCCCTTG	106
Prostaglandin E Receptor-2 (PTGER2)	NM_000956	**F:** TCCTGGCTATCATGACCATCAC **R:** AGCTTGGAGGTCCCATTTTTC	107
Cytosolic Phospholipase A2 (cPLA)	NM_001004426	**F:** GCAATGCTCGGTGCAACAT **R:** GGCTGCTGTCCATGCTGAT	108
Tumor Protein 53 (p53)	NM_000546	**F**: CCCCTCCTCAGCATCTTATCC **R**: ACCTCAGGCGGCTCATAGG	109
Prostaglandin Endoperoxide Synthase 2 (PTGS2 or COX2)	NM_000963	**F:** ATCATTCACCAGGCAAATTGC **R:** TGCCTGCTCTGGTCAATGG	99
Secretory Phospholipase A2 (sPLA2)	NM_000300	**F:** AGGAGAAGGGCTGCAACATTC **R:** CACAGAGGTTCACATGGCAGAA	102
Retinoblastoma 1 (RB1)	NM_000321	**F:** CAAGCAACCTCAGCCTTCCA **R:** GACAGAAGGCGTTCACAAAGTG	122
18S	AF176811	**F:** AGAAACGGCTACCACATCCAA **R:** CCTGTATTGTTATTTTTCGTCACTACCT	92

CHAPTER FOUR

Mitochondrial SIRT5 is Present in Follicular Cells and is altered by Reduced Ovarian Reserve and Advanced Maternal Age.

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Author Contributions

By signing the Statement of Authorship each author certifies that their stated contribution to the publication is accurate and permission is granted for the publication to be included in the candidate's thesis.

Name of Principle Author (Candidate)	Leanne Pacella-Ince		
Contribution to Paper	Experimental design and execution, data analysis and interpretation, manuscript preparation, writing and revising.		
Signature		Date	

Name of Co-Author	Deirdre Zander-Fox		
Contribution to Paper	Supervised development of work, experimental design, data discussions and interpretation and manuscript editing.		
Signature		Date	

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Contribution to Paper	Supervised development of work, experimental design, data discussions and interpretation, manuscript editing and corresponding author		
Signature		Date	

Mitochondrial SIRT5 is Present in Follicular Cells and is Altered by Reduced Ovarian Reserve and Advanced Maternal Age.

The findings from the previous chapter that there is a perturbed ovarian follicular environment in women with reduced ovarian reserve and in women with advanced maternal age, due in part to altered granulosa and cumulus cell metabolism, including mitochondrial metabolism. Interestingly, a family of proteins called the Sirtuins are able to sense the metabolic state of the cell and modify both proteins and histones post-translationally. Of particular interest is SIRT5 as it is a protein that is known to localise to the mitochondria, thus suggesting this protein may be exerting its effects within this organelle and thus possibly affecting mitochondrial metabolism. SIRT5 has previously been demonstrated to deacetylate cytochrome C, which is known to have an important function in oxidative metabolism. SIRT5 also has the ability to act as desuccinylase and desuccinylate succinyl-CoA and isocitrate dehydrogenases 2, which are both important for mitochondrial metabolism. However, the presence and function of SIRT5 within human granulosa and cumulus cells remain unknown. Therefore, this chapter will investigate the presence and function of SIRT5 in ovarian follicular cells.

Introduction

Increasing maternal age is coupled with a decline in oocyte quantity and oocyte quality as indicated by increases in oocyte aneuploidy and a reduction in metabolic output (Jansen and de Boer 1998; Pellestor *et al.* 2003; Eichenlaub-Ritter *et al.* 2004; Nagaoka *et al.* 2011). This culminates in a decline in fertility reflected by decreased pregnancy rates (Baird *et al.* 2005; Alviggi *et al.* 2009). Moreover, women with reduced ovarian reserve (independent of maternal age) have reduced fertility and suboptimal IVF outcomes. A decrease in oocyte quality is thought to be partially responsible for this decline as women with reduced ovarian reserve have lower fertilisation rates (Lekamge *et al.* 2007), higher blastocyst aneuploidy rates (Katz-Jaffe *et al.* 2013) and higher rates of miscarriage (Levi *et al.* 2001; Lekamge *et al.* 2007).

The environment within the ovarian follicle is important for support and development of a competent oocyte. Bi-directional paracrine and gonadotropin communication between the oocyte and its surrounding granulosa and cumulus cells is essential for regulating development and selection of a mature oocyte capable of ovulation, fertilisation and establishment of a viable pregnancy (Goud *et al.* 1998; Canipari 2000; Sugiura *et al.* 2005). Follicular fluid contains important components, such as proteins, steroids, hormones, electrolytes, enzymes and metabolites, which are derived from both the bloodstream and secreted by somatic cells and the oocyte (Shalgi *et al.* 1972; Schweigert *et al.* 2006; Revelli *et al.* 2009; Pinero-Sagredo *et al.* 2010). Recently, we have established that the follicular environment is altered in women with reduced ovarian reserve or advanced maternal age, suggestive of an environmental mechanism responsible for the reduced oocyte quality seen in these women (Pacella *et al.* 2012). Furthermore, gene expression is altered in granulosa and cumulus cells collected from antral follicles of women with reduced ovarian reserve, indicating that an altered transcriptome may be a contributing factor in the reduction of fertility in these women (Greenseid *et al.* 2009; May-

Panloup *et al.* 2012; Skiadas *et al.* 2012). In support of this a recent study has demonstrated that granulosa cells from women with reduced ovarian reserve have altered mRNA expression of genes associated with metabolic pathways (Skiadas *et al.* 2012). Interestingly, oocytes from women of advanced maternal age have altered gene expression of genes associated with metabolism (Grondahl *et al.* 2010) and proteomic analysis of cumulus cells has revealed metabolic proteins are differentially expressed compared to younger women (McReynolds *et al.* 2012). Together, these studies suggest that women with reduced ovarian reserve or advanced maternal age have altered follicular cell metabolism which may be a contributing factor to the reduced oocyte quality seen.

Sirtuin 5 (SIRT5), a mitochondrial protein, is a nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase which has recently been shown to possess desuccinylase activity. SIRT5 belongs to the Sirtuin family of proteins, which are mammalian homologs to the yeast silent information regulator 2 (SIR2). The Sirtuin family of proteins respond to the metabolic state of the cell and post-translationally modify proteins and histones. These proteins play a role in epigenetic gene silencing (Peng *et al.* 2011b), DNA repair and recombination (Mao *et al.* 2011; Mao *et al.* 2012), microtubule organization (North *et al.* 2003) and the regulation of aging (Haigis and Guarente 2006). Mitochondria are the primary organelle responsible for energy production and thus influence cellular metabolic state. Mitochondrial protein modifications, such as acetylation and succinylation are thought to be a mechanism behind the regulation of mitochondrial activity and overall mitochondrial function (Anderson and Hirschey 2012). Proteomic studies have established that a large proportion of mitochondrial proteins may be subjected to post-translational deacetylation or desuccinylation (Kim *et al.* 2006; Zhang *et al.* 2011). SIRT5, which alters protein function in response to changes in cellular metabolic state, is a novel candidate for

involvement in the declining oocyte quality seen in women with reduced ovarian reserve or advanced maternal age due to their perturbed follicular metabolism.

SIRT5 is localised to the mitochondrial matrix and previous animal studies have established expression in the brain (Lombard *et al.* 2007; Geng *et al.* 2011), heart (Lombard *et al.* 2007), liver (Lombard *et al.* 2007; Nakagawa *et al.* 2009) and kidney (Nakamura *et al.* 2008; Nakagawa *et al.* 2009). It was initially hypothesised that the Sirtuin family of proteins were solely histone deacetylases and SIRT5 was thought to have weak deacetylase activity and its ability to deacetylate Cytochrome C, was also suggestive of a role in oxidative metabolism and apoptosis (Schlicker *et al.* 2008). However, recent data has demonstrated the NAD^+-dependent desuccinylase activity of SIRT5 is higher compared to its deacetylase activity (Du *et al.* 2011; Peng *et al.* 2011a), with *Sirt5* knockout mice having no differences in global mitochondrial protein acetylation while global mitochondrial protein hypersuccinylation was instead detected (Lombard *et al.* 2007; Peng *et al.* 2011a). The exact biological significance of lysine desuccinylation remains largely unknown however the most characterised of the SIRT5 desuccinylate targets is carbamoyl phosphate sythase I (CPS1), an important enzyme within the urea cycle of the liver where it catalyses the reaction of ammonium to carbamoyl phosphate (Du *et al.* 2011). Other suggested desuccinylation targets of SIRT5 include isocitrate dehydrogenase 2, glutamate dehydrogenase, malate dehydrogenase 2 and, citrate sythase, which are all essential for cellular metabolism (Du *et al.* 2011; Peng *et al.* 2011a; Zhang *et al.* 2011).

Thus far, nothing is known about SIRT5 and its presence within the human ovarian follicular cells. As follicular and oocyte metabolism is essential for development of a competent oocyte, the presence of SIRT5 and its activity within granulosa and cumulus cells was investigated,

specifically in women with reduced ovarian reserve or advanced maternal age as follicular cells from these women display altered metabolic profiles.

Materials and Methods

Patient Recruitment and Cohorts

Women (n=47) undergoing infertility treatment (IVF cycles only) were recruited to participate in this study and were allocated to a cohort based on age (young maternal age: ≤35 and advanced maternal age: ≥40) and ovarian reserve (as measured by serum anti-mullerian hormone [AMH]). The serum AMH range for young women with normal ovarian reserve was 1.8-4.2µg/L and the young women with reduced ovarian reserve had serum AMH concentrations ranging 0.4-1.4µg/L. The women of advanced maternal age cohort had serum AMH concentrations ranging 0.6-1.5µg/L and these concentrations represent the 25-75% quartiles for this age group (21). Ethics for this study was gained from the Women's and Children's Hospital Research Ethics Committee *(North Adelaide, South Australia, Australia)* and from the Repromed Scientific Advisory Committee *(Dulwich, South Australia, Australia)*. Samples were de-identified and each woman was represented only once within the study period. Women undergoing donor/recipient cycles and preimplantation genetic screening cycles were excluded as were patients diagnosed with polycystic ovary syndrome.

Ovarian Simulation

Antagonist cycles using gonadotropins was used for ovarian stimulation in all women. On day 2 or 3 of the menstrual cycle recombinant FSH (*Puregon (100-375U), Schering-Plough, New South Wales, Australia* or *Gonal F (150-350U), Merck Serono, New South Wales, Australia)*, was administered and on day 7 a GnRH antagonist *(Orgalutran (250mcgms), Schering-Plough)* was administered. Once at least 2 follicles of 17mm or greater was measured via ultrasound, the hCG trigger (*Ovidrel (250mcgms)* was administered and after 36hrs oocytes were collected.

Granulosa and Cumulus Cell Collection

Granulosa cells were collected, at oocyte retrieval, from the surplus follicular fluid into Dulbecco's Phosphate Buffered Saline (PBS) *(Sigma-Aldrich, New South Wales, Australia)*. Density gradient centrifugation was utilised to purify granulosa cells by overlay of the cells on a 60%/40% silica solution *(Spermgrad, Vitrolife, Göteborg, Sweden)* followed by centrifugation for 30mins at 470g. Following oocyte collection cumulus oocytes complexes (COCs) were trimmed of excess cumulus cells and these cells were washed through Dulbecco's PBS. Cumulus cells masses were dispersed by pipetting. Both granulosa and cumulus cell counts were performed using a haemocytometer and cells were diluted to a concentration of 1 x 10^6/ml for use.

RNA Isolation and qPCR in Granulosa and Cumulus Cells

RNA from granulosa and cumulus cells was extracted using an RNeasy Kit *(Qiagen, Victoria, Australia)* according to manufacturer's instructions and the RNA concentration was verified using the Nanodrop 1000 spectrophotometer *(Thermo Scientific, Waltham, Massachusetts)*. Superscript III Reverse Transcriptase *(Invitrogen, Victoria, Australia)* was used as per manufacturer's directions to synthesize complementary DNA (cDNA), which was subsequently stored at -20°C until required. The NCBI Blast program was used to design qPCR primers to *SIRT5* human sequences (accession number: NM_012241; Amplicon size: 90; F: AAAGCAGCCGTGGAGACAAC; R: TCGCATCAGGGTTTGTCTGTAG) and these were synthesized by Geneworks *(Hindmarsh, South Australia, Australia)*. These primers were validated by gel electrophoresis and sequencing (Appendix 4). Each qPCR reaction contained 2µl of cDNA to which a master mix of 1µl of the SIRT5 forward and reverse primer at a concentration of 10pmol/µl, 10µl of SYBR Green *(Applied Biosystems, Victoria, Australia)* and 6µl of H_2O was added to, giving a total volume of 20µl. All samples were run in triplicate on the

Corbett Rotor Gene 6000 *(Corbett Life Sciences, Qiagen, Victoria, Australia)* and the PCR cycling conditions were 50°C for 2mins, 95°C for 10mins, followed by 40 amplification cycles of 95°C for 15secs and 60°C for 1min. Analysis was performed using delta, delta ct, normalized to the reference gene Ribosomal protein L19 (F: AGAAACGGCTACCACATCCAA, R: CCTGTATTGTTATTTTTCGTCACTACCT) (*Qiagen)* (Livak and Schmittgen 2001).

Immunohistochemistry of Granulosa and Cumulus Cells

Granulosa cells and cumulus cells were fixed to polysine slides using 4% paraformaldehyde. The slides were washed through Dulbecco's PBS before incubation in 0.1M glycine *(Sigma-Aldrich)* in Dulbecco's PBS for 5mins and blocked using 5% donkey serum *(Sigma-Aldrich)* for 2 hrs followed by 5% goat serum *(Sigma-Aldrich)* for 2hrs. Co-immunohistochemistry was utilised to confirm the localisation of SIRT5 to the mitochondria, using Cytochrome C as an exclusively mitochondrial protein. Thus, a 2hr incubation with both the SIRT5 primary antibody *(Abcam, Sapphire Bioscience, New South Wales, Australia)* and the Cytochrome C *(Abcam)* primary antibody in a 1:50 dilution was performed and a ThermoBrite *(Abbott Molecular, Illinois United, States of America)* set at 37°C for 2hrs was utilised for the incubation. The slides were then incubated using the ThermoBrite with the secondary antibodies Fluorescein (FITC)-conjugated AffiniPure Donkey Anti-Rabbit *(Jackson ImmunoResearch Laboratories, Abacus ALS, Queensland, Australia)* and Rhodamine (TRITC)-conjugated AffiniPure Goat Anti-Mouse *(Jackson ImmunoResearch Laboratories)* 1:100 for 2 hrs at 37°C. Nuclei were stained using 3µM 4,6-Diamidino-2-phenyindole, dilactate (DAPI) *(Sigma-Aldrich)* and was applied for 1-2mins. Granulosa and cumulus cells were imaged using the Leica TCS SP5 Spectral Scanning Confocal Microscope *(Adelaide Microscopy, Adelaide University, South Australia, Australia)*. A negative control using a SIRT5 blocking peptide *(Abgent, BioCore, New South Wales, Australia)* and a

positive control using mouse zygotes were also prepared and imaged. Image analysis was performed on a minimum of 40 cells per patient.

The primary antibody CPS1 *(Sapphire Bioscience)* was also examined in granulosa and cumulus cells using the same method as above. Human immature oocytes were used as a positive control and a no primary antibody negative control was also prepared and imaged. Image analysis was performed on a minimum of 40 cells per patient.

Western Blotting and Immunostaining of Granulosa and Cumulus Cells

To each sample of granulosa or cumulus cells, diluted to $1x10^6$/ml (a Bradford assay was performed to ensure equal amounts of protein), RIPA Buffer, a protein inhibitor cocktail (used as a 100x stock solution, *[Sigma-Aldrich]*) and loading buffer were added and heated at 70°C for 10mins. To the pre-cast polyacrylamide gel wells *(4–15% Mini-Protean TGX Precast Gel, Bio-Rad, New South Wales, Australia)* 20µl of sample was added. The gels were run at a constant 150 volts for 60mins. The gel was then transferred onto a 100% pure nitrocellulose membrane *(GE Healthcare Amersham, Thermo Fisher Scientific, Victoria, Australia)* by running the gel and membrane at a constant 100 volts for 60mins on ice. Once the transfer was complete the membrane was immunostained, by first blocking the membrane in a blocking solution (supplied with the GE Healthcare Amersham ECL Advance Western Blotting Detection Kit *[Thermo Fisher Scientific])* overnight at 4°C. Incubation with the SIRT5 antibody (used for immunohistochemistry) diluted to 1:1000 for 3 hrs at room temperature, the secondary antibody donkey anti rabbit HRP (*Jackson ImmunoResearch Laboratories, Pennsylvania, United States of America)* diluted to 1:100,000 was incubated with the membrane for 1hr. The ECL detection reagents (GE Healthcare Amersham ECL Advance Western Blotting Detection Kit) were applied to the

membrane for 5mins and then the membrane was exposed to Hyerfilm-ECL (*GE Healthcare Amersham)* in a darkroom. A protein standard *(Precision Plus Protein Dual Colour Standards, Bio-Rad)*, alpha tubulin loading control, positive control (recombinant SIRT5 *[Abnova, Sapphire Bioscience]*) and negative control (no primary antibody) were also prepared. Recombinant SIRT5 corresponds to all 310 amino acids and a 60kDa band was observed (Figure 4.3A) whereas, the SIRT5 antibody corresponds to amino acids 30-46 only thus a smaller 36kDa band was observed (Figure 4.3B). Quantitation of the bands was performed by image analysis using densitometry normalised to the loading control.

SIRT5 Desuccinylation Activity in Granulosa and Cumulus Cells

SIRT5 protein desuccinylation activity in granulosa and cumulus cells was assessed according to manufacturers' instructions using the Enzo Life Sciences SIRT5 Fluorimetric Drug Discovery Kit *(Enzo Life Sciences, Sapphire Bioscience)*. To granulosa or cumulus cells ($1x10^6$/ml) containing 2µl of enzyme extraction buffer 5µl of assay buffer was added and incubated at 37°C for 10mins. To each of the samples 3µl of substrate solution containing 0.5mM Fluor-de-lys – Succinyl and 5.5mM NAD^+ was added and incubated at 37°C for 60mins. The reaction was stopped by adding 10µl of developer solution, containing 2mM Nicotinamide to each of the samples and incubated in the dark at room temperature for 15mins. SIRT5 desuccinylation activity was measured by fluorescence using an excitation wavelength of 360nm. Each sample was run in triplicate and a human recombinant SIRT5 standard curve was run with each analysis (r^2>0.98). Negative controls were run for each experimental run which included a sample without enzyme, a sample without substrate, nicotinamide and extraction buffer controls were also run with each analysis.

Granulosa and Cumulus Cell Culture

Granulosa and cumulus cells, diluted to $1x10^6$/ml, were collected and purified as outlined above and then cultured in G-1 media *(Vitrolife)* with either 10mM nicotinamide or 10mM resveratrol added at 37°C in $5\%O_2/6\%CO_2$ for 24 or 48hrs. A G-1 media only control was included.

Ammonium Concentration in Follicular Fluid and Spent Culture Media

Ammonium concentration in follicular fluid and spent granulosa and cumulus cells culture media was assessed using a microfluorometric assay (Gardner and Lane 1993). The concentration of ammonium was determined using an enzyme-linked reaction to NADH. An ammonium standard curve (r^2>0.98) was run with each analysis and samples were assessed in triplicate.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism Software version 6 and data are expressed as mean ± standard error of the mean (SEM). To determine significance between groups a Chi-square was used for pregnancy rates and infertility diagnoses. Significance for correlation data was determined using Pearson's correlation. For the remainder of the analyses a one-way ANOVA with Tukey's post-hoc test was used. A p-value <0.05 was considered statistically significant.

Results

Table 4.1: Patient Demographics and Cycle Outcomes[1]

		Young Maternal Age with Normal Ovarian Reserve	Reduced Ovarian Reserve	Advanced Maternal Age
Age		31.2±0.9[a]	33.2±0.71[a]	41.9±0.53[b]
Serum AMH (µg/L)		3.5±0.4[a]	0.79±0.2[b]	1.2±1.0[c]
BMI		23.9±1.3[a]	25.7±2.1[a]	23.6±2.2[a]
Starting FSH Dose (IU)		183.8±15.6[a]	298.3±16.9[b]	310.2±10.6[b]
# previous fresh cycles (IVF/ICSI)		0.59±0.17[a]	2.89±0.88[b]	3.00±0.71[b]
# oocytes collected		12.4±0.80[a]	7.63±1.64[b]	8.54±1.57[b]
Fertilisation Rate (%)		70.6±5.6[a]	70.9±6.5[a]	48.1±7.7[b]
Mean # Embryos Transferred		1.1±0.1[a]	1.3±0.2[ab]	1.6±0.2[b]
Mean # Embryos Frozen		3.6±0.7[a]	1.7±0.7[b]	0.5±0.2[b]
Pregnancy Rate (%)		56.3[a]	33.3[b]	19.1[b]
Implantation Rate (%)		42.3[a]	28.4[b]	10.6[c]
Utilisation of Oocytes (%)[2]		62.7[a]	38.2[b]	24.6[b]
Infertility Diagnosis (%)[3]	**Ovulatory**	5.9[a]	0.0[a]	14.3[a]
	Tubal	11.8[a]	0.0[a]	0.0[a]
	Endometriosis	5.9[a]	0.0[a]	14.3[a]
	Unexplained	11.8[a]	21.5[a]	0.0[a]
	Male Factor	70.6[a]	69.2[a]	85.7[a]

[1] n(Young Maternal Age with Normal Ovarian Reserve)=22 n(Reduced Ovarian Reserve)=12, n(Advanced Maternal Age)=13, data expressed as mean±SEM, a-c indicate significant differences between cohorts, $p<0.05$. Exclusion criteria: donor/recipient cycles, preimplantation genetic screening and PCOS patients

[2] Utilization of Oocytes was calculated as follows: (frozen embryos + embryos transferred) / oocytes

[3] Infertility diagnosis: multiple diagnoses for some patients

Patient Demographics

Mean maternal age, serum AMH levels, IVF cycle details and outcomes are indicated in Table 4.1. Young women with normal ovarian reserve had a significantly lower starting FSH dose, had undergone fewer fresh IVF cycles, had more oocytes collected and embryos frozen compared to both the reduced ovarian reserve and advanced maternal age groups ($p<0.05$, Table 4.1). In addition, they had an increase in both pregnancy rate and implantation rate compared to the reduced ovarian reserve and advanced maternal age groups ($p<0.05$, Table 4.1). The reduced ovarian reserve cohort had an increase in fertilisation and implantation rates compared to the advanced maternal age group ($p<0.05$, Table 4.1). No significant differences were found between groups for maternal BMI or infertility diagnosis.

Granulosa and Cumulus Cell SIRT5 Gene Expression

SIRT5 gene expression was detected in granulosa and cumulus cells from all women (Figure 4.1). The levels of *SIRT5* were significantly increased in granulosa cells compared to cumulus cells in young women with normal ovarian reserve and in women with reduced ovarian reserve ($p<0.05$, Figure 4.1). No significant difference was found between granulosa and cumulus cells in the advanced maternal age group (Figure 4.1). In addition, *SIRT5* gene expression was significantly decreased in both granulosa and cumulus cells from women with reduced ovarian reserve and in women of advanced maternal age compared to the young women with normal ovarian reserve ($p<0.05$, Figure 4.1). Granulosa cell *SIRT5* expression was significantly decreased in the women of advanced maternal age compared to the women with reduced ovarian reserve ($p<0.05$, Figure 4.1). However, no difference was detected between cumulus cells in the reduced ovarian reserve and advanced maternal age groups (Figure 4.1).

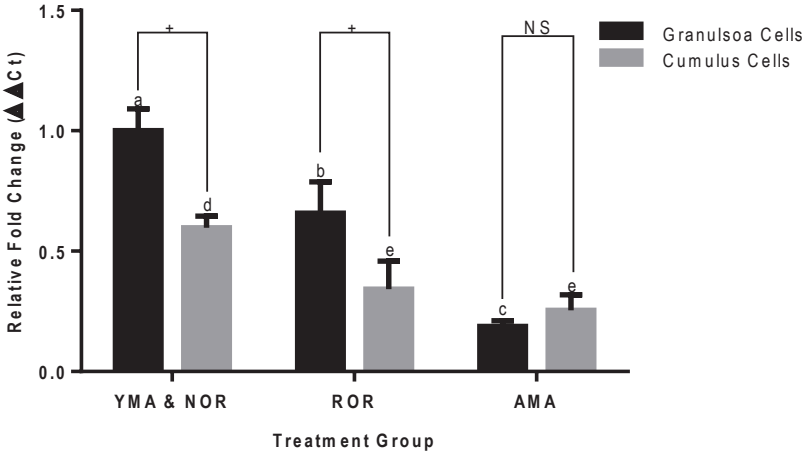


Figure 4.1: Relative fold change in gene expression of *SIRT5* in granulosa and cumulus cells.
n(YMA & NOR) = 17, n(ROR) = 7, n(AMA) = 13 and all samples were run in triplicate. a – e indicate significant differences between cohorts for cell type ($p<0.05$), + indicate significant differences between cell type within cohorts ($p<0.05$), NS indicates not significant. YMA – young maternal age, NOR – normal ovarian reserve, ROR – reduced ovarian reserve and AMA – advanced maternal age.

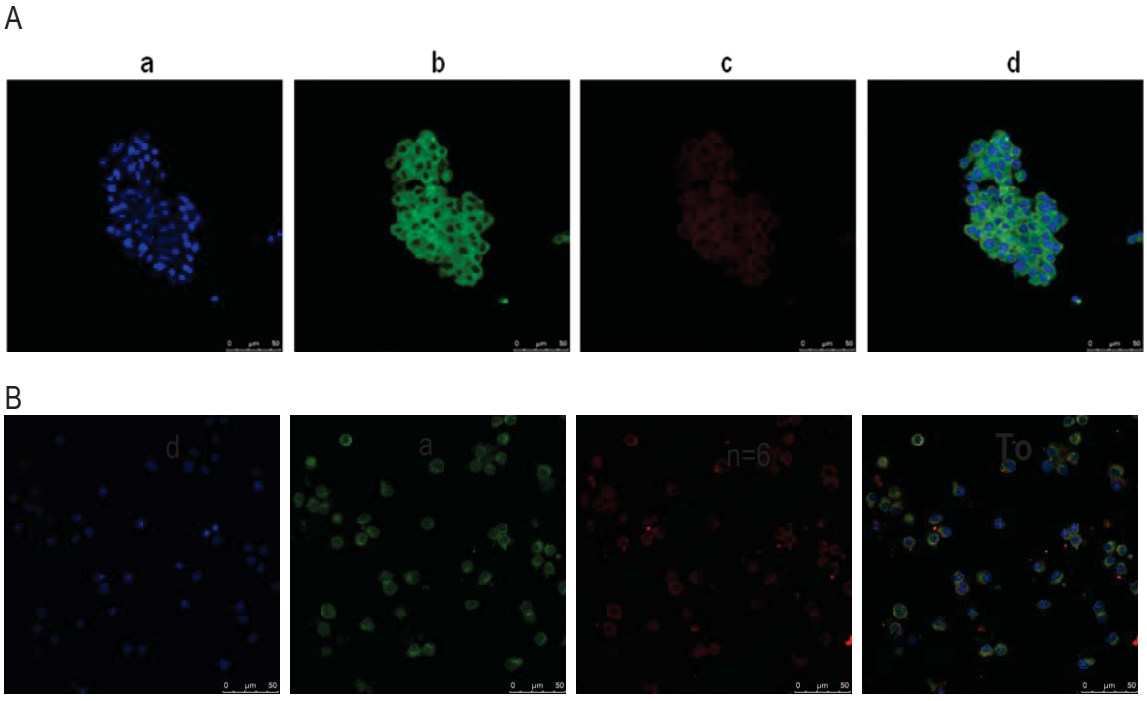


Figure 4.2: Co-localisation of SIRT5 protein to the mitochondria in the A) granulosa and B) cumulus cells
n = 7 patients per cohort and all samples were run in duplicate. a – blue indicates DAPI or nuclear staining, b – green indicates SIRT5 protein, c – red indicates Cytochrome C and was used as a mitochondrial marker and d – merged image.

SIRT5 Protein Localisation and Levels in Granulosa and Cumulus Cells

The presence of the SIRT5 protein in granulosa and cumulus cells was assessed in the three cohorts by immunohistochemistry and image analysis was performed to determine differences between cohorts. SIRT5 was found to co-localise with the mitochondria in all groups in both granulosa and cumulus cells (Figure 4.2).

Similar to gene expression, SIRT5 protein levels were significantly increased in granulosa cells compared to cumulus cells in young women with normal ovarian reserve ($p<0.05$, Figure 4.3C) and no difference was detected between granulosa and cumulus cells in the advanced maternal age group (Figure 4.3C). In contrast to gene expression, SIRT5 protein levels were similar between granulosa and cumulus cells in women with reduced ovarian reserve (Figure 4.3C). SIRT5 protein levels were highest in granulosa cells of the young women with normal ovarian reserve compared to the reduced ovarian reserve and advanced maternal age groups ($p<0.05$, Figure 4.3C). In cumulus cells protein levels were significantly decreased in the advanced maternal age women compared to the young women with normal ovarian reserve and women of advanced maternal age ($p<0.05$, Figure 4.3C). In contrast, to gene expression and no differences were established in SIRT5 protein levels in cumulus cells between the young women with normal ovarian reserve and women with reduced ovarian reserve (Figure 4.3C).

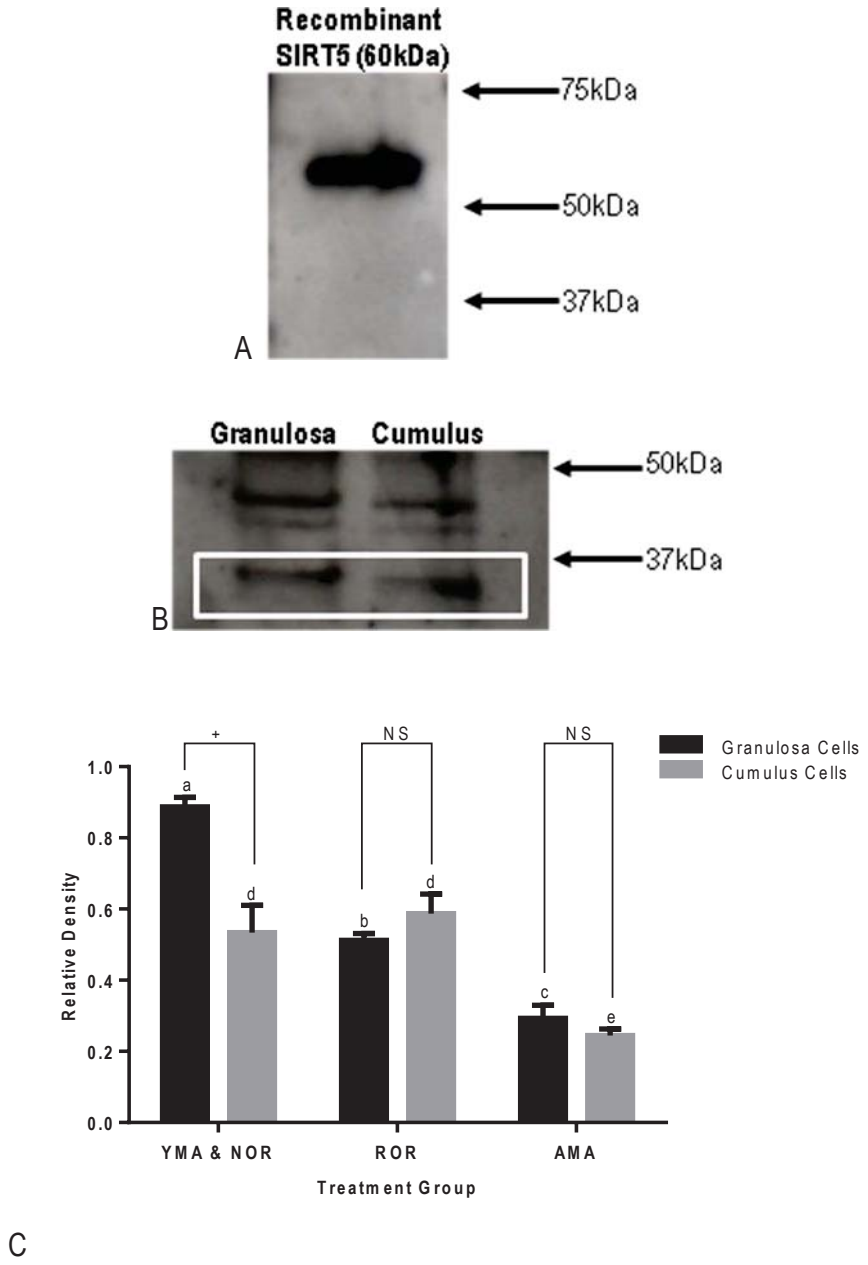


Figure 4.3: Gel displaying A) recombinant SIRT5 at 60kDa and B) in granulosa and cumulus cells at 36kDa. C) Relative SIRT5 protein density in granulosa and cumulus cells

A) 60kDa recombinant SIRT5 corresponds to all amino acids and B) 36kDa SIRT5 in granulosa and cumulus cells corresponds to amino acids 30-46. C) n(YMA & NOR) = 17, n(ROR) = 7, n(AMA) = 13 and all samples were run in duplicate. a – e indicate significant differences between cohorts for cell type ($p<0.05$), + indicate significant differences between cell type within cohorts ($p<0.05$), NS indicates not significant. YMA – young maternal age, NOR – normal ovarian reserve, ROR – reduced ovarian reserve and AMA – advanced maternal age.

SIRT5 Protein Desuccinylation Activity in Granulosa and Cumulus Cells

SIRT5 protein desuccinylation activity was detected in granulosa and cumulus cells of women from all three groups. SIRT5 protein desuccinylation activity was highest in granulosa cells compared to cumulus cells in young women with normal ovarian reserve ($p<0.05$, Figure 4.4). In women with either reduced ovarian reserve or advanced maternal age SIRT5 protein desuccinylation activity were similar between granulosa and cumulus cells (Figure 4.4). There was a significant decrease in SIRT5 protein desuccinylation activity in granulosa cells of women with either reduced ovarian or advanced maternal age compared to the young women with normal ovarian reserve ($p<0.05$, Figure 4.4). In addition, there was a significant reduction in SIRT5 protein desuccinylation activity in cumulus cells from women of advanced maternal age compared to both the young women with normal ovarian reserve and the women with reduced ovarian reserve ($p<0.05$, Figure 4.4). No difference was found in SIRT5 protein desuccinylation activity in cumulus cells between the young women with normal ovarian reserve and the young women with reduced ovarian reserve (Figure 4.4).

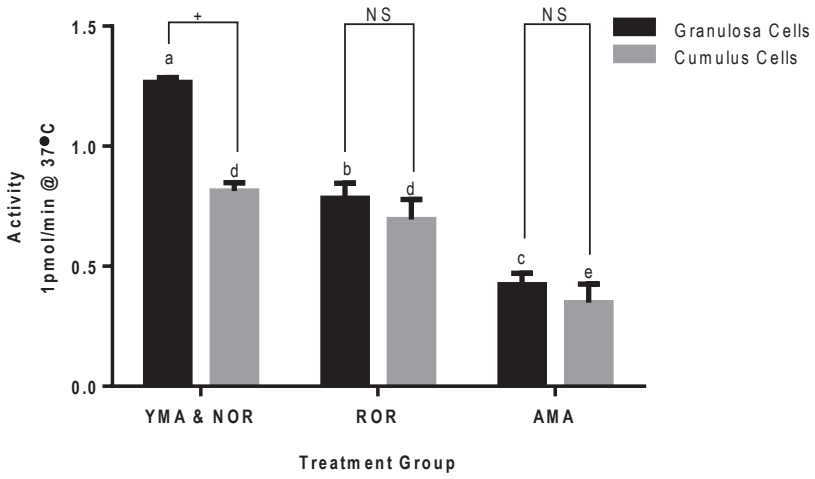


Figure 4.4: SIRT5 protein desuccinylation activity in granulosa and cumulus cells.

n = 5 patients per cohort and all samples were run in triplicate. a – e indicate significant differences between cohorts for cell type ($p<0.05$), + indicate significant differences between cell type within cohorts ($p<0.05$), NS indicates not significant. YMA – young maternal age, NOR – normal ovarian reserve, ROR – reduced ovarian reserve and AMA – advanced maternal age.

Assessment of the SIRT5 target CPS1 in Granulosa and Cumulus Cells

CPS1 protein is an enzyme that catalyses the first reaction the conversion of ammonium to carbamoyl phosphate. CPS1 was found to be present in both granulosa and cumulus cells of all cohorts, with significantly increased protein levels present in granulosa cells ($p<0.05$, Figure 4.5). Localisation of CPS1 was not different between granulosa and cumulus cells (Figure 4.5). If CPS1 was a desuccinylation target for SIRT5, it would be expected that in the presence of decreased SIRT5 protein desuccinylation activity there would be increased succinylation, resulting in decreased CPS1 activity.

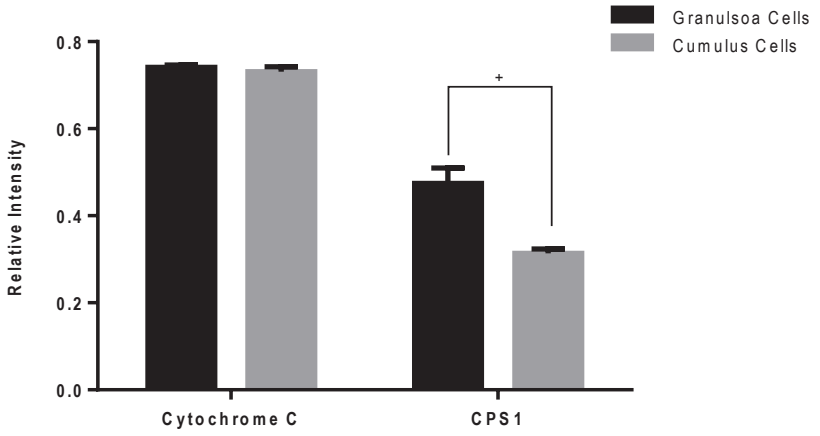
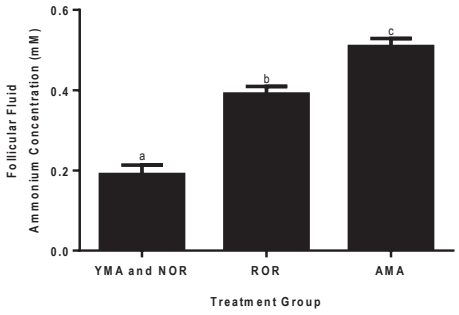


Figure 4.5: Relative CPS1 protein intensity in granulosa and cumulus cells.
n = 5 patients (YMA & NOR), all samples were run in duplicate and the fluorescence of 40 cells were analysed per patient. + indicate significant differences between cell type ($p<0.05$), NS indicates not significant. YMA – young maternal age, NOR – normal ovarian reserve.

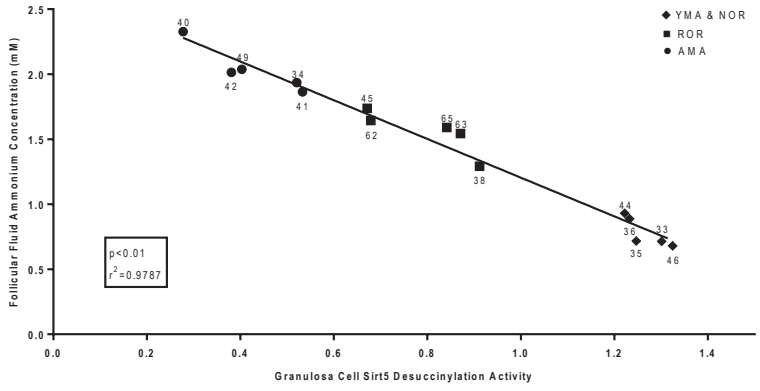
This decrease in CPS1 activity would result in ammonium accumulation as a smaller amount would be converted to carbamoyl phosphate. The concentration of ammonium was therefore assessed in follicular fluid of women from all 3 cohorts as a surrogate marker for CPS1 activity and desuccinylation by SIRT5 in vivo. Ammonium levels in follicular fluid were significantly increased in both the reduced ovarian reserve and advanced maternal age groups compared to the young women with normal ovarian reserve ($p<0.05$, Figure 4.6A). In addition, compared to the reduced ovarian reserve group ammonium concentration was significantly increased in the

advanced maternal age group ($p<0.05$, Figure 4.6A). A negative correlation between SIRT5 protein desuccinylation activity and follicular fluid ammonium concentration was established ($p<0.01$, Figure 4.6B).

To establish if the increase in the production of ammonium may be related to changes in SIRT5 desuccinylation activity granulosa and cumulus cells from women with normal ovarian reserve were cultured for 48 hrs in the presence of either Resveratrol (a Sirtuin stimulator) or Nicotinamide (a Sirtuin inhibitor). Ammonium concentrations in both granulosa and cumulus cells were decreased in the presence of Resveratrol and increased in the presence of Nicotinamide ($p<0.05$, Figure 4.6C), thus confirming that altering SIRT5 protein activity results in an alteration to ammonium levels.



A



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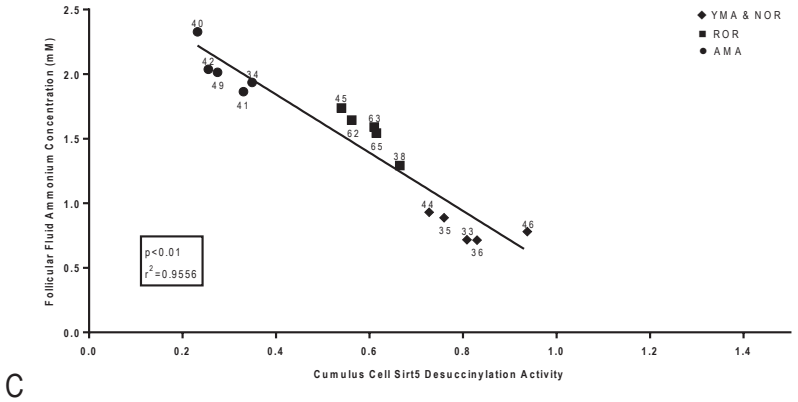
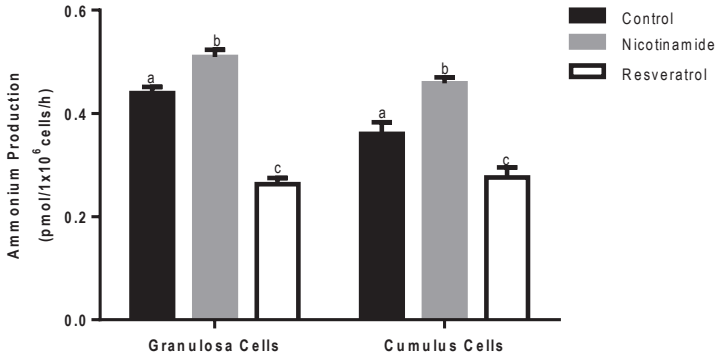


C



D

Figure 4.6: A) Ammonium concentration in follicular fluid and correlation between follicular fluid ammonium concentration and SIRT5 protein succinylation activity in B) granulosa cells and C) cumulus cells. D) Ammonium production in spent culture media from granulosa and cumulus cells after a 48 hr culture with either Nicotinamide or Resveratrol.

A) n = 5 patients per cohort and all samples were run in triplicate. a – c indicate significant differences between cohorts ($p<0.05$). B) and C) n = 5 patients per cohort, 33 – 65 are individual patient identification numbers. D) n = 5 patients, samples were run in triplicate, a – c: indicate significant differences between treatments within cell type. YMA – young maternal age, NOR – normal ovarian reserve, ROR – reduced ovarian reserve and AMA – advanced maternal age.

Discussion

The microenvironment within the follicle is essential for the growth and development of the oocyte, thus changes within this environment may serve as a marker of oocyte developmental competence. During the process of folliculogenesis the cells within the follicle differentiate into two cells types, each with specific functions. The granulosa cells, which line the antrum of the follicle, are involved in steroidogenesis, whereas cumulus cells, which surround the oocyte, are important for support, growth and maturation of the oocyte. Previous studies have established that women with reduced ovarian reserve have altered gene expression in granulosa and cumulus cells (May-Panloup *et al.* 2012; Skiadas *et al.* 2012). In particular, granulosa cells from women with reduced ovarian reserve have altered expression of genes involved in metabolic pathways compared to women with normal ovarian reserve and similarly, proteomic analysis of cumulus cells from women of advanced maternal age have established metabolic proteins that are differentially expressed compared to cumulus cells from younger women (McReynolds *et al.* 2012; Skiadas *et al.* 2012). Therefore, there are changes in metabolic profile of granulosa and cumulus cells that may adversely affect follicle and oocyte development. As SIRT5 is a metabolic sensing protein its activity, and thus its ability to post-translationally modify proteins involved in mitochondrial metabolism, is of significant interest. This study has established that *SIRT5* gene expression and active protein is present in granulosa and cumulus cells and co-localises to the mitochondria consistent with what has been reported in other tissues (Michishita *et al.* 2005; Nakamura *et al.* 2008; Schlicker *et al.* 2008). Furthermore, *SIRT5* mRNA in granulosa and cumulus cells was decreased with reduced ovarian reserve and advanced maternal age, indicating that SIRT5 function may be down-regulated in these cell types, which may have implications for cellular functions that require SIRT5, specifically metabolism or ammonium clearance.

The Sirtuin family of proteins are of interest as they sense the metabolic state of the cell and subsequently modify proteins and histones. Histone post-translational deacetylation modifications influence functions such as silencing gene transcription, chromatin remodelling, DNA repair, cell proliferation, apoptosis and cell cycle checkpoints (Gallinari *et al.* 2007; Wang *et al.* 2008; Barneda-Zahonero and Parra 2012; Ferrari *et al.* 2012). Mammalian Sirtuins also have the ability to deacetylate proteins and due to varied cellular locations they target many different substrates and have a broad range of cellular functions including cell survival and metabolism, cell cycle regulation, thermogenesis, insulin secretion, DNA repair and transcription. Interestingly, SIRT5 possesses two post-translational protein modifications actions as it has the ability to both deacetylate and desuccinylate proteins.

During the process of folliculogenesis the transition of granulosa into cumulus cells is dependent on endocrine signals from the follicle and paracrine signalling from the oocyte (Eppig *et al.* 1997). After this differentiation has occurred granulosa cells and cumulus cell have specific functions and respond differently to extracellular signals. For example in response to FSH, cumulus cells undergo a process of expansion and secrete hyaluronic acid while in comparison granulosa cells do not undergo expansion and are more steroidogenically active. In young women with normal ovarian reserve the levels of *SIRT5* mRNA and protein were higher in granulosa cells compared to cumulus cells, suggesting *SIRT5* levels are down-regulated following somatic cell differentiation. However, in contrast in women with either reduced ovarian reserve or advanced maternal age SIRT5 protein levels are similar between the two cell types possibly indicative of a diminished level of differentiation. This hypothesis is supported by our previous study demonstrating that cumulus cells from women with reduced ovarian reserve or advanced maternal age do not undergo the metabolic or steroidogenic changes observed in cumulus cells

from young women with normal ovarian reserve and instead displayed characteristics more commonly associated with granulosa cells (Pacella *et al.* 2012).

The oocyte preferentially utilises pyruvate as an energy source and as such is reliant on cumulus cells to metabolise glucose into pyruvate hence the communication between the oocyte and surrounding somatic cells is essential for the development of a viable oocyte (Eppig 2001; Sugiura *et al.* 2005). Factors produced by the oocyte and granulosa and cumulus cells are secreted into follicular fluid, altering the biosynthetic and metabolic activity of this fluid and the ovarian follicular cells (Schweigert *et al.* 2006; Revelli *et al.* 2009; Pinero-Sagredo *et al.* 2010). The bi-directional communication between the oocyte and cumulus cells is essential for oocyte viability and alterations in SIRT5 may be indicative of altered follicular cell metabolic profile.

SIRT5 has been demonstrated to have significant protein desuccinylate activity as opposed to deacetylate activity as seen in the other Sirtuins (Du *et al.* 2011; Peng *et al.* 2011a). *Sirt5* knockout mice have an increase in global protein succinylation, with little or no differences in global protein acetylation (Lombard *et al.* 2007; Du *et al.* 2011). Further, it has been reported that several mitochondrial metabolic enzymes are susceptible to desuccinylation, due to the presence of succinylation sites, such as Succinyl-CoA, which is an intermediate within the TCA cycle, as well as isocitrate dehydrogenase and glutamate dehydrogenase. In this study, SIRT5 protein desuccinylation activity was detected in both granulosa and cumulus cells, with SIRT5 protein desuccinylation activity reduced in granulosa cells from women with reduced ovarian reserve and women of advanced maternal age which may have implications on the activity of target proteins which are involved in pathways such as ammonium clearance and the tricarboxcylic acid cycle.

CPS1, a recently confirmed desuccinylation target of SIRT5, is a 165kDa mitochondrial protein primarily expressed in liver and intestine but has also been found at low levels in kidney and fibroblast (Martinez *et al.* 2010). In the liver, CPS1 catalyses the rate-limiting synthesis of carbamoyl phosphate from ammonium and bicarbonate, suggesting a reduction in CPS1 activity (such as what would occur with increased succinylation) would lead to ammonium accumulation (Martinez *et al.* 2010). Therefore the decrease in SIRT5 as seen in the reduced ovarian reserve and advanced maternal age women would likely result in a decrease in CPS1 activity and an accumulation of ammonium in follicular fluid. As confirmed in this study follicular fluid ammonium concentration was inversely related to SIRT5 desuccinylation activity in granulosa and cumulus cells.

In vivo studies in cows examining the effect of a high protein diet on oocyte development have established an increase in follicular fluid ammonia concentration, decreased oocyte cleavage and after culture a decrease rate of blastocyst formation was also detected (Blanchard *et al.* 1990; Sinclair *et al.* 2000). Using a bovine model, exposure to ammonium in vitro decreased cumulus cells viability and perturbed hormone production and metabolism resulting in impaired oocyte maturation (Rooke *et al.* 2004). Furthermore, increased ammonium concentration during in vitro maturation of porcine oocytes resulted in decreased oocyte nuclear maturation, embryo cleavage rates and blastocyst development (Yuan and Krisher 2010). The presence of ammonium in embryo culture media in the mouse, cow and human also impairs blastocyst development, increases apoptosis (Hammon *et al.* 2000; Lane and Gardner 2003; Virant-Klun *et al.* 2006; Zander *et al.* 2006) and reduces fetal development after embryo transfer (Lane and Gardner 1994; Zander *et al.* 2006). Thus, this increase in follicular fluid ammonium levels may be partially related to the decline in oocyte viability and utilisation and ultimately the decline in pregnancy rates in women with reduced ovarian reserve or advanced maternal age. Further, increased

ammonium concentration in follicular fluid may also increase follicular fluid pH. As oocytes are highly sensitive to pH changes (Bagger *et al.* 1987), an increase in pH throughout the final stages of oocyte development may have detrimental effects to the oocyte and reduce its ability to be fertilised and develop into a viable embryo (Dale *et al.* 1998).

To confirm that accumulation of ammonium in follicular fluid in women with reduced ovarian reserve or advanced maternal age may be attributed to reduced SIRT5 desuccinylation activity, we investigated the impact of culturing granulosa and cumulus cells with an inhibitor and stimulator of SIRT5. In both granulosa and cumulus cells, treatment with the Sirtuin stimulator, Resveratrol resulted in a decrease in ammonium concentration while treatment with a Sirtuin inhibitor, Nicotinamide resulted in an increase in ammonium concentration, suggesting that SIRT5 in granulosa and cumulus cells is partially responsible for the regulation of ammonium clearance via regulation of CPS1. However, nicotinamide may also be exerting its effects on NAD^+ which play important roles in energy metabolism and electron transfer, cell signalling pathways and Ca^{2+} signalling (Ziegler 2000; Koch-Nolte *et al.* 2009; Houtkooper *et al.* 2010).

This study demonstrates that SIRT5 protein levels and desuccinylation activity decreased in women with reduced ovarian reserve or advanced maternal age. Interestingly *SIRT5* mRNA in cumulus cells from women with reduced ovarian reserve was significantly lower than granulosa cells however this difference was lost at the SIRT5 protein level suggestive of a possible altered SIRT5 protein translation in cumulus cells. In contrast, women with reduced ovarian reserve display a down-regulation of RNA transcription. Due to the desuccinylation activity of SIRT5 and the ability of mitochondrial proteins to be desuccinylated other mitochondrial metabolism proteins

may be targeted and may also contribute to the decline in oocyte quality which warrants further investigation.

The association between the decline in oocyte quality coupled with reduced pregnancy rates in women of increasing maternal age is well established and further highlighted by the high pregnancy rates seen in women over the age of 40 receiving donor oocytes (Navot *et al.* 1994; Legro *et al.* 1995). However, the association of pregnancy rates with declining ovarian reserve remains subject to controversy. In our study, young women with reduced ovarian reserve had a 23% decrease in pregnancy rates compared to young women with normal ovarian reserve. Similar studies have shown an association between reduced ovarian reserve and reduced pregnancy rates (Hazout *et al.* 2004; Lekamge *et al.* 2007; Wunder *et al.* 2008; Brodin *et al.* 2013; Hattori *et al.* 2013; Honnma *et al.* 2013; Li *et al.* 2013; Lin *et al.* 2013). Perturbations in post-translational modifications of proteins by SIRT5 may be contributing to the decline in oocyte viability that is seen in women with reduced ovarian reserve or women of advanced maternal age by altering key metabolic pathways. Knowledge of these perturbations may lead to novel therapies to improve mitochondrial metabolism in the oocyte and follicular cells in women undergoing IVF treatment.

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CHAPTER FIVE

Mitochondrial SIRT3 and its Target Glutamate Dehydrogenase are Altered in Follicular Cells of Women with Reduced Ovarian Reserve or Advanced Maternal Age

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Mitochondrial SIRT3 and its Target Glutamate Dehydrogenase are Altered in Follicular Cells of Women with Reduced Ovarian Reserve or Advanced Maternal Age.

The previous chapter demonstrated that SIRT5 was differentially expressed in granulosa and cumulus cells and this may be involved in the perturbed ovarian follicular environment in women with reduced ovarian reserve and in women of advanced maternal age. However, there is also another mitochondrial sirtuin, SIRT3, which is also known to localise to the mitochondria. SIRT3 has been established to possess strong deacetylation activity and is known to deacetylate the mitochondrial protein glutamate dehydrogenase, which is involved in mitochondrial metabolism. However, the presence and function of SIRT3 in human granulosa and cumulus cells remains unknown and considering the findings of SIRT5, SIRT3 is also a possible candidate for the perturbed ovarian follicular environment seen in women with reduced ovarian reserve and in women of advanced maternal age. Studies in this chapter will investigate the presence and function of SIRT3 in ovarian follicular cells.

Introduction

It is well understood that oocyte quality and quantity declines with increasing maternal age, as indicated by increased oocyte aneuploidy and a reduction in metabolic output (Christopikou, Tsorva *et al.* 2013; Eichenlaub-Ritter 2012; Eichenlaub-Ritter, Vogt *et al.* 2004; Pellestor, Andreo *et al.* 2003) which translates to decreased pregnancy rates (Alviggi, Humaidan *et al.* 2009; Baird, Collins *et al.* 2005; Dew, Don *et al.* 1998; Yan, Wu *et al.* 2012). However, a decline in fertility and pregnancy is also observed with a decline in ovarian reserve independent to maternal age. Oocytes from women with reduced ovarian reserve have lower fertilisation rates, higher blastocyst aneuploidy rates (including a significantly increased risk of trisomy) and higher rates of miscarriage (Haadsma, Mooij *et al.* 2010; Katz-Jaffe, Surrey *et al.* 2013; Lekamge, Barry *et al.* 2007; Levi, Raynault *et al.* 2001). Yet the reason for this decline in oocyte quality in women with reduced ovarian reserve is unclear.

Oocyte developmental competence is highly dependent on the bi-directional dialogue between the granulosa/cumulus cells and the oocyte though the presence of transzonal projections, gap junctions and the secretion of paracrine factors (Gilchrist, Lane *et al.* 2008). Interruption of the transfer of nutrients (glucose, amino acids and nucleotides), proteins and ions as well as second messenger molecules between the follicular support cells and the oocyte results in a loss in oocyte viability (Buccione, Schroeder *et al.* 1990; Gershon, Plaks *et al.* 2008; Sugiura, Pendola *et al.* 2005). Alterations in the molecular physiology of the granulosa and cumulus cells will likely impact on the dialogue between these cells and the oocyte and disrupt development. Proteomic analysis of cumulus cells from women of advanced maternal age has revealed several metabolic proteins are differentially expressed compared to younger women (Grondahl, Yding Andersen *et al.* 2010; McReynolds, Dzieciatkowska *et al.* 2012). Further, granulosa cells from women with reduced ovarian reserve have altered mRNA expression of genes associated with metabolic

pathways (Greenseid, Jindal *et al.* 2009; May-Panloup, Ferre-L'Hotellier *et al.* 2012; Skiadas, Duan *et al.* 2012). Together, these studies suggest that women with reduced ovarian reserve or advanced maternal age have altered follicular cell metabolism possibly contributing to the decline in oocyte quality seen.

A family of metabolic sensor proteins, called the Sirtuins, which are mammalian homologs to the yeast silent information regulator 2 (SIR2), post-translationally modify histones and proteins in response to changes in metabolic state and have been implicated in the regulation of aging and mitochondrial function as well as playing a role in epigenetic gene silencing, DNA repair and recombination and microtubule organization (Mao, Hine *et al.* 2011; North, Marshall *et al.* 2003; Peng, Yuan *et al.* 2011). These proteins are nutrient sensing nicotinamide adenine dinucleotide (NAD^+) – dependent deacetylases or desuccinylases. They are characteristically up-regulated by NAD^+ and down-regulated by NADH and nicotinamide, thus are able to respond to the metabolic state of the cell (Schwer and Verdin 2008). Proteomic analysis has established that a large proportion of mitochondrial proteins may be subjected to post-translational deacetylation (Kim, Sprung *et al.* 2006) and recently, SIRT5, a mitochondrial based protein, has been shown to be present in granulosa and cumulus cells and through the desuccinylation of carbomoyl phosphate synthetase I (CPS1) modulates the levels of ammonium present in follicular fluid (Pacella et al., 2013). Interestingly, its activity in follicular cells is reduced in women of both advanced maternal age and also reduced ovarian reserve.

Sirtuin 3 (SIRT3) is reported to be a second mitochondrial-based protein, which is able to sense metabolic state and post-translationally alter mitochondrial function. The exact location of SIRT3 within mitochondria differs between tissues types; with one study demonstrating exclusive

localisation to the mitochondria while another demonstrating localisation to both the nucleus and mitochondria (Lombard, Alt *et al.* 2007; Scher, Vaquero *et al.* 2007). Its expression is particularly high in mitochondrial rich tissues such as the brain, heart, liver and brown adipose tissue (Ahn, Kim *et al.* 2008; Lombard, Alt *et al.* 2007; Shi, Wang *et al.* 2005). Surprisingly, *Sirt3* null mice appear phenotypically normal despite tissues (liver, brown adipose tissue, brain and heart) showing hyperacetylation (Ahn, Kim *et al.* 2008; Lombard, Alt *et al.* 2007). SIRT3 has been shown to target mitochondrial metabolic enzymes, such as glutamate dehydrogenase (GDH) and its deacetylase activity is stimulated upon caloric restriction (Lombard, Alt *et al.* 2007; Schlicker, Gertz *et al.* 2008). SIRT3 activation increases oxidative phosphorylation by deacetylation of enzymes in Complexes I, II and IV which are involved in the electron transport chain (Ahn, Kim *et al.* 2008; Bao, Lu *et al.* 2010; Cimen, Han *et al.* 2010; Finley, Haas *et al.* 2011; Kendrick, Choudhury *et al.* 2011; Schlicker, Gertz *et al.* 2008). Together these studies demonstrate that SIRT3 may play a central role in the regulation of cellular metabolism however to date nothing is known about SIRT3 and its presence and role in ovarian follicular cells. As follicular metabolism is essential for the development of a competent oocyte, alterations in SIRT3, as well as it targets in granulosa and cumulus cells may alter the follicular environment and impact on oocyte health. Therefore, the presence and activity of SIRT 3 in follicular cells was investigated, in good prognosis women attending for IVF and compared to that in women with reduced ovarian reserve and advanced maternal age as cells from these women display altered metabolic profiles.

Materials and Methods

Patient Recruitment and Cohorts

Informed consent to participate in this study was obtained from women (n=72) undergoing routine IVF/ICSI treatment. They were allocated to a cohort based on maternal age (young maternal age: ≤35 or advanced maternal age: ≥40) and ovarian reserve (as measured by serum anti-mullerian hormone [AMH] levels) and randomly allocated to an experimental protocol (Supplementary Figure 5.1). Previous studies have established that serum AMH correlates well with antral follicle count and thus can be utilised as a marker of ovarian reserve (Hansen, Hodnett *et al.* 2011; Rosen, Johnstone *et al.* 2012; Tremellen and Kolo 2010; Wiweko, Prawesti *et al.* 2013). The serum AMH age range for young women with normal ovarian reserve and young women with reduced ovarian reserve were 1.8-5.6µg/L and 0.4-1.5µg/L respectively. Women of advanced maternal age had serum AMH concentrations ranging between 0.6-1.5µg/L and these concentrations were representative of the 25^{th}-75^{th} percentage quartiles for this age group (Tremellen and Kolo 2010). Exclusion criteria were donor/recipient cycles, preimplantation genetic screening/diagnosis cycles and patients diagnosed with polycystic ovary syndrome. Samples were de-identified and each woman was represented once within the study period. Ethics was obtained from the Women's and Children's Hospital Research Ethics Committee *(North Adelaide, South Australia, Australia)* and from Repromed's Institutional Review Board *(Dulwich, South Australia, Australia)*.

Collection of Granulosa and Cumulus Cells

All women underwent antagonist ovarian stimulation cycles using gonadotrophins as previously described (Pacella, Zander-Fox *et al.* 2012). HCG trigger (Ovidrel (250mcg) Merck Serono, Frenchs Forest, NSW, Australia) was administered when at least 2 follicles at least ≥17mm and

cumulus-enclosed oocytes collected 36 hrs later. Immediately after oocyte retrieval, granulosa cells were collected from the surplus follicular fluid into Dulbecco's Phosphate Buffered Saline (PBS) (Sigma-Aldrich, Castle Hill, New South Wales, Australia). The granulosa cells were purified utilizing density gradient centrifugation and were first overlaid on a 60%/40% silica solution (Spermgrad, Vitrolife, Göteborg, Sweden) followed by 30min centrifugation at 470g at room temperature. Using an insulin needle, following oocyte retrieval cumulus oocyte complexes (COCs) were manually trimmed of excess cumulus cells, granulosa cells and blood clots attached to cumulus cells were avoided. These were washed through Dulbecco's PBS and cumulus cell masses were dispersed by gentle pipetting. Granulosa and cumulus cells were diluted to a concentration of 1 x 10^6/ml for use and cell counts were performed using a haemocytometer.

RNA Isolation and qPCR in Granulosa and Cumulus Cells

Granulosa and cumulus cell RNA was extracted according to manufacturer's instructions using an RNeasy Kit (Qiagen, Doncaster, Victoria, Australia). RNA quality and concentration was verified using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts, United States of America) and RNA was considered to be of acceptable quality if absorbance for A260/A280 was between 1.8-2.0. Complementary DNA (cDNA) was synthesized using Superscript III Reverse Transcriptase (Invitrogen, Mulgrave, Victoria, Australia), according to manufacturer's instructions, diluted to 5ng/µl and subsequently stored at -20°C until required. Human *SIRT3* qPCR primers were designed using the NCBI Blast Program (accession number: NM_012239; Amplicon size: 104; F: TGCCCCAGAGGTTCTTGCT: R: CTCGGTCAAGCTGGCAAAAG) and were synthesized by Geneworks (Hindmarsh, South Australia, Australia). Primers were validated by gel electrophoresis and sequenced (Appendix 4).

Each 20µl qPCR reaction contained 2µl cDNA and a master mix containing 10µl SYBR Green (Applied Biosystems, Mulgrave, Victoria, Australia), 1µl of each the *SIRT3* forward and reverse primers at a concentration of 10pmol/µl and 6µl H_2O. Samples were run in triplicate and the PCR cycling conditions were 50°C for 2mins, 95°C for 10mins, followed by 40 amplification cycles of 95°C for 15secs and 60°C for 1min and on the Corbett Rotor Gene 6000 (Corbett Life Sciences, Qiagen). Analysis was performed using delta, delta ct, normalized to the reference gene Ribosomal protein L19 (F: AGAAACGGCTACCACATCCAA, R: CCTGTATTGTTATTTTTCGTCACTACCT) (Qiagen) (Livak and Schmittgen 2001).

Immunohistochemistry of Granulosa and Cumulus Cells

Granulosa and cumulus cells were fixed to polysine slides (Thermo Scientific) using 4% paraformaldehyde and then washed through Dulbecco's PBS. The slides were incubated in 0.1M glycine (Sigma-Aldrich) in Dulbecco's PBS for 5 mins and blocked using 5% donkey serum (Sigma-Aldrich) followed by 5% goat serum (Sigma-Aldrich) each for 2 hrs. The localisation of SIRT3 to the mitochondria was confirmed by co-immunohistochemistry with Cytochrome C as a marker of mitochondrial protein. A ThermoBrite (Abbott Molecular, Des Plaines, Illinois United States of America) set at 37°C for 2 hrs was utilised for incubation of the SIRT3 primary antibody (Abcam, Sapphire Bioscience, Waterloo, New South Wales, Australia) and the Cytochrome C (Abcam) primary antibody in a 1:50 dilution. Incubation of the secondary antibodies, Fluorescein (FITC)-conjugated AffiniPure Donkey Anti-Rabbit (Jackson ImmunoResearch Laboratories, Abacus ALS, Brisbane Queensland, Australia) and Rhodamine (TRITC)-conjugated AffiniPure Goat Anti-Mouse (Jackson ImmunoResearch Laboratories) diluted to 1:100 using the ThermoBrite for 2 hrs at 37°C. Nuclei were stained for 1-2 mins using 3µM 4,6-Diamidino-2-phenyindole, dilactate (DAPI) (Sigma-Aldrich) and imaged using a Leica TCS SP5 Spectral

Scanning Confocal Microscope (Adelaide Microscopy, Adelaide University, Adelaide, South Australia, Australia). A negative control using a SIRT3 blocking peptide (Abgent, BioCore, Alexandria, New South Wales, Australia) and a positive control using mouse zygotes were also prepared and imaged. Image analysis was performed on a minimum of 40 cells per patient.

Western Blotting of Granulosa and Cumulus Cells

A protein inhibitor cocktail (used as a 100x stock solution, [Sigma-Aldrich]), radio immunoprecipitation assay (RIPA) buffer and a loading buffer were added to each sample of granulosa or cumulus cells, diluted to $1x10^6$/ml (a Bradford assay was performed to ensure equal amounts of protein) and were subsequently heated for 10mins at 70°C. A 20µl aliquot of the sample was added to the pre-cast polyacrylamide gel wells (4–15% Mini-Protean TGX Precast Gel, Bio-Rad, Gladsville, New South Wales, Australia) and run at a constant 150 volts for 60 mins. Transfer of the gel onto a 100% pure nitrocellulose membrane was performed by running the gel and membrane at a constant 100 volts for 60 mins on ice. The membrane was then immunostained using a blocking solution (supplied with the GE Healthcare Amersham ECL Advance Western Blotting Detection Kit (Thermo Fisher Scientific, Scoresby, Vic, Australia) overnight at 4°C followed by incubation with the SIRT3 antibody (used for immunohistochemistry) diluted to 1:1000 for 3 hrs at room temperature. The secondary antibody donkey anti-rabbit HRP (Jackson ImmunoResearch Laboratories) diluted to 1:100,000 was incubated with the membrane for 1 hr. This was followed by application of the ECL detection reagents (GE Healthcare Amersham ECL Advance Western Blotting Detection Kit) for 5mins. The membrane was exposed to Hyperfilm ECL (GE Healthcare Amersham) in a darkroom. A protein standard (Precision Plus Protein Dual Colour Standards, Bio-Rad), alpha tubulin loading control, positive control (recombinant SIRT3 [Abnova, Sapphire Bioscience]) and negative control (no primary antibody)

were also prepared. Quantitation of the bands was performed by image analysis using densitometry normalised to the loading control. The 69kDa recombinant SIRT3 protein corresponds to all amino acids (1 – 400) and 44kDa SIRT3 antibody corresponds to amino acids 203 – 221 only. For assessment of acetylated levels of GDH, acetylated mitochondrial proteins were probed with primary antibody diluted to 1:1000 and secondary antibody Donkey anti-goat HRP (Santa Cruz Biotechnology, ThermoFisher Scientific), diluted to 1:5000. An anti-COX IV antibody loading control (Abcam), positive control (Cytochrome C) and negative control (no primary antibody) were also prepared.

SIRT3 Deacetylation Activity in Granulosa and Cumulus Cells

SIRT3 deacetylation activity in granulosa and cumulus cells was assessed using the Life Sciences SIRT3 Fluorimetric Drug Discovery Kit (Enzo Life Sciences, Sapphire Bioscience), according to manufacturer's instructions. Each sample was run in triplicate and a human recombinant SIRT3 was run with each analysis (r^2>0.98). Negative controls including, a sample without enzyme, a sample without substrate, nicotinamide and extraction buffer controls were also run with each analysis.

For the inhibition and stimulation of SIRT3 protein activity the inhibitors used were Nicotinamide (0mM, 5mM, 10mM, 25mM and 50mM [Sigma-Aldrich] and Suramin (0mM, 5mM, 10mM, 20mM and 40mM [supplied with activity kit]). The stimulators of SIRT3 used were Resveratrol (0mM, 5mM, 10mM 50mM and 100mM [Sigma-Aldrich]) and SRT1720 (0µM, 75µM, 150µM, 300µM and 600µM [Selleckchem, Life Research]). A dose response for the SIRT3 inhibitors and stimulators was performed using the SIRT3 Fluorimetric Drug Discovery Activity Kit. Granulosa and cumulus cells ($1x10^6$/ml), were collected and purified as outlined above and cultured in G-1

media (Vitrolife) with either 10 mM Nicotinamide or 10mM Resveratrol at 37°C in 5%O_2/6%CO_2 for 24 hrs. A G-1 media only control was included.

Enzyme Activity of Glutamate Dehydrogenase in Granulosa and Cumulus Cells

Enzyme activity (Vmax) of GDH was assessed using quantitative fluorescent assays (Passonneau and Lowry 1993) using a buffer containing 50mM Imidazole (Sigma-Aldrich), 25mM ammonium acetate (Sigma-Aldrich), 100µM ADP (Roche Diagnostic Australia, Dee Why, New South Wales, Australia), 100µM NADPH (Roche Diagnostic Australia) and 0.05% BSA (Sigma-Aldrich) with 0.2µl of sample added to each reaction. Reactions were initiated by addition of 2mM α-ketoglutaric acid (GDH), fluorescence was assessed after 10min at 37°C and each sample was run in triplicate. A standard curve (r^2>0.98) was obtained for each analysis. A positive control of glutamate dehydrogenase (Roche Diagnostics, Mannheim, Germany), and a negative control of lactate dehydrogenase (Roche Diagnostics) and no sample was also included.

Immunoprecipitation for Acetylated Proteins in the Mitochondrial Extracted Granulosa and Cumulus Cells

Mitochondria were extracted from granulosa and cumulus cells, following manufacturers' instructions (mitochondria isolation kit for cultured cells; MitoSciences, Abcam). To verify the mitochondrial fraction a western blot for cytochrome C (mitochondrial) and α-tubulin (cytoplasmic) was performed on both the extracted mitochondria sample and the neat cells and purity of >80% in the mitochondrial fraction was confirmed by image analysis. Immunoprecipitation of mitochondrial isolated granulosa and cumulus cells was performed using the Dynabead Protein G Kit (Invitrogen, Life Technologies, Mulgrave, Victoria, Australia)

according to manufacturers' instructions. The anti-acetylated antibody (Abcam) was first bound to the Dynabeads by incubation of the beads and antibody (1:100) for 10mins. The mitochondrial isolated sample was added to the beads and incubated for a further 10mins. The Dynabeads bound to the acetylated proteins was resuspended in 50µl of washing buffer and 10µl of elution buffer was added. The Dynabeads (bound to the antibody and sample) were then stored at -80°C until needed for western blot analysis. For western blot analysis the Dynabeads were thawed and 10µl of loading buffer with protein inhibitor cocktail was added and denatured by heating the sample for 10mins at 70°C. The supernatant (containing the acetylated proteins) was removed and loaded into the prepared gel. The Western blot protocol as outlined above was then followed.

Statistical Analysis

GraphPad Prism Software version 6 was used for all statistical analyses and data are expressed as mean ± standard error of the mean (SEM). To determine significance between groups a Chi-square was used for pregnancy rates and infertility diagnoses. For the remainder of the analyses a one-way ANOVA with Tukey's post-hoc test was used. A p-value <0.05 was considered statistically significant.

Results

Table 5.1: Patient Demographics and Cycle Outcomes[1]

		Young Maternal Age with Normal Ovarian Reserve (n=32)	Young Maternal Age with Reduced Ovarian Reserve (n=17)	Advanced Maternal Age (n=23)
Age		31.2±0.6[a]	32.4±0.5[a]	41.8±0.5[b]
Serum AMH (µg/L)		3.3±0.2[a]	0.9±0.1[b]	1.2±0.1[c]
Antral Follicle Count		16.9±1.0[a]	6.8±0.9[b]	9.2±0.6[c]
BMI		23.6±0.9[a]	24.6±1.3[a]	25.4±1.8[a]
Starting FSH Dose (IU)		181.6±10.3[a]	291.9±8.4[b]	305.8±6.8[b]
# previous fresh cycles (IVF/ICSI)		0.9±0.3[a]	2.5±0.6[b]	2.3±0.5[b]
# oocytes collected		12.3±0.9[a]	8.2±0.9[b]	7.2±0.1[b]
Fertilisation Rate (%)		69.2±4.6[a]	67.0±4.4[a]	53.0±5.9[b]
Mean # Embryos Transferred		1.1±0.1[a]	1.3±0.3[ab]	1.7±0.2[b]
Mean # Embryos Frozen		3.0±0.5[a]	1.9±0.4[b]	0.6±0.2[c]
Pregnancy Rate (%)		56.2[a]	32.3[b]	20.6[b]
Implantation Rate (%)		44.4[a]	28.4[b]	13.6[c]
Utilisation of Oocytes (%)[2]		64.1[a]	35.3[b]	22.9[b]
Infertility Diagnosis (%)[3]	**Ovulatory**	8.7[a]	0.0[a]	9.4[a]
	Tubal	9.8[a]	0.0[a]	0.0[a]
	Endometriosis	7.3[a]	0.0[a]	12.6[a]
	Unexplained	14.7[a]	19.1[a]	1.0[a]
	Male Factor	72.5[a]	70.3[a]	83.1[a]

[1] Data expressed as mean±SEM, a-c indicate significant differences between cohorts, $p<0.05$. Exclusion criteria: donor/recipient cycles, preimplantation genetic screening and PCOS patients

[2] Utilization of Oocytes was calculated as follows: (frozen embryos + embryos transferred) / oocytes

[3] Infertility diagnosis: multiple diagnoses for some patients

Patient Demographics

Mean maternal age, serum AMH levels, antral follicle count, IVF cycle details and outcomes are outlined in Table 5.1. Similar to previous studies, serum AMH correlated with antral follicle count (Supplementary Figure 5.2) (Hansen, Hodnett *et al.* 2011; Rosen, Johnstone *et al.* 2012; Tremellen and Kolo 2010; Wiweko, Prawesti *et al.* 2013). Young women with normal ovarian reserve had lower starting FSH doses compared to women with reduced ovarian reserve and women of advanced maternal age ($p<0.05$, Table 5.1). Compared to both the reduced ovarian reserve and advanced maternal age groups, young women with normal ovarian reserve had undergone fewer fresh IVF cycles, had more oocytes collected and more embryos frozen ($p<0.05$, Table 5.1). The young women with normal ovarian reserve also had an increase in pregnancy rate and implantation rate compared to the reduced ovarian reserve and advanced maternal age groups ($P<0.05$, Table 5.1). The women with reduced ovarian reserve had an increase in fertilisation rate and implantation rate compared to the advanced maternal age women ($p<0.05$, Table 5.1). No significant differences were found for either BMI or infertility diagnosis.

Granulosa and Cumulus Cell SIRT3 Gene Expression

SIRT3 gene expression levels were significantly elevated in granulosa cells compared to cumulus cells in young women with normal ovarian reserve ($p<0.05$, Figure 5.1). Interestingly, this differentiation in *SIRT3* gene expression between granulosa and cumulus cells was not evident in either young women with reduced ovarian reserve or advanced maternal age women (Figure 5.1). Granulosa cell *SIRT3* gene expression was significantly decreased in women with reduced ovarian reserve and was further decreased in women of advanced maternal age compared to young women with normal ovarian reserve ($p<0.05$, Figure 5.1). Similarly, cumulus cell *SIRT3*

gene expression levels in women with advanced maternal age were reduced compared to young women with normal ovarian reserve ($p<0.05$, Figure 5.1).

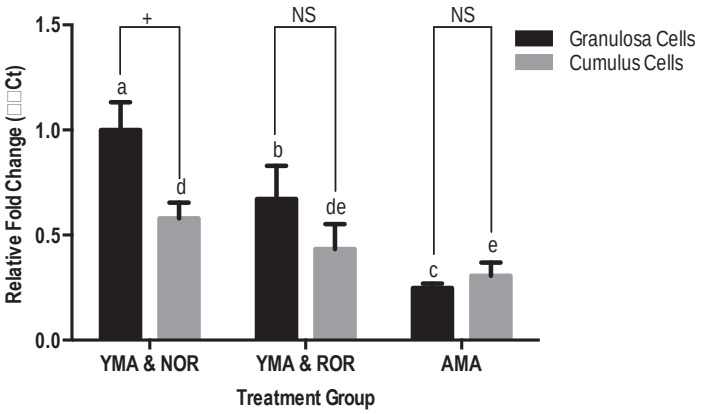


Figure 5.1: Relative fold change in *SIRT3* gene expression in granulosa and cumulus cells.
n(YMA & NOR) = 17, n(ROR) = 7, n(AMA) = 13 and all samples were run in triplicate. Samples expressed relative to granulosa cells from young women with normal ovarian reserve. a – e indicate significant differences between cohorts for cell type ($p<0.05$), + indicate significant differences between cell type within cohorts ($p<0.05$), NS indicates not significant. YMA – young maternal age, NOR – normal ovarian reserve, ROR – reduced ovarian reserve and AMA – advanced maternal age.

SIRT3 Protein Localisation and Levels in Granulosa and Cumulus Cells

SIRT3 was found to co-localise with the mitochondria in both granulosa and the cumulus cells (Supplementary Figure 5.3). Similar to the gene expression data, granulosa cell SIRT3 protein levels detected by western blot analysis were elevated compared to the cumulus cells in young women with normal ovarian reserve (Figure 5.2A) with this difference again not evident in young women with reduced ovarian reserve or the advanced maternal age women. Compared to young women with normal ovarian reserve granulosa cell SIRT3 protein levels were significantly decreased in the young women with reduced ovarian reserve and the advanced maternal age women ($p<0.05$, Figure 5.2B). Granulosa cell SIRT3 protein levels were further decreased in the advanced maternal age cohort compared to young women with reduced ovarian reserve.

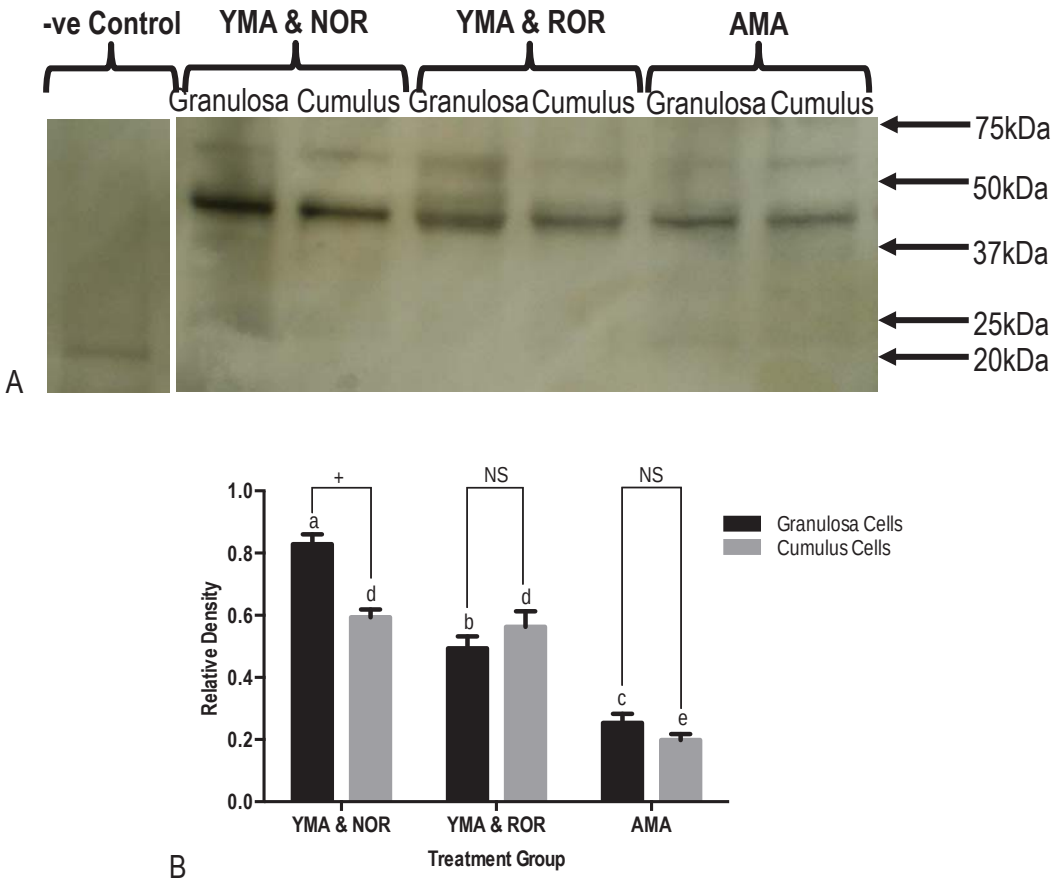


Figure 5.2: A) Western Blot displaying SIRT3 in granulosa and cumulus cells at 44kDa. B) Relative SIRT3 protein density in granulosa and cumulus cells.
A) 44kDa SIRT3 in granulosa and cumulus cells corresponds to amino acids 203 – 221. The weak band at 60kDa in all samples (including negative control) corresponds to albumin due to the presence of high concentrations of Human Serum Albumin (HSA) in the media used to collect granulosa and cumulus cells. B). n(YMA & NOR) = 17, n(ROR) = 7, n(AMA) = 13 and all samples were run in duplicate. a – e indicate significant differences between cohorts for cell type ($p<0.05$), + indicate significant differences between cell type within cohorts ($p<0.05$), NS indicates not significant. YMA – young maternal age, NOR – normal ovarian reserve, ROR – reduced ovarian reserve and AMA – advanced maternal age.

Cumulus cell SIRT3 protein levels were significantly decreased in women of advanced maternal age compared to young women with normal ovarian reserve ($p<0.05$, Figure 5.2B). In contrast to the gene expression data, women of advanced maternal age had decreased SIRT3 protein levels compared to young women with reduced ovarian reserve ($p<0.05$, Figure 5.2B). No significant difference was found in cumulus cell SIRT3 protein levels between the young women with normal ovarian reserve and the young women with reduced ovarian reserve (Figure 5.2B).

SIRT3 Protein Deacetylation Activity in Granulosa and Cumulus Cells

The main enzymatic function of SIRT3 is the deacetylation of protein targets to post-translationally alter target protein functional activity. SIRT3 deacetylase activity was assessed in Vmax conditions and found to be present in both granulosa and cumulus cells from all women. SIRT3 protein deacetylation activity was highest in granulosa cells compared to cumulus cells in young women with normal ovarian reserve ($p<0.05$, Figure 5.3). Granulosa cell SIRT3 protein deacetylation activity was reduced in young women with reduced ovarian reserve and further decreased in women of advanced maternal age compared to young women with normal ovarian reserve ($p<0.05$, Figure 5.3). Women of advanced maternal age had decreased cumulus cell SIRT3 protein deacetylation activity compared to young women with normal ovarian reserve or young women with reduced ovarian reserve ($p<0.05$, Figure 5.3). No difference in cumulus cell SIRT3 protein deacetylation activity was detected between the young women with normal ovarian reserve and young women with reduced ovarian reserve (Figure 5.3).

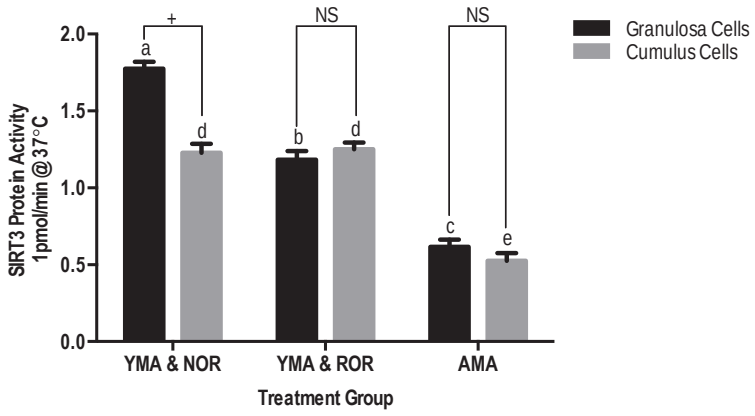


Figure 5.3: SIRT3 protein deacetylation activity in granulosa and cumulus cells.
n = 5 patients per cohort and all samples were run in triplicate. a – e indicate significant differences between cohorts for cell type ($p<0.05$), + indicate significant differences between cell type within cohorts ($p<0.05$), NS indicates not significant. YMA – young maternal age, NOR – normal ovarian reserve, ROR – reduced ovarian reserve and AMA – advanced maternal age.

Acetylation of Glutamate Dehydrogenase in Mitochondria of Granulosa and Cumulus Cells

SIRT3 is able to deacetylate glutamate dehydrogenase (GDH) therefore acetylation of GDH in granulosa and cumulus cells was determined by Western Blot analysis of mitochondrial acetylated protein fraction. Compared to young women with normal ovarian reserve GDH acetylation in granulosa cells was increased in young women with reduced ovarian reserve and was further increased in advanced maternal age women ($p<0.05$, Figure 5.4). Young women with normal ovarian reserve and young women with reduced ovarian reserve had similar GDH acetylation levels in cumulus cell (Figure 5.4). GDH acetylation in cumulus cell was highest in the advanced maternal age women compared to young women with normal ovarian reserve and young women with reduced ovarian reserve ($p<0.05$, Figure 5.4).

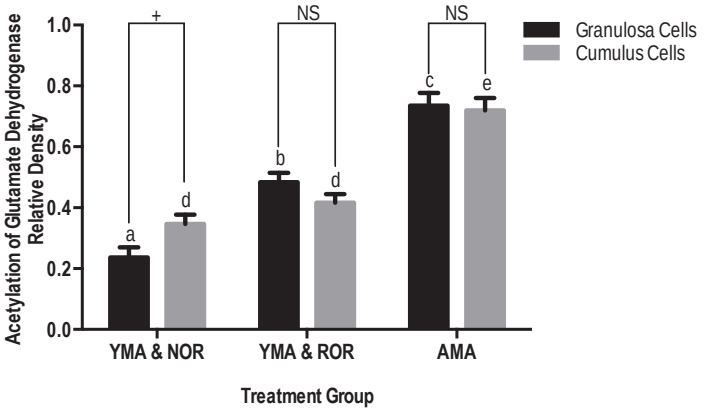
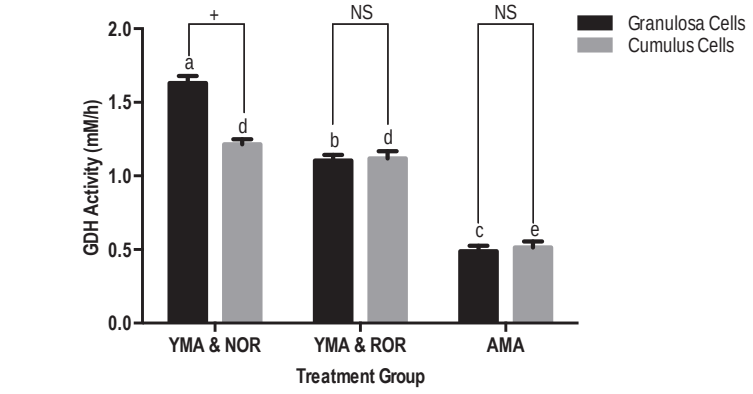


Figure 5.4: GDH acetylation in the mitochondrial of granulosa and cumulus cells
n= 10 patients per cohort and all samples were run in duplicate. a – e indicate significant differences between cohorts for cell type ($p<0.05$), + indicate significant differences between cell type within cohorts ($p<0.05$), NS indicates not significant. YMA – young maternal age, NOR – normal ovarian reserve, ROR – reduced ovarian reserve and AMA – advanced maternal age.

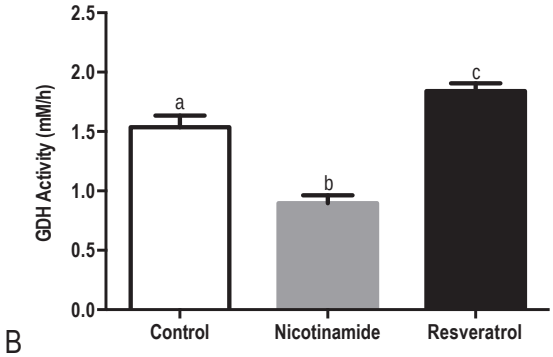
Activity of the SIRT3 Target Glutamate Dehydrogenase in Granulosa and Cumulus Cells

To assess the impact of increased GDH protein acetylation on activity, GDH Vmax activity which catalyses the reaction between glutamate and α-ketoglutarate, was determined. In agreement with SIRT3 deacetylation activity, granulosa cell GDH activity was decreased in young women

with reduced ovarian reserve and in women of advanced maternal age compared to young women with normal ovarian reserve ($p<0.05$, Figure 5.5A). Granulosa cell GDH activity in women of advanced maternal age was decreased compared to young women with reduced ovarian reserve, reflective of what was observed in granulosa cell SIRT3 deacetylation activity ($p<0.05$, Figure 5.5A). Similar to cumulus cell SIRT3 deacetylation activity, cumulus cell GDH activity was decreased in women of advanced maternal age compared to young women with normal ovarian reserve and young women with reduced ovarian reserve ($p<0.05$, Figure 5.5A). No significant differences were detected in cumulus cell GDH activity between young women with normal ovarian reserve and young women with reduced ovarian reserve (Figure 5.5A).



A



B

Figure 5.5: A) Enzyme activity of GDH B) GDH activity in granulosa cells after a 2 hr culture with either 10mM Nicotinamide or 10mM Resveratrol.

A) GDH activity in granulosa and cumulus cells. n = 10 patients per cohort and all samples were run in duplicate. a – e indicate significant differences between cohorts for cell type ($p<0.05$), + indicate significant differences between cell type within cohorts ($p<0.05$), NS indicates not significant. B) n(YMA & NOR) = 5 patient samples were run in triplicate, a – c: indicate significant differences between treatments. YMA – young maternal age, NOR – normal ovarian reserve, ROR – reduced ovarian reserve and AMA – advanced maternal age.

To further establish if the changes in GDH are directly related to SIRT3 deacetylation activity, granulosa cells from young women with normal ovarian reserve were cultured in the presence of either Resveratrol (a Sirtuin stimulator) or Nicotinamide (a Sirtuin inhibitor). Nicotinamide and Resveratrol were used as they were established to be the most effective inhibitor and stimulator of SIRT3 protein deacetylation activity compared to the other inhibitors AGK7, Suramin and Sirtinol and the other activator SRT170 (Supplementary Figure 5.4). The dose response established that 10mM Nicotinamide and 10mM Resveratrol were the optimal concentrations in altering SIRT3 protein deacetylation activity. GDH activity in granulosa cells was increased in the presence of Resveratrol ($p<0.05$, Figure 5.5B). In contrast, Nicotinamide caused a decrease in GDH activity in granulosa cells ($p<0.05$, Figure 5.5B). Together this data confirms that pharmacologically altering SIRT3 activity results in an alteration to GDH activity.

Discussion

The sole source by which ATP is produced by the oocyte is through the process of oxidative phosphorylation via the electron transport chain (also known as the mitochondrial respiratory chain) within the mitochondria. However, metabolism within the human oocyte is specialised with pyruvate being the main energy substrate, unlike somatic cells where energy is produced via aerobic respiration of glucose (Downs and Utecht 1999; Wilding, Dale *et al.* 2001). Thus, cumulus cells have an essential role as they metabolise glucose into pyruvate which is transported into the oocyte, where it is able to be utilised. Thus, the communication between the oocyte and its surrounding cells is essential for the development of a viable oocyte (Canipari 2000; Goud, Goud *et al.* 1998; Sugiura, Pendola *et al.* 2005). Therefore, changes to mitochondrial metabolism within the follicular cells may ultimately affect oocyte development. Previous studies have established that women with reduced ovarian reserve have altered expression of genes involved in metabolic pathways in granulosa and cumulus cells compared to young women with normal ovarian reserve (Greenseid, Jindal *et al.* 2009; May-Panloup, Ferre-L'Hotellier *et al.* 2012; Skiadas, Duan *et al.* 2012). Similarly, proteomic analysis of cumulus cells from women of advanced maternal age have differentially expressed metabolic proteins compared to cumulus cells from younger women (McReynolds, Dzieciatkowska *et al.* 2012) and oocytes from women of advanced maternal age have altered expression of genes involved in the electron transport chain (Grondahl, Yding Andersen *et al.* 2010). Therefore, there are changes to oocyte, granulosa and cumulus cell metabolism that may affect development of the follicle and oocyte. As SIRT3 is a metabolic sensing protein, its ability to post-translationally alter proteins involved in mitochondrial metabolism is of significant interest. This study has established that *SIRT3* gene expression and active protein is present in granulosa and cumulus cells and co-localises to the mitochondria consistent with what has been reported in other tissues (Hallows, Albaugh *et al.* 2008; Michan and Sinclair 2007; Schwer, North *et al.* 2002). Furthermore, *SIRT3*

gene expression in granulosa and cumulus cells is decreased in women with reduced ovarian reserve and advanced maternal age indicative of a down-regulation of SIRT3 function which may have implications for cellular functions that require SIRT3, specifically mitochondrial metabolism.

Post-translational acetylation of mitochondrial proteins is fast becoming recognised as a fundamental mechanism for regulating the activity of mitochondrial proteins and overall mitochondrial function (Hirschey, Shimazu *et al.* 2009). This modification can influence multiple protein functions including cell survival and metabolism, cell cycle regulation, thermogenesis, insulin secretion, DNA repair and transcription (Hirschey, Shimazu *et al.* 2009). A proteomic study has revealed that approximately 20% of all mitochondrial proteins are targets of reversible acetylation and suggest that enzymes involved in the TCA cycle may be targets of deacetylation (Hirschey, Shimazu *et al.* 2009). Mitochondrial protein acetylation is sensitive to metabolic state as mitochondrial protein acetylation increased in the liver of mice during fasting, with 14% of acetylated mitochondrial proteins unique to fed mice and 24% unique to fasted mice (Lombard, Alt *et al.* 2007). Interestingly, the acetylation of mitochondrial proteins also increased in mice during long-term high fat diet feeding (Hirschey, Shimazu *et al.* 2010). Therefore, an altered metabolic state such as nutrient deficiency or nutrient excess is able to alter the amount of mitochondrial proteins that are acetylated (Anderson and Hirschey 2012). Thus, in oocytes from women with advanced maternal age where it has been demonstrated that mitochondrial function is impaired (Van Blerkom, Davis *et al.* 1995; Wilding, Dale *et al.* 2001), SIRT3 may be implicated as it plays an important role in the deacetylation of mitochondrial proteins that are involved in energy metabolism.

During antral follicle development the granulosa cells surrounding the oocyte differentiate into cumulus cells, this process is dependent on endocrine signalling from the follicle and paracrine

signalling from the oocyte (Eppig, Chesnel *et al.* 1997). After this differentiation has occurred granulosa and cumulus cells each have specific functions and respond differently to extracellular signals. Even though this study may not be reflective of the non-stimulated "natural" microenvironment, all the women were exposed to the same type of ovarian stimulation. In young women with normal ovarian reserve the levels of *SIRT3* mRNA and protein were higher in the granulosa cells compared to the cumulus cells, suggesting that the transition from granulosa in cumulus cells results in down-regulation of SIRT3. In contrast, in young women with reduced ovarian reserve and in women of advanced maternal age *SIRT3* mRNA and protein levels are similar between granulosa and cumulus cells suggestive of altered differentiation. These findings are reflective of cumulus cells from women with reduced ovarian reserve and from women of advanced maternal age as they did not undergo the same quantum of metabolic or steroidogenic changes associated with differentiation and instead demonstrated traits associated with granulosa cell function (Pacella, Zander-Fox *et al.* 2012). This altered cumulus cell function may have implications for COC metabolism and on pyruvate supply to the oocyte thereby altering oocyte metabolism and affecting its developmental potential.

One of the most well characterised confirmed targets of SIRT3 is glutamate dehydrogenase, a mitochondrial enzyme involved in both TCA activity and also maintenance of oxidative state. Activation of GDH is thought to promote the synthesis of ATP by allowing amino acids to be utilised as fuel for the TCA cycle (Frigerio, Casimir *et al.* 2008). GDH has been demonstrated to co-localise with SIRT3 in the mitochondrial matrix and in *SIRT3* knockout mice GDH is hyperacetylated compared to wild-type (Lombard, Alt *et al.* 2007). SIRT3 can directly deacetylate GDH thus controlling mitochondrial metabolism, with incubation of SIRT3 with NAD^+ increased deacetylation of GDH therefore increasing its activity, with activity being positively correlated with SIRT3 levels (Schlicker, Gertz *et al.* 2008). These studies demonstrate that SIRT3 is able to

deacetylate GDH and as a result GDH is activated. In our study we determined that granulosa cell activity of GDH was decreased in young women with reduced ovarian reserve and in women of advanced maternal age in line with the changes in SIRT3 levels, suggestive of SIRT 3 mediated alteration in mitochondrial metabolism.

To confirm if this reduction in GDH activity may be attributed to reduced SIRT3 deacetylation activity, we investigated the impact of culturing granulosa cells with inhibitors or stimulators of SIRT3. Treatment with the inhibitor Nicotinamide resulted in decreased GDH activity while treatment with the Sirtuin stimulator resulted in increased GDH activity. Furthermore, the acetylation profile of GDH in mitochondria of granulosa and cumulus cells revealed increased acetylation of GDH in both the granulosa and cumulus cells of women of advanced maternal age compared to young women with normal ovarian reserve while young women with reduced ovarian reserve had increased granulosa cell GDH activity in the mitochondria compared to young women with normal ovarian reserve. Together, this suggests that SIRT3 in granulosa and cumulus cells may be partially responsible for regulation of GDH activity and thus for the decline in oocyte metabolism and oocyte viability. However, SIRT3 is also involved in the regulation of multiple other targets including; isocitrate dehydrogenase, ATP5A, an important subunit on the respiratory chain complex required for ATP synthesis and HSP70 (heat shock protein 70), which is involved in the regulation of protein unfolding as well as protection against adverse environmental insults such as temperature variations and oxidative stress. Furthermore, SIRT3 is also involved in regulation of MnSOD (manganese superoxide dismutase), which has been implicated in aging and involved in the regulation of ROS (reactive oxygen species) (Chen, Zhang *et al.* 2011; Law, Liu *et al.* 2009; Miao and St Clair 2009; Schlicker, Gertz *et al.* 2008). Due to the importance of these cellular processes in oocyte and embryo development as well as their

link to aging it is likely that SIRT3 may be acting on multiple biological pathways and further investigation into its relationship with these targets is warranted.

The association between the decline in oocyte quality and pregnancy rates with increasing maternal age is well established and was again demonstrated in this study with a 35% decrease in pregnancy rates compared to young women with normal ovarian reserve. However, the decline in oocyte quality and pregnancy rates with reduced ovarian reserve remains subject to controversy. In our study young women with reduced ovarian reserve had a 24% decline in pregnancy rates compared to young women with normal ovarian reserve. Despite the small cohort of patients in this study the results presented here are rather compelling and are consistent with other studies examining IVF outcome in women with reduced ovarian reserve as they have found an association with decreased pregnancy rates (Brodin, Hadziosmanovic *et al.* 2013; Hattori, Sato *et al.* 2013; Hazout, Bouchard *et al.* 2004; Honnma, Baba *et al.* 2013; Lekamge, Barry *et al.* 2007; Li, Lee *et al.* 2013; Lin, Yao *et al.* 2013; Wunder, Guibourdenche *et al.* 2008). Perturbations by SIRT3 via post-translational protein modification may be a causative factor in the decline in oocyte viability in women with reduced ovarian reserve and advanced maternal age, possibly via alteration of metabolic pathways. Knowledge of these perturbations may lead to novel therapies to improve mitochondrial metabolism, such as specifically targeting deacetylation of GDH, in the oocyte and follicular cells in women undergoing IVF treatment.

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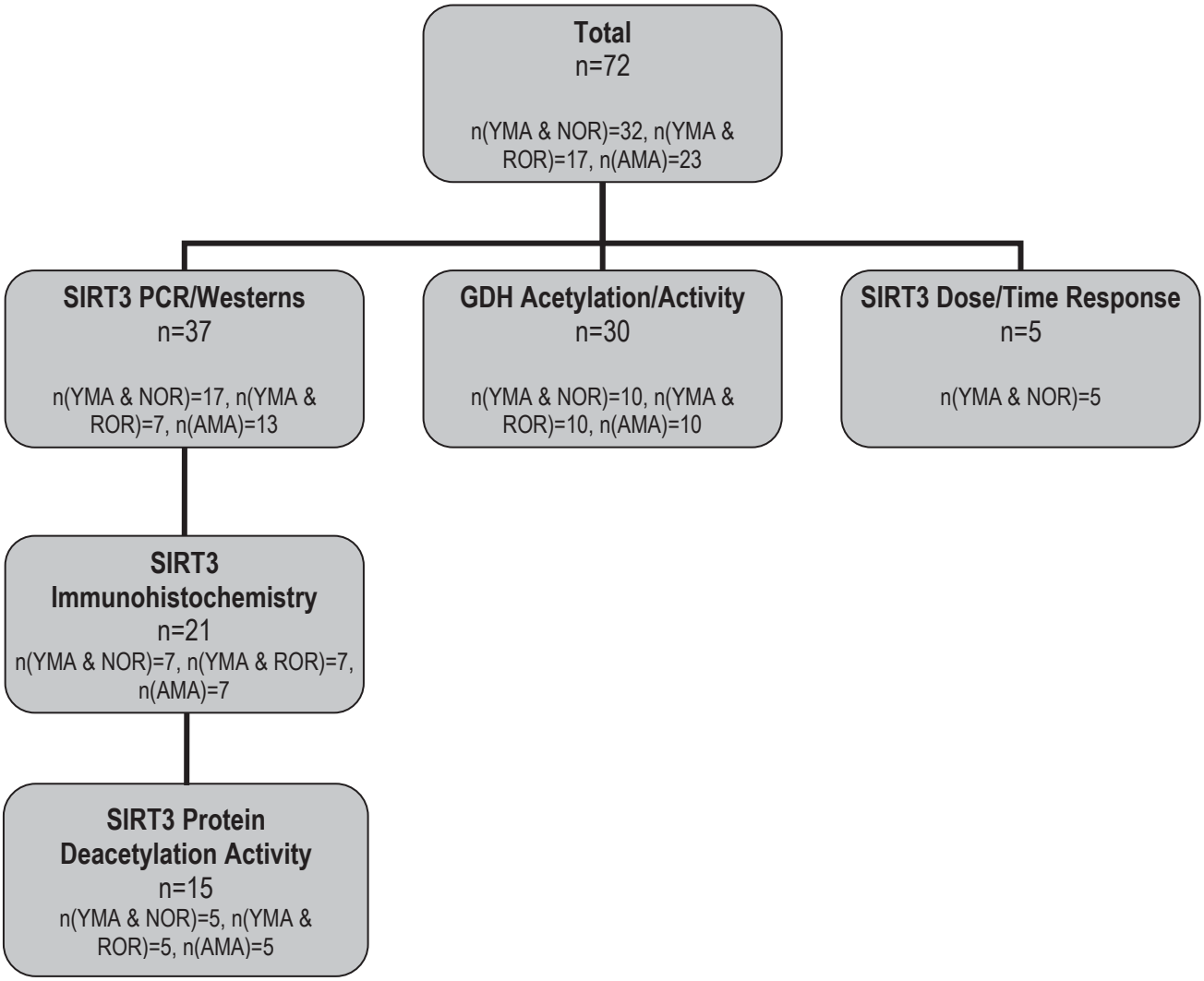
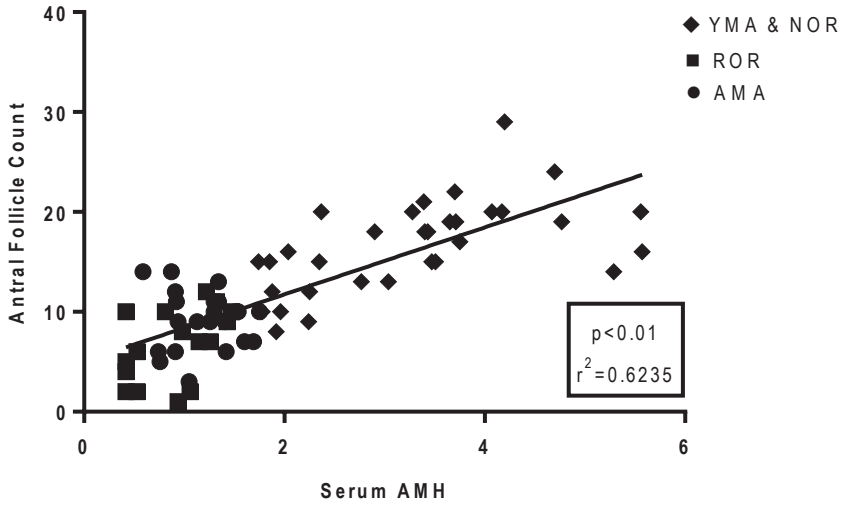


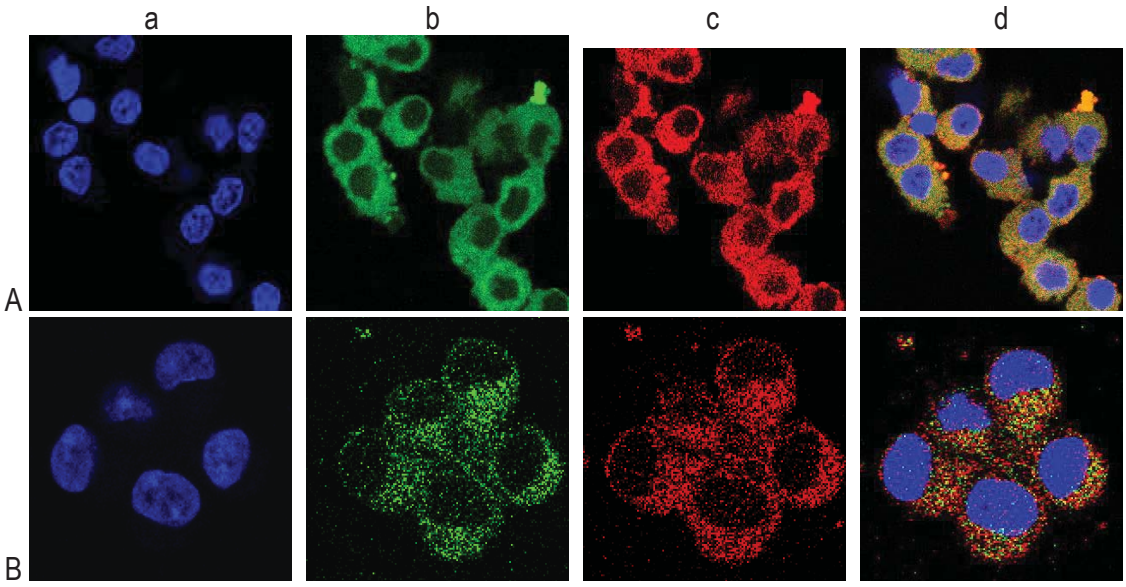
Supplementary Figure 5.1: Random allocation of patients to each experimental protocol.

Patients were randomly allocated to each treatment (e.g. SIRT3 PCR/Westerns, GDH Acetylation/Activity or SIRT3 Dose/Time Response). Out of the n=37 patients allocated to the SIRT3 PCR/Western group n = 21 were also allocated to the SIRT3 Immunohistochemistry group and of these n=21 patients n=15 were also allocated to the SIRT3 Protein Deacetylation Activity group (thus, 15 patients had PCR/Westerns, Immunohistochemistry and Protein Deacetylation activity performed on their granulosa and cumulus cells).

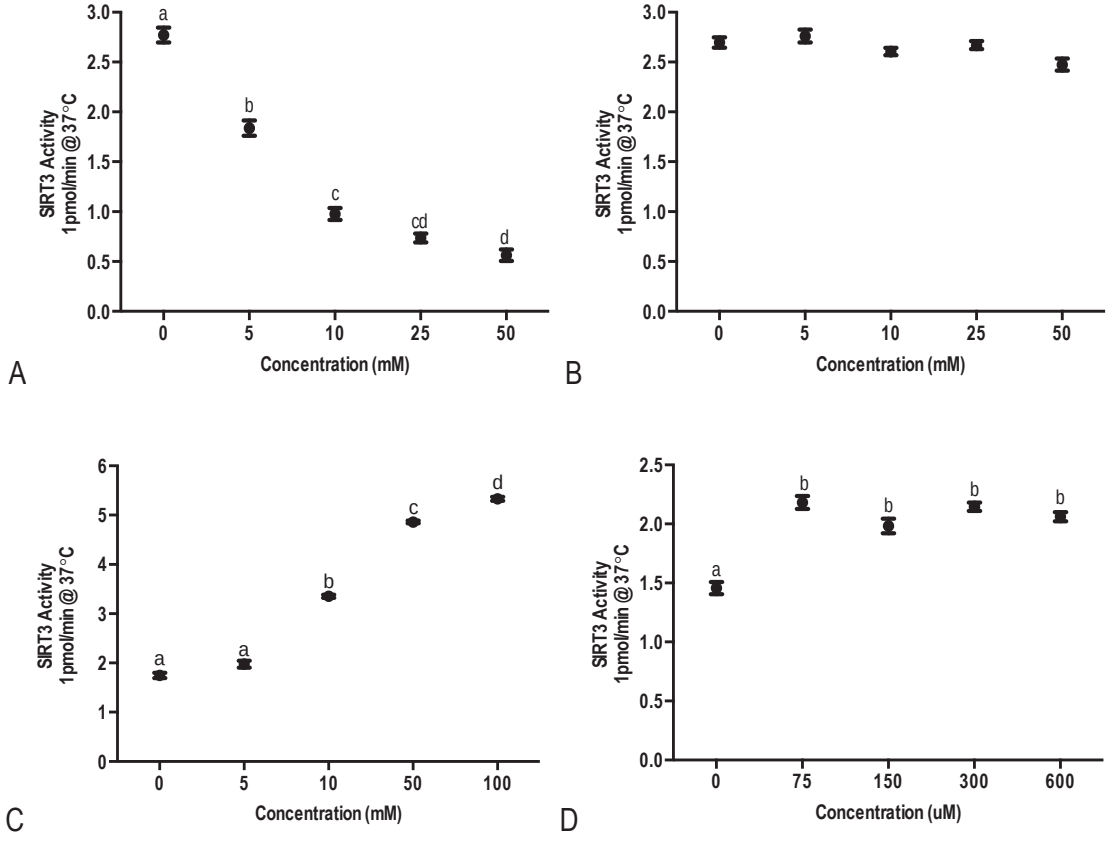


Supplementary Figure 5.2: Correlation between serum AMH and antral follicle count in patient cohort.

n(YMA & NOR) = 32, n(YMA & ROR) = 17, n(AMA) = 23. YMA – young maternal age, NOR – normal ovarian reserve, ROR – reduced ovarian reserve and AMA – advanced maternal age.



Supplementary Figure 5.3: Co-localisation of SIRT3 protein to the mitochondria in A) granulosa cells and B) cumulus cells.
n = 7 patients per cohort and all samples were run in duplicate. a – blue indicates DAPI or nuclear staining, b – green indicates SIRT3 protein, c – red indicates Cytochrome C and was used as a mitochondrial marker and d – merged image.



Supplementary Figure 5.4: Dose response in SIRT3 protein deacetylation activity for the SIRT3 inhibitors A) Nicotinamide and B) Suramin and the SIRT3 activators C) Resveratrol and D) SRT1720

Samples were run in triplicate, n(YMA&NOR)=5. The SIRT3 inhibitors and activators were utilised in the SIRT3 protein deacetylation activity kit. Note: the inhibitors AGK7 and Sirtinol did not dissolve in assay buffer and thus were unable to be tested. a – d: indicate significant differences between treatments, NB: B) no significant difference detected for Suramin at the concentrations examined.

CHAPTER SIX

Final Discussion

Final Discussion

It is well established that a woman's fertility declines with increasing maternal age, with an exponential loss in fertility after a women turns 40 [1-5]. It is also well established that pregnancy rates after IVF are significantly lower as a woman's maternal age increases compared to younger women [6]. The data presented in chapter 2 of this thesis supports that IVF outcomes are significantly reduced in women >40 years. However, in addition to chronological aging, AMH serum levels are increasingly used to establish the "ovarian" age of a women. Women with low serum AMH levels for age and therefore a reduced ovarian reserve for their chronological age, often have pregnancy rates that are reduced compared to women with normal ovarian reserve for age [7-9]. In this study the cohort of women that were ≤35 years but had a reduced ovarian reserve, similarly had reduced pregnancy rates and implantation rates compared to equivalent aged women who had normal serum AMH levels or normal ovarian reserve (chapter 2). Furthermore, as a consequence of their reduced ovarian reserve, during an IVF cycle they also had less oocytes collected, poorer blastocyst development and as a consequence less embryos frozen (chapter 2). These data add further evidence to the view that serum AMH levels can be utilised as a measure of a woman's reproductive potential independent to her maternal age, in particular in predicting outcomes after IVF.

The microenvironment within the ovarian follicle is an important factor that affects the growth and developmental competence of the oocyte, with bi-directional communication occurring between follicular cells and the oocyte that are essential for the maintenance of viability [10, 11]. Thus, any changes within this environment either to the ovarian follicular cells or the fluid of the antrum (follicular fluid) may serve as an indicator of oocyte health and developmental competence. Therefore, chapter 3 examined the composition of fluid collected from the antral follicles of older

women (≥40 years) and also from women ≤35 years with reduced ovarian reserve and compared this to young women with a normal ovarian reserve for their age. This study established that the levels of the carbohydrates glucose, lactate and pyruvate, and the hormones progesterone, estrogen and AMH are altered in follicular fluid from women with reduced ovarian reserve and in women of advanced maternal age, which in this study are the groups of women with reduced pregnancy outcomes. Interestingly, the glycolytic activity of the cumulus cells (a key energy generating pathway) in women of advanced maternal age and in women with reduced ovarian reserve was decreased and this may be in part due to increased expression of phosphofructokinase platelet and decreased expression of lactate dehydrogenase A. Similarly the follicular fluid progesterone changes may be related to an increase in cumulus cell expression of the progesterone receptor and prostaglandin E receptor-2 observed in young women with reduced ovarian reserve and in women of advance maternal age. In young women with reduced ovarian reserve and in women of advanced maternal age these results suggest there are metabolic and molecular changes to granulosa and cumulus cells which appeared to be indicative of changes to cellular differentiation with cumulus cells appearing to maintain a phenotype more typically observed in granulosa cells. It is therefore likely that these metabolic and molecular perturbations in follicular cells result in the observed changes to the follicular fluid content (as described in chapter 3), which maybe responsible for the reduced developmental potential of the oocyte, as demonstrated by the decrease in clinical pregnancy rates and decrease in oocyte utilisation. Interestingly, the reductions in the metabolism of the ovarian cells, with maternal ageing and reduced ovarian reserve may implicate changes in mitochondrial metabolism in the follicular cells as glucose is required for mitochondrial metabolism, thus a decrease in the availability of glucose may lead to a decrease in mitochondrial metabolism and thus a decrease in the amount of ATP available for cellular processes required for ovarian follicle development [12].

The family of Sirtuin proteins are able to sense the metabolic state of the cell and subsequently modify both proteins and histones post-translationally. Histone post-translational deacetylation is able to influence many cellular functions including the cell cycle, silencing gene transcription, chromatin remodelling, DNA repair, cell proliferation and apoptosis [13-15]. However, evidence has also determined that the mammalian Sirtuins are able to deacetylate and more recently desuccinylate proteins. Due to the diverse functions and locations of proteins within the cell, the Sirtuins are able to target various substrates and thus have different cellular functions. Mitochondrial protein acetylation is recognised as a fundamental mechanism for regulating the activity of mitochondrial proteins as well as overall mitochondrial function [16]. This relatively simple modification to proteins can infuence many protein functions including cell survival, cellular metabolism, cell cycle regulation, thermogenesis, insulin secretion, DNA repair and transcription [16]. A proteomic study of cellular proteins revealed a large number of mitochondrial proteins are targets of reversible post-translational acetylation, futhermore demonstrating that this modification could occur in mitochondrial proteins [16]. Interestingly, approximately 20% of all mitochondrial proteins were acetylated [16]. Furthermore, it has been established that several mitochondrial metabolic enzymes are sucesptible to desuccinylation due to the presence of succinylation sites, such as succinyl-CoA, suggesting that the enzymes invovled in glycolysis, the TCA cycle and the electron transport chain may be targets of deacetylation and desuccinylation. Considering that SIRT3 and SIRT5 are mitochondrial proteins able to post-translationally alter protein function in response to changes in cellular metabolic state, these proteins pose as novel candidates for linking cellular metabolism and molecular changes within cells. The role of these proteins in ovarian cells is not well understood, and therefore the hypotheses tested in chapters 4 and 5 was whether these proteins may be involved in the changes observed in ovarian follicular cells and have an involvement in the maintenance of oocyte quality.

Chapter 4 details that *SIRT5* mRNA levels are in fact decreased in both granulosa and cumulus cells of women ≥40 years and young women with reduced ovarian reserve compared to young women with normal ovarian reserve, suggestive of a down-regulation of SIRT5 function in these cells. This study also established SIRT5 protein in granulosa and cumulus cells co-localised with the mitochondria, a localisation similar to that reported in other tissues [17-19]. The decline in *SIRT5* mRNA mirrored a decrease in granulosa cell SIRT5 protein desuccinylation activity in women with reduced ovarian reserve and in older women. This decrease in granulosa and cumulus cell *SIRT5* mRNA and protein desuccinylation activity suggests that cellular functions regulated by SIRT5 may be altered. Interestingly, these alterations in SIRT5 function were associated with changes in the follicular fluid ammonium concentration, which was increased in young women with reduced ovarian reserve and in women of advanced maternal age compared to young women with normal ovarian reserve (chapter 4). This accumulation of ammonium in the follicular fluid may increase follicular fluid pH. Oocytes are sensitive to changes in pH with increases in pH during the final stages of oocyte development leading to a reduction in oocyte fertilisation and a decline in embryo viability [20, 21]. Furthermore, previous animal studies have established that exposure to increased ammonium concentrations may lead to deceased cumulus cell viability, perturbed hormone production [22], decreased oocyte nuclear maturation rates, embryo cleavage rates, blastocyst development [23-26], and a reduction in fetal development after embryo transfer [23, 27].

This increase in follicular fluid ammonium concentration seen in young women with reduced ovarian reserve and in women of advanced maternal age is likely a result of SIRT5 protein desuccinylation activity on CPS1, which is the enzyme known to be involved in ammonium clearance [28]. A decline in granulosa and cumulus cell SIRT5 protein desuccinylation activity

may cause an accumulation of ammonium in the follicular fluid, via its actions on CPS1, which may result in altered granulosa and cumulus cell metabolism or steroidogenesis, thus altering follicular fluid concentration of carbohydrates and hormones, leading to the decline in oocyte viability (Figure 6.1). Furthermore, the observed granulosa and cumulus cell decreases in *SIRT5* mRNA and in SIRT5 protein desuccinylation may also have implications for other cellular functions, such as metabolism via the TCA cycle or ammonium clearance, that require SIRT5 regulation. One of the limitations of this study is the lack of causality, specifically the effect on oocyte development and competence. Thus, further studies on the inhibition of SIRT5 which results in granulosa and cumulus cells secreting more ammonium are warranted and the effect that this may have on the oocyte also requires further investigation. Further investigations assessing ammonium concentration of individual follicles and tracking the viability of the associated oocyte would be beneficial. Whilst technically challenging this would provide a more precise relationship between ammonium levels and oocyte viability as well as embryo development and pregnancy. Further, there is yet to be comprehensive analysis of the targets of SIRT5 and it maybe that there are additional targets which further alter the physiology of the ovarian cells affecting development and the follicle in ways not contemplated in this thesis. Although *SIRT5* knockout mice display no differences in global acetylation of mitochondrial proteins, they instead display global mitochondrial protein hypersuccinylation thus indicating that SIRT5 is the main mitochondrial protein desuccinylase in the mitochondria [29, 30]. Therefore, it would be valuable to further examine the role of SIRT5 in animal models such as a mouse model null for *SIRT5* in ovarian cells. This would enable the pathways related to SIRT5 to be directly established as well as establish the cellular and molecular consequences. Furthermore, SIRT5 not only has the ability to act as a protein desuccinylase but also as a protein demalonylase and a weak protein deacetylase and the potential roles that both of these actions have on proteins requires further investigation.

Having established that SIRT5 was differentially expressed in the ovarian cells of women ≥40 years and in women of reduced ovarian reserve, chapter 5 examined the role of SIRT3, another mitochondrial SIRT protein which contains the conserved catalytic core domain as SIRT5. In concordance with previous studies on different tissue types, within granulosa and cumulus cells SIRT3 protein was found to co-localise with the mitochondria [31-33]. Similar to SIRT5, *SIRT3* mRNA was decreased in young women with reduced ovarian reserve and in women of advanced maternal age, indicative of a down-regulation of SIRT3 function in these cell types. Granulosa cell SIRT3 protein deacetylation activity was decreased in young women with reduced ovarian reserve, and in women of advanced maternal age both granulosa and cumulus cell SIRT3 protein deacetylation activity was decreased is similar to what was found for SIRT5 (chapter 4). Thus, the decrease in SIRT3 mRNA and protein deacetylation activity may also affect cellular functions that require SIRT3 regulation, specifically mitochondrial metabolism.

It has previously been established that SIRT3 is able to deacetylate glutamate dehydrogenase (GDH) and therefore regulate mitochondrial metabolism [19]. A decline in SIRT3 protein deacetylation activity would result in an increase in acetylated GDH and thus its enzymatic role in mitochondrial metabolism would be diminished. The effect of SIRT3 on GDH in granulosa and cumulus cells has previously been discussed (chapter 5), however the observed decrease in GDH activity would result in a decrease in α-ketoglutarate, as GDH is the enzyme responsible for the conversion of glutamate to α-ketoglutarate. A decline in granulosa and cumulus cell SIRT3 protein deacetylation activity increases the amount of acetylated GDH, which reduces its activity. This reduction in GDH activity may impair the TCA cycle and as a consequence glycolysis and oxidative phosphorylation are altered, this in turn may serve as an explanation for the increased granulosa and cumulus cell lactate production and glucose uptake observed in Chapter 3. This

up-regulation of glycolysis by granulosa and cumulus cells may be the follicle's way of trying to compensate for decrease in TCA cycle activity and the decrease in energy production (Figure 6.1). Interestingly this type of plasticity and metabolic compensation has been reported previously in oocytes and embryos [34-38]. This altered follicle metabolism may be in part contributing to the decline in oocyte quality, as the metabolites in the follicular fluid are altered. Considering that both SIRT3 and SIRT5 have other targets such as succinate dehydrogenase, complex 1 of the electron transport chain, acetyl CoA sythetase 2, malate dehydrogenase and isocitrate dehydrogenase, which are all important for the production of ATP, their function on these enzymes warrants further investigation. Similar, to the comments regarding SIRT5, there are still many unknown targets of SIRT3 and therefore it remains that the alterations in SIRT3 levels and activity observed in this study may also affect many other pathways important for oocyte viability.

One of the key questions still to be answered is; are SIRT3 and SIRT5 also involved in the regulation of the oocyte and not just the supporting cells of the follicle? For example, α-ketoglutarate is involved in the TCA cycle, which is responsible for the production of energy within the oocyte, thus if SIRT3 alters the activity of GDH the production of energy within the oocyte is also likely impaired. Interestingly, previous animal studies have established that impairment of oocyte metabolism results in impaired oocyte nuclear and cytoplasmic maturation, embryo development and blastocyst formation [39-42]. Preliminary investigations into the presence of SIRT3 and SIRT5 protein determined that the proteins were found to be present in the oocyte (Appendix 5). However, studies are required to determine if the SIRT3 and SIRT5 proteins are altered in the oocyte and whether the changes in SIRT3 and SIRT5 as a result of ageing or reduction in ovarian reserve observed in granulosa and cumulus cells are also present in the oocyte.

It must also be acknowledged that the SIRT family consists of 7 members, where at least SIRT1, 2 and 6 all respond to changes in metabolic state in a similar fashion as SIRT3 and SIRT5 as they all contain a NAD^+ binding domain, which is conserved across mammals. While this study focussed on the mitochondrial based SIRTs it should be recognised that the other members of the SIRT family, which are usually cytoplasmic in location may also have a role in the reduced oocyte viability that we reported in Chapter 2 with ovarian ageing. To date, aside from SIRT1, there are no human studies on the role of the remaining SIRTs in granulosa or cumulus cell biology or the human oocyte.

Furthermore, the experimental paradigm that was investigated in this thesis was limited to ovarian ageing. However, there are other lifestyle and fertility aetiologies (such as PCOS and obesity) that involve changes to the metabolic state of the individual and also the follicle. Obesity is known to impair female fertility at least in part by actions directly on the oocyte [43, 44]. Interestingly, a previous studies in follicular fluid of obese women observed increased levels of the metabolites glucose and lactate compared to women with normal BMI, thus it is possible that the SIRTs may be exerting their effect in these women [45]. The SIRTs are responsive to metabolic state and have been shown to be altered in other tissues with obesity [46-49]. Therefore, it remains to be elucidated whether SIRT3 and SIRT5 are also involved in the reduction in oocyte viability in these other circumstances.

In conclusion, altered granulosa and cumulus cell SIRT3 protein deacetylation and SIRT5 protein desuccinylation activities may contribute to altered metabolic and steroidogenic function, which is reflected in the altered follicular environment. Due to the intimate relationship between this environment and the oocyte this decline in protein activity may be partially responsible for the

decline in oocyte developmental competence seen in young women with reduced ovarian reserve and in women of advanced maternal age. The results presented in this thesis have for the first time established a function for the Sirtuins in human granulosa and cumulus cells which maybe contributing the reduced oocyte viability and thus reduced pregnancy rates seen in women with reduced ovarian reserve and in women of advanced maternal age.

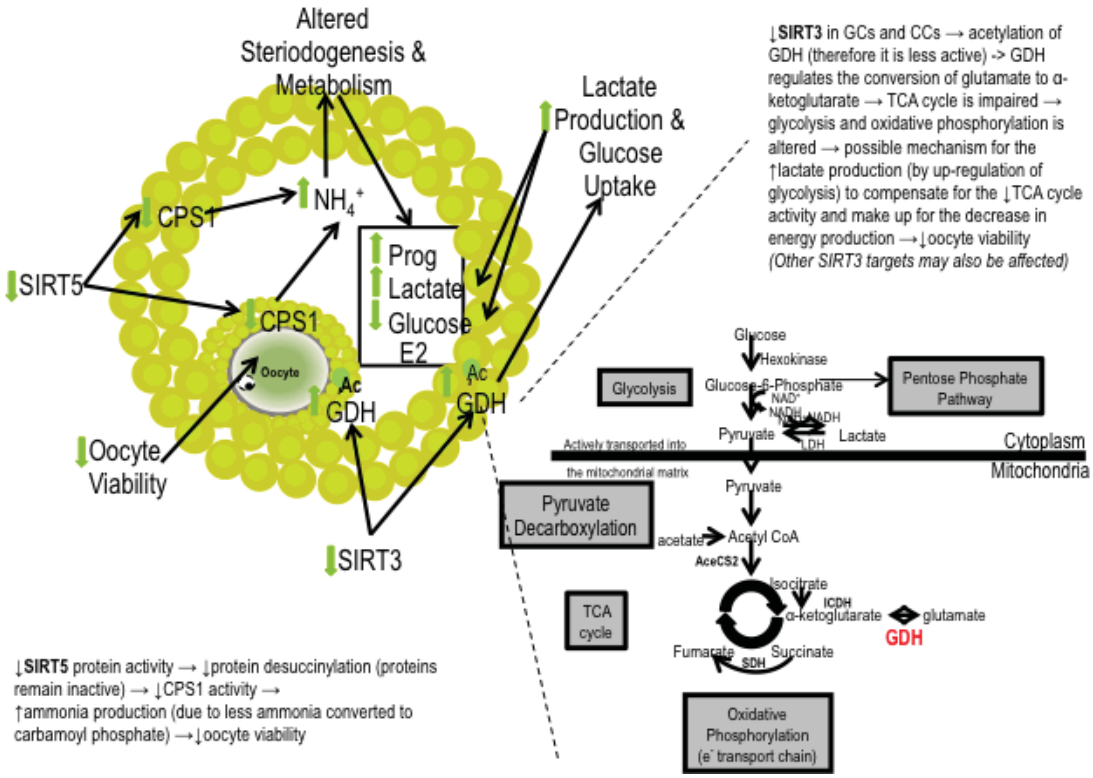


Figure 6.1: Working hypothesis for the role of granulosa and cumulus cell SIRT3 and SIRT 5 and the relationship with the changes in follicular fluid composition, metabolism and oocyte viability.

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Appendices

Appendix 1: Consent Form

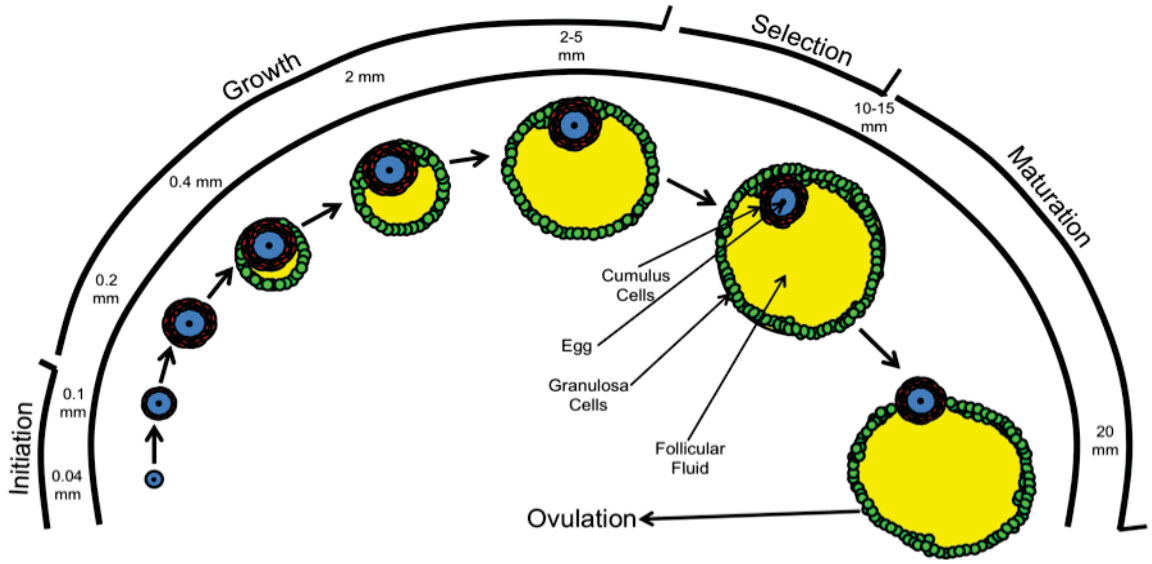
SAGE Study: Participant Information Sheet and Consent Form

Scientific Title: Oocyte-somatic cells interactions regulating embryo development in aging oocytes.

Lay Title: A study to investigate the aging of human eggs to improve pregnancy rates in older women.

Why do we want to do the study?

As women get older the pregnancy rates in their IVF cycles decreases. This is largely attributable to a progressive decline of egg quality and quantity. As eggs mature, specialized cells (cumulus cells) surrounding each egg also develop forming a unit called a cumulus-egg complex. Egg-cumulus interaction is very important to produce good quality eggs that are able to make embryos that develop to the blastocyst stage. Proteins produced and secreted by the egg help maintain the quality of the egg. It is important to gain better understanding of the mechanism and consequence of reproductive aging. By increasing our understanding of how human eggs interact with cumulus cells, we may be able to improve the viability of eggs that would be beneficial for treating older infertile women.



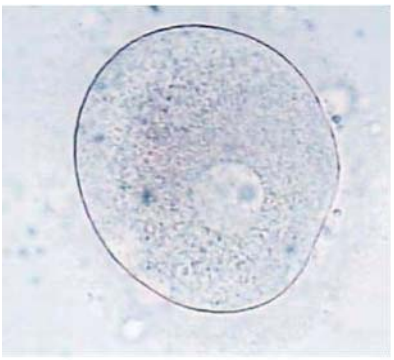
Who can take part?

If you are having eggs collected as a part of an IVF/ICSI cycle, you will be invited to join this study.

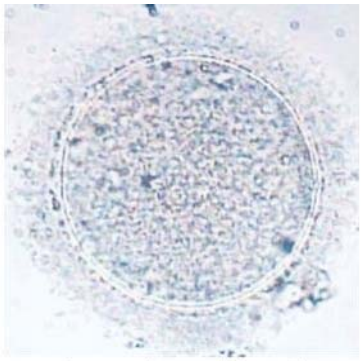
What happens during the study?

The decrease in fertility with female aging and diminished ovarian reserve is mainly due to a decrease in egg quality and quantity. This study will compare older and younger women with regard to low and normal ovarian reserves. The following surplus materials, which are routinely discarded as a part of IVF treatment, will be collected and used in this study:

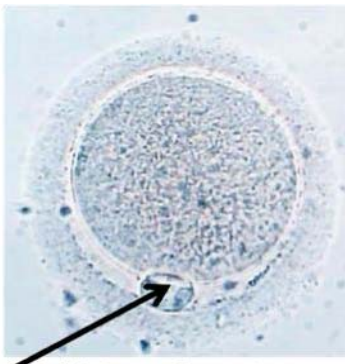
- **Follicular Fluid:** a by product if the egg retrieval, collected during egg collection and routinely discarded after pick up of the eggs from the follicular fluids.
- **Granulosa Cells:** tissues found in the follicular fluid and routinely discarded together with the follicular fluid.
- **Cumulus Cells:** cell routinely removed from the egg before fertilisation to assess the maturity of the egg and then discarded.
- **Immature Eggs:** these are not full-grown eggs, and are not able to be fertilised are form an embryo. They are normally discarded after the embryologist has assessed them as immature (see pictures below).
- **Unfertilised Eggs:** a mature egg, which failed to fertilise following IVF, and are then discarded.
- **Spent Culture Media:** nutrient solutions in which eggs/embryos are bathed, and normally disposed of after removing eggs/embryos from it.



Immature Oocyte
(GV Stage)



Immature Oocyte
(0 polar body)



Mature Oocyte
(1 polar body)

In this study, we will examine some of the changes occurring within the egg and will look at the interactions taking place between the egg and follicular cells using biochemical and metabolic measurements. We will also measure energy substrate usage of eggs and embryos by analysis of the spent culture media. This study will only access the fluids and cells that have completed their job of nurturing the egg and have been declared as surplus by the embryologist involved in your treatment. All of the surplus material will be discarded following the completion of this study and will not be used in any other research projects. This study will not interfere with the fertilisation or development of your embryos.

What are the risks and/or discomforts involved in the study?

This study will not cause any additional risks or discomforts other than those performed as routine aspects of your treatment. No additional tissue or cells will be removed at egg collection.

What happens if I do not want to take part?

Your participation is voluntary. Saying no will not affect your treatment in any way. You are free to change our mind at any time and ask to be removed from the study you had previously consented. If you withdraw from the study any sample collected from you will be discarded.

What will I get out of this study?

There will be no immediate benefits for you. However, we hope the knowledge we gain from this study will increase our understanding of egg development and improve our management of infertility problems.

What happens to the results?

Your identification number only will be used instead of you name to protect your privacy.

After the study is completed, all results will be pooled together to be analysed and published in a scientific journal. Your details will not be identifiable in any publication as a result of the study.

What if I have a question about the study?

If you are interested in this study or have any questions you are welcome to call the

- Principle Investigator, Dr. Tamer Hussein, on (08) 8333 8104

Assurances of Confidentiality

No identifying information about your medical history will be taken from Repromed. Your identity will be kept confidential, and nothing will be published which could reveal your identity. Your information will remain confidential except in the case of a legal requirement to pass on personal information to authorise third parties. This requirement is standard and applies to information collected both in research and non-research situations. Such requests to access information are rare, however we have an obligation to inform you of this possibility.

The Woman's and Children's Hospital Ethics Committee has approved this study.

Should you wish to speak to a person not directly involved, in particular in relation to matters concerning policies, information about the approval process, or your right as a participant, or have any concern or complaint, you may contact the

- Research Secretariat, Ms. Brenda Penny, on (08) 8161 6521.

Thank you for reading this information sheet.

SAGE Study: Participant Information Sheet and Consent Form

Scientific Title: Oocyte-somatic cells interactions regulating embryo development in aging oocytes.

Lay Title: A study to investigate the aging of human eggs to improve pregnancy rates in older women.

1. The nature and purpose of the research project described on the attached participation information sheet has been explained to me. I understand it, and agree to take part.
2. The privacy and confidentiality of any information I provided will be safeguarded as explained in the participation information sheet.
3. I understand that I may not directly benefit by taking part in this study.
4. I acknowledge that the possible risk and/or side effects, discomforts and inconveniences, as outlined in the participation information sheet, have been explained to me.
5. I understand that while information gained in the study may be published, I will not be identified and information will be confidential.
6. I understand I can withdraw from the study at any stage and that this will not affect medical care or any other aspects of my relationship with Repromed.
7. I understand that there will be no payment to me for taking part in this study.
8. I have had the opportunity to discuss taking part in this research project with a family member or friend and/or have had the opportunity to have a family member or friend present whilst the research project was being explained by the researcher.
9. I am aware that I should retain a signed copy of the consent form, when completed, and the participant information sheet.
10. I consent to samples of eggs (immature and unfertilised), follicular fluid, follicular cells and media being taken during my treatment cycle for use in the above project.

Signed:_____ Date:_____

Full name of patient:_____

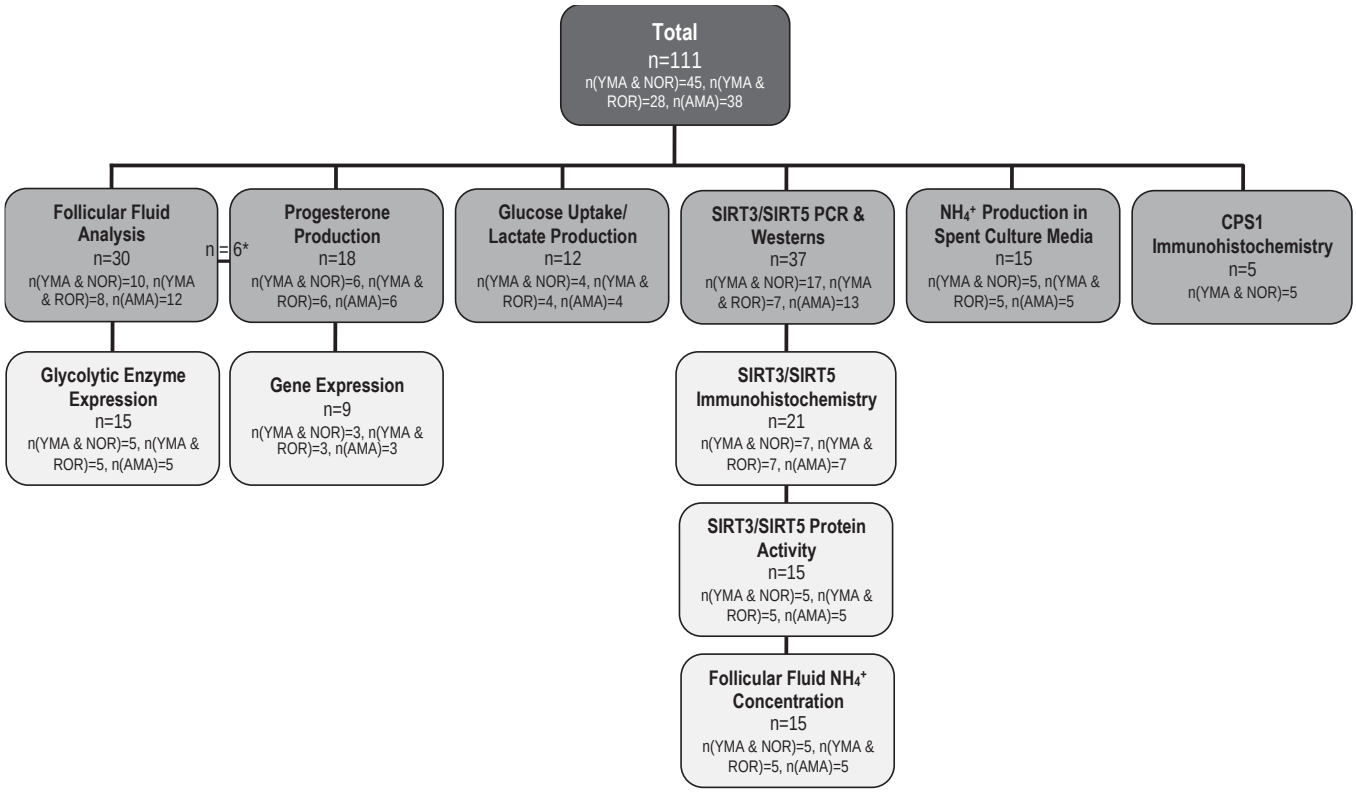
I certify that I have explained the study to the patient and consider that she understands what is involved.

Signed:_____ Date:_____

Full name of patient:_____

Title:_____

Appendix 2: Allocation of Patients to Experimental Protocols



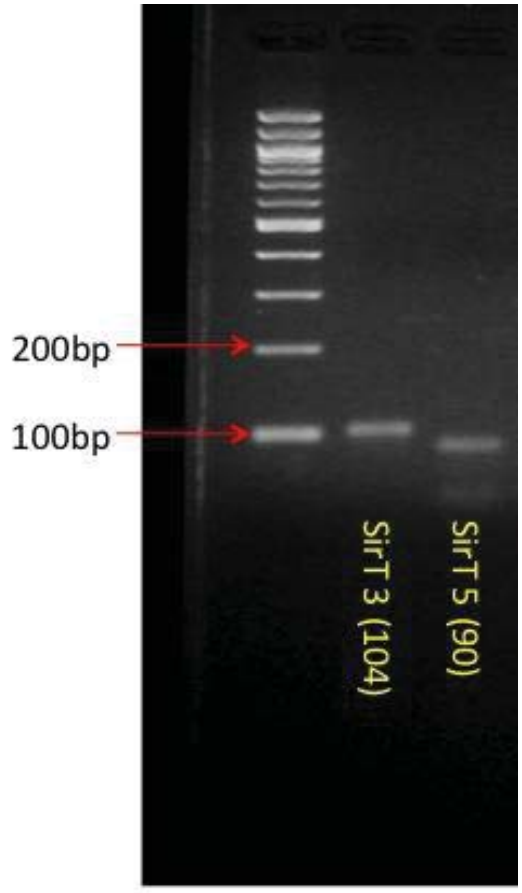
* 6 patients were randomly allocated to both the follicular fluid analysis and progesterone production experimental protocols

Appendix 3: Follicular Fluid Concentrations of Hormones and Metabolites from follicles of different sizes[1]

		Leading Follicle	2nd Follicle	2 Follicles Combined
Estradiol (pmol/L)	**Patient A**	1492.3±9.9[a]	1514.0±6.1[a]	1501.3±2.2[a]
	Patient B	1536.0±4.7[a]	1558.0±2.9[a]	1539.0±9.1[a]
	Patient C	1526.3±12.7[a]	1561.3±13.5[a]	1510.0±12.6[a]
Progesterone (nmol/L)	**Patient A**	3923.3±37.0	3795.3±81.4[a]	3961±12.1[a]
	Patient B	4190.0±40.6[a]	4368.7±130.9[a]	4228.0±150.4[a]
	Patient C	3541.0±58.7[a]	3404.3±76.9[a]	3269.3±147.8[a]
LH (IU/L)	**Patient A**	<1.0[a]	<1.0[a]	<1.0[a]
	Patient B	<1.0[a]	<1.0[a]	<1.0[a]
	Patient C	<1.0[a]	<1.0[a]	<1.0[a]
FSH (IU/L)	**Patient A**	11.7±0.1[a]	11.5±0.1[a]	11.3±0.1[a]
	Patient B	4.3±0.1[a]	4.4±0.0[a]	4.2±0.1[a]
	Patient C	4.6±0.1[a]	4.5±0.2[a]	4.9±0.1[a]

[1] n = 3 (3 aliquots of follicular fluid from each collection tube), data expressed as mean±SEM, a indicates no significant difference between cohorts (p>0.05).

Appendix 4: SIRT3 and SIRT5 Validation



Gel Electrophoresis of SIRT3 and SIRT5 Primers
Validation of the human *SIRT3* and *SIRT5* primers used for PCR, as demonstrated the *SIRT3* primer is 104bp and *SIRT5* is 90bp.

SIRT3

Chromosome 11, Location 11p15.5

Primers

SirT3 Primer (F)	t g c c c c a g a g g t t c t t g c t	19
SirT3 Primer (R)	c t c g g t c a a g c t g g c a a a a g	20
SirT3 Primer (long) (F)	c c a g g g c a t t c c g g a g g c a g	20
SirT3 Primer (long) (R)	c a g g c t c t g g c c c g a a t c a g c	21

Genomic Sequence

SirT3 Isoform A (long)	g c g a g t c c g g a g g a c t c c t t g g a c t g c g c g g a a c a t g g c g t t c t g g g g t t	50
>NM_012239 exon 1	g g c g c g c c g c g g c a g c c c t c c g g c t g t g g g g c c g g g t a g t t g a a c g g g t c	100
	g a g g c c g g g g g a g g c g t g g g g c c g t t t c a g g c c t g c g g c t g t c g g c t g g t	150
	g c t t g g c g g c a g g g a c g a t g t g a g t g c g g g g c t g a g a g g c a g c c a t g g g g	200
	c c c g c g g t g a g c c c t t g g a c c c g g c g c g c c c c t t g c a g a g g c c t c c c a g a	250
	c c c g a g g t g c c c a g g g c a t t c c g g a g g c a g c c g a g g g c a g c a g c t c c c a g	300
	t t t c t t c t t t t c g a g	315
exon 2	t a t t a a a g g t g g a a g a a g g t c c a t a t c t t t t t c t g t g g g t g c t t c a a g t g	365
	t t g t t g g a a g t g g a g g c a g c a g t g a c a a g g g g a a g c t t t c c c t g c a g g a t	415
	g t a g c t g a g c t g a t t c g g g c c a g a g c c t g c c a g a g g g t g g t g g t c a t g g t	465
	g g g g g c c g g c a t c a g c a c a c c c a g t g g c a t t c c a g a c t t c a g	507
exon 3	a t c g c c g g g g a g t g g c c t g t a c a g c a a c c t c c a g c a g t a c g a t c t c c c g t	557
	a c c c c g a g g c c a t t t t t g a a c t c c c a t t c t t c t t t c a c a a c c c c a a g c c c	607
	t t t t t c a c t t t g g c c a a g g a g c t g t a c c c t g g a a a c t a c a a g c c c a a c g t	657
	c a c t c a c t a c t t t c t c c g g c t g c t t c a t g a c a a g g g g c t g c t t c t g c g g c	707
	t c t a c a c g c a g a a c a t c g a t g g g c t t g a g a g a g	740
exon 4	t g t c g g g c a t c c c t g c c t c a a a g c t g g t t g a a g c t c a t g g a a c c t t t g c c	790
	t c t g c c a c c t g c a c a g t c t g c c a a a g a c c c t t c c c a g g g g a g g a c a t t c g	840
	g	841
exon 5	g c t g a c g t g a t g g c a g a c a g g g t t c c c c g c t g c c c g g t c t g c a c c g g c g t	891
	t g t g a a g c c c g a c a t t g t g t t c t t t g g g g a g c c g c t g c c c c a g a g g t t c t	941
	t g c t g c a t g t g g t t g a t t t c c c c a t g g c a g a t c t g c t g c t c a t c c t t g g g	991
	a c c t c c c t g g a g	1003
exon 6	g t g g a g c c t t t t g c c a g c t t g a c c g a g g c c g t g c g g a g c t c a g t t c c c c g	1053
	a c t g c t c a t c a a c c g g g a c t t g g t g g g g c c c t t g g c t t g g c a t c c t c g c a	1103
	g c a g g g a c g t g g c c c a g c t g g g g g a c g t g g t t c a c g g c g t g g a a a g c c t a	1153
	g t g g a g c t t c t g g g c t g g a c a g a a g a g a t g c g g g a c c t t g t g c a g c g g g a	1203
	a a c t g g g a a g	1213

exon 7	c t t g a t g g a c c a g a c a a a t a g g a t g a t g g c t g c c c c c a c a c a a t a a a t g g	1263
	t a a c a t a g g a g a c a t c c a c a t c c c a a t t c t g a c a a g a c c t c a t g c c t g a a	1313
	g a c a g c t t g g g c a g g t g a a a c c a g a a t a t g t g a a c t g a g t g g a c a c c c g a	1363
	g g c t g c c a c t g g a a t g t c t t c t c a g g c c a t g a g c t g c a g t g a c t g g t a g g	1413
	g c t g t g t t t a c a g t c a g g g c c a c c c c g t c a c a t a t a c a a a g g a g c t g c c t	1463
	g c c t g t t t g c t g t g t t g a a c t c t t c a c t c t g c t g a a g c t c c t a a t g g a a a	1513
	a a g c t t t c t t c t g a c t g t g a c c c t c t t g a a c t g a a t c a g a c c a a c t g g a a	1563
	t c c c a g a c c g a g t c t g c t t t c t g t g c c t a g t t g a a c g g c a a g c t c g g c a t	1613
	c t g t t g g t t a c a a g a t c c a g a c t t g g g c c g a g c g g t c c c c a g c c c t c t t c	1663
	a t g t t c c g a a g t g t a g t c t t g a g g c c c t g g t g c c g c a c t t c t a g c a t g t t	1713
	g g t c t c c t t t a g t g g g g c t a t t t t t a a t g a g a g a a a a t c t g t t c t t t c c a	1763
	g c a t g a a a t a c a t t t a g t c t c c t c a a a g g g a c t g c a g g t g t t g a c a t g a g	1813
	t t g g a a a g g g a a c c c t g g g a t a c g t g g c g t c c c c t c t a t t g g a a c a g t c t	1863
	g a g g a c t g a a g g c a t t t g t c c c t g g a t t t a t t g g a g a c g g c c c a g c t c c t	1913
	c c c t c t g a a g g t g g t c a c a t t c t g t t g a c t c t c c a t a c t c a g c c t c t c c t	1963
	c c a g a a a c a g a t c t g t t c c a g a a c a t t c c a g c a c t t t c t a t c t g g c c t c c	2013
	t t g t c c c c a c a c t a c g c c c c c c c a c c c t c g c c a g g g c t t c c t c t a g t g a c	2063
	a c t g t t a g a g c t a a t c t c t g a g a c a g g g a a g g c a t t a c t c a c t t a a a a c c	2113
	c a g g c t g a g t c c t g g c c a c c t g c t g g a t t g t g a c a t a g g a g g t g g a a t c c	2163
	a c t g a a c t g c t a c t t c t g c a c a g g c t c c t t c t c c t g g g g c t g t a c c c a g g	2203
	c c c a g c c c t g a t g g c t c a c c c t g t c a g g c a c c a g c t g c t c c c t c c t g g g c	2263
	t c t c a c c c a c c t g c a c a t c c t c c t t c c t a g c a t c a c a t t a c c t g c g t g t t	2303
	t c c c c a g a c a a a a g c a c t t c c c a t t c t t g a a c c t t g c c t a c c c t g g g c t g	2363
	a g c t g a c g g c a a t a g a t t t a a t g a c a g t g a c t c c c a g g a a g g g g g t c c t g	2403
	t g a c t t t g c g c c t t a a t a a g a a c a a a a a g g t g g a a t t g g t g a c c t a g g a a a	2463
	a c t g t t g a a t t c t a a a a a g a a t g a a g t t a g t t t c t a a c c c t a g t t a a t g t	2503
	t c c t t t t t t a t t t t t t g a g t c t t g c c c t g t c a c t c a g g g t g g a g t g c g g t	2563
	g t t a t g a t c t c a g c t c a c t g c a a c t t c c g c c t c c c c g g g t t t a a g c g a t t c	2603
	t c c t g g g t a g c t g g g a t t a c a g g t g t g t c c c a c c a c a c c t a g c a c a t g g g	2663
	c a t a t t t g t a a t a g a g a c a a g g t t t t g c t a t g t t g g c c a g g c t g g t c t c g	2703
	a a c t c c t g g c t t c a a g t g a t c c a c c c a c c t c g g c c t c c c a a a g t g c t g g g	2763
	a t t a c a g g c a t g a g c c a c t g t g c c t g g c c c c t t t a t t t g a t a a t t t a c a c	2803
	a t a c a t t t t t g t c c a a a a c t c t t c t t t a t t t c a a g a t g a t g t t t c t g t g g	2863
	c t a t g t g t g g t a t g t g g t a t a a a t c t c a a t c t a t g g t c	2901
SirT3 Isoform B (short)	g c g a g t c c g g a g g a c t c c t t g g a c t g c g c g g a a c a t g g c g t t c t g g g g t t	50
>NM_001017524 exon 1	g g c g c g c c g c g g c a g c c c t c c g g c t g t g g g g c c g g g t a g t t g a a c g g g t c	100
	g a g g c c g g g g g a g g c g t g g g g c c g t t t c a g g c c t g c g g c t g t c g g c t g g t	150
	g c t t g g c g g c a g g g a c g a t	169


exon 2
t a t t a a aggtg g a a g a a g g t c c a t a t c t t t t t c t g t g g g t g c t t c a a g t g 219
t t g t t g g a a g t g g a g g c a g c a g t g a c a a g g g g a a g c t t t c c c t g c a g g a t 269
g t a g c t g a g c t g a t t c g g g c c a g a g c c t g c c a g a g g g t g g t g g t c a t g g t 319
g g g g g c c g g c a t c a g c a c a c c c a g t g g c a t t c c a g a c t t c a g 361
exon 3
a t c g c c g g g g a g t g g c c t g t a c a g c a a c c t c c a g c a g t a c g a t c t c c c g t 411
a c c c c g a g g c c a t t t t t g a a c t c c c a t t c t t c t t t c a c a a c c c c a a g c c c 465
t t t t t c a c t t t g g c c a a g g a g c t g t a c c c t g g a a a c t a c a a g c c c a a c g t 511
c a c t c a c t a c t t t c t c c g g c t g c t t c a t g a c a a g g g g c t g c t t c t g c g g c 561
t c t a c a c g c a g a a c a t c g a t g g g c t t g a g a g a g 594
exon 4
t g t c g g g c a t c c c t g c c t c a a a g c t g g t t g a a g c t c a t g g a a c c t t t g c c 644
t c t g c c a c c t g c a c a g t c t g c c a a a g a c c c t t c c c a g g g g a g g a c a t t c g 694
g 695
exon 5
g c t g a c g t g a t g g c a g a c a g g g t t c c c c g c t g c c c g g t c t g c a c c g g c g t 745
t g t g a a g c c c g a c a t t g t g t t c t t t g g g g a g c c g c t g c c c c a g a g g t t c t 795
t g c t g c a t g t g g t t g a t t t c c c c a t g g c a g a t c t g c t g c t c a t c c t t g g g 845
a c c t c c c t g g a g 855
exon 6
g t g g a g c c t t t t g c c a g c t t g a c c g a g g c c g t g c g g a g c t c a g t t c c c c g 907
a c t g c t c a t c a a c c g g g a c t t g g t g g g g c c c t t g g c t t g g c a t c c t c g c a 957
g c a g g g a c g t g g c c c a g c t g g g g g a c g t g g t t c a c g g c g t g g a a a g c c t a 1007
g t g g a g c t t c t g g g c t g g a c a g a a g a g a t g c g g g a c c t t g t g c a g c g g g a 1057
a a c t g g g a a g 1067
exon 7
c t t g a t g g a c c a g a c a a a t a g g a t g a t g g c t g c c c c c a c a c a a t a a a t g g 1117
t a a c a t a g g a g a c a t c c a c a t c c c a a t t c t g a c a a g a c c t c a t g c c t g a a 1167
g a c a g c t t g g g c a g g t g a a a c c a g a a t a t g t g a a c t g a g t g g a c a c c c g a 1217
g g c t g c c a c t g g a a t g t c t t c t c a g g c c a t g a g c t g c a g t g a c t g g t a g g 1267
g c t g t g t t t a c a g t c a g g g c c a c c c c g t c a c a t a t a c a a a g g a g c t g c c t 1317
g c c t g t t t g c t g t g t t g a a c t c t t c a c t c t g c t g a a g c t c c t a a t g g a a a 1367
a a g c t t t c t t c t g a c t g t g a c c c t c t t g a a c t g a a t c a g a c c a a c t g g a a 1417
t c c c a g a c c g a g t c t g c t t t c t g t g c c t a g t t g a a c g g c a a g c t c g g c a t 1467
c t g t t g g t t a c a a g a t c c a g a c t t g g g c c g a g c g g t c c c c a g c c c t c t t c 1517
a t g t t c c g a a g t g t a g t c t t g a g g c c c t g g t g c c g c a c t t c t a g c a t g t t 1567
g g t c t c c t t t a g t g g g g c t a t t t t t a a t g a g a g a a a a t c t g t t c t t t c c a 1617
g c a t g a a a t a c a t t t a g t c t c c t c a a a g g g a c t g c a g g t g t t g a c a t g a g 1667
t t g g a a a g g g a a c c c t g g g a t a c g t g g c g t c c c c t c t a t t g g a a c a g t c t 1717
g a g g a c t g a a g g c a t t t g t c c c t g g a t t t a t t g g a g a c g g c c c a g c t c c t 1767

```
c c c t c t g a a g g t g g t c a c a t t c t g t t g a c t c t c c a t a c t c a g c c t c t c c t   1817
c c a g a a a c a g a t c t g t t c c a g a a c a t t c c a g c a c t t t c t a t c t g g c c t c c   1867
t t g t c c c c a c a c t a c g c c c c c c c c a c c c t c g c c a g g g c t t c c t c t a g t g a c   1917
a c t g t t a g a g c t a a t c t c t g a g a c a g g g a a g g c a t t a c t c a c t t a a a a a c c   1967
c a g g c t g a g t c c t g g c c a c c t g c t g g a t t g t g a c a t a g g a g g t g g a a t c c   2017
a c t g a a c t g c t a c t t c t g c a c a g g c t c c t t c t c c t g g g g c t g t a c c c a g g   2067
c c c a g c c c t g a t g g c t c a c c c t g t c a g g c a c c a g c t g c t c c c t c c t g g g c   2117
t c t c a c c c a c c t g c a c a t c c t c c t t c c t a g c a t c a c a t t a c c t g c g t g t t   2167
t c c c c a g a c a a a a g c a c t t c c c a t t c t t g a a c c t t g c c t a c c c t g g g c t g   2217
a g c t g a c g g c a a t a g a t t t a a t g a c a g t g a c t c c c a g g a a g g g g g t c c t g   2267
t g a c t t t g c g c c t t a a t a a g a a c a a a a a g g t g g a a t t g g t g a c c t a g g a a a   2317
a c t g t t g a a t t c t a a a a a g a a t g a a g t t a g t t t c t a a c c c t a g t t a a t g t   2367
t c c t t t t t t t a t t t t t t g a g t c t t g c c c t g t c a c t c a g g g t g g a g t g c g g t   2417
g t t a t g a t c t c a g c t c a c t g c a a c t t c c g c c t c c c c g g g t t t a a g c g a t t c   2467
t c c t g g g t a g c t g g g a t t a c a g g t g t g t c c c a c c a c a c c t a g c a c a t g g g   2517
c a t a t t t g t a a t a g a g a c a a g g t t t t g c t a t g t t g g c c a g g c t g g t c t c g   2567
a a c t c c t g g c t t c a a g t g a t c c a c c c a c c t c g g c c t c c c a a a g t g c t g g g   2617
a t t a c a g g c a t g a g c c a c t g t g c c t g g c c c c t t t a t t t g a t a a t t t a c a c   2667
a t a c a t t t t t g t c c a a a a c t c t t c t t t a t t t c a a g a t g a t g t t t c t g t g g   2717
c t a t g t g t g g t a t g t g g t a t a a a t c t c a a t c t a t g g t c                           2755
```

SIRT5

Chromosome 6, Location 6p23

Isoform 1 (A)
N terminus → C terminus
length=310

```
mrplqivpsrlisqlycgl kppastrnqi clkmarpsss madfrkf fakakhiviisgag  60
vsaesgvptfrgaggywrk wqaqdlatpl afahnpsrvwefyhyrrevmgskepnaghra  120
iaecetrlgkqgrrvvvit qnidelhrkagtknlleihgslfktrctscgvvaenykspi  180
cpalsgkgapepgtqdasi pvekl prceeagcg gllrphvvwfgenldpaileevdrela  240
hcdlclvvgtssvvypaamf apqvaargvpvae fntettpatnrf rfahqgpcgttlpea  300
lachenetvs                                                      310
```

Isoform 2 (B)
N terminus → C terminus
length=299

```
mrplqivpsrlisqlycgl kppastrnqi clkmarpsss madfrkf fakakhiviisgag  60
vsaesgvptfrgaggywrk wqaqdlatpl afahnpsrvwefyhyrrevmgskepnaghra  120
iaecetrlgkqgrrvvvit qnidelhrkagtknlleihgslfktrctscgvvaenykspi  180
cpalsgkgapepgtqdasi pvekl prceeagcg gllrphvvwfgenldpaileevdrela  240
hcdlclvvgtssvvypaamf apqvaargvpvae fntettpatnrf shlisiddliiikn  299
```

Isoform 3 (C)
N terminus → C terminus
length=292

```
mrplqivpsrlisqlycgl kppastrnqi clkmarpsss madfrkf fakakhiviisgag  60
vsaesgvptfrgaggywrk wqaqdlatpl afahnpsrvwefyhyrrevmgskepnaghra  120
iaecetrlgkqgrrvvvit qnidelhrkagtknlleihgslfktrctscgvvaenykspi  180
cpalsgkg                  ceeagcg gllrphvvwfgenldpaileevdrela  222
hcdlclvvgtssvvypaamf apqvaargvpvae fntettpatnrfrf hf qgpcgttlpea  282
lachenetvs                                                      292
```

SirT 5 Antibody
(ab13697) 36kDa
sythetic peptide corresponding to amino acids 30-46 of Human SirT5 (abcam)

```
clkmarpsss madfrkf
```

Blocking Peptide

```
                  pepgtqdasi pvekl prceeagcg gllrphvvwfgenldpaileevdrela
hcdlclvvgtssvvypaamf apqvaargvpvae fntettpatnrfrfahqgpcgttlpea
lachenetvs
```

sythetic blocking peptide corresponding to amino acids 190-310 of Human SirT5 of ISOFORM 1 (abgent)

Recombinant SirT5
Abnova (H00023408-P01)
60.17kDa

```
mrplqivpsrlisqlycgl kppastrnqiclkmarpsss madfrkf fakakhiviisgag
vsaesgvptfrgaggywrk wqaqdlatpl afahnpsrvwefyhyrrevmgskepnaghra
iaecetrlgkqgrrvvvit qnidelhrkagtknlleihgslfktrctscgvvaenykspi
cpalsgkgapepgtqdasi pvekl prceeagcg gllrphvvwfgenldpaileevdrela
hcdlclvvgtssvvypaamf apqvaargvpvae fntettpatnrfrfahqgpcgttlpea
lachenetvs
```

full-length recombinant protien with GST tag corresponding to amino acids 1-310 of Human SirT5 of LONG ISOFORM (abnova)

Primers

SirT5 Primer (F)	a a a g c a g c c g t g g a g a c a a c	20
SirT5 Primer (R)	t c g c a t c a g g g t t t g t c t g t a g	22
SirT5 Primer (isoform 1) (F)	g c a t g t g c g g g g c c c a a g t a	20
SirT5 Primer (isoform 2) (F)	g c g c g a g c t g c c c c a g t a a a t g	22
SirT5 Primer (isoform 1 and 2) (R	t g g t t g t c t c c a c g g c t g c t	20

Genomic Sequence

SirT5 Isoform A (1)

>NM_012241 exon 1	c c g g a g c g c g g t c g g g a c a c a g c g c c t c t a g g a g a a a g c c t g g a a g g c g c	50
	t c c g g g g g t a c c c a g a g c t c t t a g c g g g c c g g c a g c a t g t g c g g g g c c c a	100
	a g	102
exon 2	t a a a t g g a a a t g t t t t c t a a c a t a t a a a a a c c t a c a g a a g a a g a a a a t a a	152
	t t t t c t g g a t c a a a t t a g a a g t c t g t a t t a t a t t g a t g t c t c c a g a t t c a	202
	a a t a t a t t a g a a a g c a g c c g t g g a g a c a a c c a t c t t c a t t t g g g a g a a a	252
	t a a c t a a a g	261
exon 3	c c c g c c t c a a g c a t t a g a a c t a c a g a c a a a c c c t g a t g c g a c c t c t c c a g	311
	a t t g t c c c a a g t c g a t t g a t t t c c c a g c t a t a t t g t g g c c t g a a g c c t c c	361
	a g c g t c c a c a c g a a a c c a g a t t t g c c t g a a a a t g g c t c g g c c a a g t t c a a	411
exon 4	g t a t g g c a g a t t t t c g a a a g t t t t t t g c a a a a g c a a a g c a c a t a g t c a t c	461
	a t c t c a g g a g c t g g t g t t a g t g c a g a a a g t g g t g t t c c g a c c t t c a g a g g	511
	a g c t g g a g g t t a t t g g a g a a a a t g g c a a g c c c a g	545
exon 5	g a c c t g g c g a c t c c c c t g g c c t t t g c c c a c a a c c c g t c c c g g g t g t g g g a	595
	g t t c t a c c a c t a c c g g c g g g a g g t c a t g g g g a g c a a g g a g c c c a a c g c c g	645
	g g c a c c g c g c c a t a g c c g a g t g t g a g a c c c g g c t g g g c a a g c a g g g c c g g	695
	c g a g t c g t g g t c a t c a c c c a g a a c a t c g a t g a g c t g c a c c g c a a g g c t g g	745
	c a c c a a g a a c c t t c t g g a g a t c c a t g	771
exon 6	g t a g c t t a t t t a a a a c t c g a t g t a c c t c t t g t g g a g t t g t g g c t g a g a a t	821
	t a c a a g a g t c c a a t t t g t c c a g c t t t a t c a g g a a a a g g	859
exon 7	t g c t c c a g a a c c t g g a a c t c a a g a t g c c a g c a t c c c a g t t g a g a a a c t t c	909
	c c c g	913

exon 8	g t g t g a a g a g g c a g g c t g c g g g g g c t t g c t g c g a c c t c a c g t c g t g t g g t	963
	t t g g a g a a a a c c t g g a t c c t g c c a t t c t g g a g g a g g t t g a c a g a g a g c t c	1013
	g c c c a c t g t g a t t t a t g t c t a g t g	1037
exon 9	g t g g g c a c t t c c t c t g t g g t g t a c c c a g c a g c c a t g t t t g c c c c c c a g g t	1087
	g g c t g c c a g g g g c g t g c c a g t g g c t g a a t t t a a c a c g g a g a c c a c c c c a g	1137
	c t a c g a a c a g a t t c a g	1153
exon 10	g t t t c a t t t c c a g g g a c c c t g t g g a a c g a c t c t t c c t g a a g c c c t t g c c t	1203
	g t c a t g a a a a t g a a a c t g t t t c t t a a g t g t c c t g g g g a a g a a a g a a a t t a	1253
	c a g t a t a t c t a a g a a c t a g g c c a c a c g c a g a g g a g a a a t g g t c t t a t g g g	1303
	t g g t g a g c t g a g t a c t g a a c a a t c t a a a a a t a g c c t c t g a t t c c c t c g c t	1353
	g g a a t c c a a c c t g t t g a t a a g t g a t g g g g g t t t a g a a g t a g c a a a g a g c a	1403
	c c c a c a t t c a a a a g t c a c a g a a c t g g a a a g t t a a g g c a t a t t a t t t g g t t	1453
	t g a a c t g a a a c g t g a g g t a t c t t t g a t g t g t a t g g t t g g t t a t t g g g a g g	1503
	g a a a a a t t t t g t a a a t t a g a t t g t c t a a a a a a a a t a g t t a t t c t g a t t a t	1553
	a t t t t t g t t a t c t g g g c a a a g t a g a a g t c a a g g g g t a a a a a c c c t a c t a t	1603
	t c t g a t t t t t g c a c a a g t t t t a g t g g a a a a t a a a a t c a c a c t c t a c a g t a	1653
	g g t a a t t t a t t g t a t a a a g a c a t t a c c c c a c g a t a t g g c t t t a t t a g g g a	1703
	c t t t t t t t t t t t t t t t t t t t g a g a c a g a g t t t c a c t c t t g t t g c c c a g g c t g	1753
	g a g t g c a g t g g t g c g a t c t c a g c t c a c a g c a a c c t c c g c c t c c c g g g t t c	1803
	a a g a g a t t c t c c t g c c t c a g c c t c a t g a g t a g c t g g g a t t a c a g g t a t g t	1853
	a c c a c c a c a c c c a g c t a a t t t t g t a t t t t t a g t a g a g a c g g g g t t t c t c c	1903
	a t g t t g g t c a g g c t g g t c t t a a a c t c t c g a c c t c a g g t g a t c t g c c c g c c	1953
	t c g g c c t c c c a a a g t g c t g a g a t t a c g g g c a t g a g c c a c c g c a c c c g g c t	2003
	t a c t g g g g g c t t t t t a a c c t t g t t t g g c t a c a t t a c c t c a g t g a a c a a g g	2053
	g g a a g c t c a c a a c a g g a a c t c a c a c a a a g a a g g a a g a g a a c a g t a c c a a c	2103
	a a g g t a g a a c a t c a c g a t g a g a a g a a a g a a t g a t g c a a a t a t g t g g a c c c	2153
	c c a g g a t a a a c a c a g c t g c c c a a a a t t t a g c t c t g g c c c c c t t a g a t g g g	2203
	g g a a g t c a a c a g t g a a g t g t t t c t c a g c a t c a t a a a g g c a g a a g c t g t g g	2253
	t t c c t g t g g g g c c t g c c a a c c g t t c c c a g a t g c t g a a c t c t g c t g g g a g t	2303
	t t t t t c c a t g g g a c t t t a a a a a a t g a t g c c c t t a g g t t g g g c c a g a c c t c	2353
	t g t t a a c t t c a g t a g g g a t g g c a c c a g g t t c a a g a g g c c a a a g a a g a g a c	2403
	c t g g a g c t a g t g a a g g a a a c a t a g g g t t t a t t t g g g g a a c c t t a c a g g g t	2453
	g g t c c a g t g g c c g c g g g c t g g a c a g a a c t g c a a c c a c t t a t a a a a a g c a t	2503
	g c a g t t t a c a t a g c a c t t t c a c t c a g c a c c c t c c c c t c a g c a g c c t c c a c	2553
	g t g g c a a c c c t c a c t t c t t a a g t t a t t g c t g t c a g a t g c a t c t g c c a t a c	2603
	a g g g t c a t t c t c a g g g g a t g c t t a a g t t a t t t c t g t c a g g t a c a t c t t c c	2653
	a t a c a c t t t a c t a c c t t g g a g t a a a g t a g t a a g a a t a c a g c t t t t t c c t t	2703

```
a a c c t t t a c c a g c t a a c t c a g t g c t t a g g g g c c t t g g a a t g c c t g c t g t c   2753
c a g c a g g t g t c a c a g g c c t g a c t g g g a g g c a t g g c c a t t a t c a a c a g t g t   2803
a t g a a g g t c a c a t a t g g c t c c c t g a a g t g a t t a c a t a c t t g g a c c a c a t c   2853
a c c t c a g c c c c c t t g c a a a a a t t g c a t t t a a t g t t a c a c c c t t g g c t t t t g t   2903
a g a a a c a g g c a a c a a g a c a c t a t c a t a t a a a a c t t t g t a c t g c a t t g c a a   2953
g g c a t a a c c t t t a a t a a a t c c c a g t g g t c c t t t g t g t a g g g a a c g g g g a t   3003
g c t c a t a g c c t a t g g g g c g g c t g g a g a a c c a g t c a g g g a c c c t g a g g g c t   3053
c g a t g t a c a c t t t a c a t g g g t g g g c c a a g g a g t t t a c a c t g a g g g c a c t g   3103
g t a a t a c c t g t a a t a g t c t t a t a g t a c t t a t a a a g c a g t t t t g c a c a t a a   3153
a a t a c c a t c a t g c a c t t a t a a g t t t g t t c t t g g g c t g c t c c a a g t t a a c t   3203
t t a t t c a t t t t c c t a g t g t t a g t g t c t c a g g g a g t c t g a t t a t a t t t t t g   3253
a t t t g t a a t t t c t a t c t g a c t a a g g c c t a g a g a t t t c a a a a c t g t t c t t t t   3303
t t g t t t t c a a g t t t t a t c a t t t t t g t t c c t a t t t g g t t t t g t c g t t t t t a   3353
a a t t g a g a a t t g c t t c t a a a a c a g a a g a c a t g a a a a g a g a a t t a a a a a t a   3403
c a a t a t a t g t g t a a g a t a g a a t t a t t c a a c t t a g c a t t t a t t a a a c a t c t   3453
a c c a g a t g a t a g a c a t t a g a a a t a a a a t g a c t a g c a a g a c c t g g c c c t t c   3503
c c c t c a g g g g t t c a c a g a a t g g c t g g a g c a a c t g t c a t a t a a g c t c t t a t   3553
g a a g t g c a g a a a c t a c a c a g a c a t t t g t g c g g g t t c a g a c c a g c g c a a t t   3603
a g a g a t g g g c a g a g t g g g a t g g g g t a g g g g t a a g g t g c t t g a a a t t t g c c   3653
t g g g t g g g a a t t t t t g a a g t a t a a a a a a g g a c t t c t a c c t g g g g g g c t g c g   3703
g c a g t a g a g g g a a g a g c a c g g t c g g t a c a a a t g c a t g g c a g g t g t g a a a c   3753
a g t t t g a t t t g t t c a a a g a a t g a t g a a t c a c t t a g t g t t g t a t a a t g g a t   3803
g g g a g g a g a g a a a c a c a g c g a t c a c a a a g g g c c a t g t t t g c c a a g a a a t a   3853
a a a t a t a c t t g g                                                                         3865
```

SirT5 Isoform B (2)

exon 1

```
t g g g a g g g a g g c a c c c c g g g g g g c g g g g c g t g g g a g a c t g t a t t c g g g g g   50
c g c g a g c t g c c c c a g                                                                    65
```

exon 2

```
t a a a t g g a a a t g t t t t c t a a c a t a t a a a a a c c t a c a g a a g a a g a a a a t a a   115
t t t t c t g g a t c a a a t t a g a a g t c t g t a t t a t a t t g a t g t c t c c a g a t t c a   165
a a t a t a t t a g a a a g c a g c c g t g g a g a c a a c c a t c t t c a t t t t g g g a g a a a   215
t a a c t a a a g                                                                              224
```

exon 3

```
c c c g c c t c a a g c a t t a g a a c t a c a g a c a a a c c c t g a t g c g a c c t c t c c a g   274
a t t g t c c c a a g t c g a t t g a t t t c c c a g c t a t a t t g t g g c c t g a a g c c t c c   324
a g c g t c c a c a c g a a a c c a g a t t t g c c t g a a a a t g g c t c g g c c a a g t t c a a   374
```

exon 4	g t a t g g c a g a t t t t c g a a a g t t t t t t g c a a a a g c a a a g c a c a t a g t c a t c	424
	a t c t c a g g a g c t g g t g t t a g t g c a g a a a g t g g t g t t c c g a c c t t c a g a g g	474
	a g c t g g a g g t t a t t g g a g a a a a t g g c a a g c c c a g	508
exon 5	g a c c t g g c g a c t c c c c t g g c c t t t g c c c a c a a c c c g t c c c g g g t g t g g g a	558
	g t t c t a c c a c t a c c g g c g g g a g g t c a t g g g g a g c a a g g a g c c c a a c g c c g	608
	g g c a c c g c g c c a t a g c c g a g t g t g a g a c c c g g c t g g g c a a g c a g g g c c g g	658
	c g a g t c g t g g t c a t c a c c c a g a a c a t c g a t g a g c t g c a c c g c a a g g c t g g	708
	c a c c a a g a a c c t t c t g g a g a t c c a t g	734
exon 6	g t a g c t t a t t t a a a a c t c g a t g t a c c t c t t g t g g a g t t g t g g c t g a g a a t	784
	t a c a a g a g t c c a a t t t g t c c a g c t t t a t c a g g a a a a g g	822
exon 7	t g c t c c a g a a c c t g g a a c t c a a g a t g c c a g c a t c c c a g t t g a g a a a c t t c	872
	c c c g	876
exon 8	g t g t g a a g a g g c a g g c a g c g g g g g c t t g c t g c g a c c t c a c g t c g t g t g g t	926
	t t g g a g a a a a c c t g g a t c c t g c c a t t c t g g a g g a g g t t g a c a g a g a g c t c	956
	g c c c a c t g t g a t t t a t g t c t a g t g	980
exon 9	g t g g g c a c t t c c t c t g t g g t g t a c c c a g c a g c c a t g t t t g c c c c c c a g g t	1030
	g g c t g c c a g g g g c g t g c c a g t g g c t g a a t t t a a c a c g g a g a c c a c c c c a g	1080
	c t a c g a a c a g a t t c a g	1096
exon 10	t c a t t t g a t c t c c a t c t c a t c t c t a a t t a t t a t a a a g a a t t a a a a c a a g t	1146
	c a t c a t t g t a g a a a a g c a a g a a a a t g c a g a t a g a g a a a a a g a a g a a a a t a	1196
	a a a c t g g a g t a t t t c c a c a a c c c a a g t t t a g a g t t g g c c c c c c a c c t c c c a	1246
	t g c c a t g g a c t g a g c a g c a g g g g c c c a g c a t c c c t t g g a t a t g g t g g c t g	1296
	t g t c t t c a t g t g a a a g a a a c t g a a c t t g g t g g t t t t t c c t g c c a g t t c a g	1346
	g a g a g a t t c t t g g c a t g t a a t a t a t a t c a c t g c t c a a g t c a a g c c t c c t a	1396
	a a a c c a c a g a c c t g t t t c a g c t g c t a c t t c a g c c a a a a t t c t t c a g c t t c	1546
	a t a t t g t c t t g a a a a c c t a t g a t t g t c t c t a a c a a a c a g g c t a c t t g c t a	1596
	g t t a g a a a t t c t t a t c a a t t t g g c a a g c t a c t t a t c a a c c a g a c t g a c c a	1646
	c a a g a a c t g t c a t c t c a t c a a t g a a g g a g t a a c t g a t c a a t g a a g c c a g c	1656
	a a t g c t t t t t t c t t g g c a t c a t c a a a g c t g a c a t t t a g a a g a g a t g c t g g	1746
	t g a t a g t c a t c t c a t c c t a c t c a a t t t t t c a a a g g c a g a a a c c a a c c c t g	1796
	g a g c a a t t g a g a g g a c t g t t t a a a c a c a g a g c t t a a c a a t g g c a g a a t t g	1846
	t a t a t c t c g t g c t t a a c a g a t t t t g g t t g a a c t t t a c c c t a g g t c a g g g g	1896
	t c a g c a a a c t a c t g c c t g t g g g c c a a a t t t g c c c a c c a c c t g t a t c t g t a	1946

```
a at aaggttt catt ggaac acagct gtggc cat at gttt gtatatt gt gt    1996
g t g g c t g c t t t t g c a t t a g g a t g a c a g a g g t g a a t a g t t g c a a c a g a g a c    2046
t g g c t g g t c t g c a a a g c c t a a a a t a t g t c c t g t g t g g c c c t t t a c a g a a a    2096
a a g t t t t c t a a c c c c t g c t c t a g g t t a c g g a g a a a a a a a a a a t g g a a t a a    2146
t g t t c t c t g c t a c t t t t a a c c t g a t t t t c t t t g t t a c c t a a a t a g g c a g c    2196
t a g a a t g c t g c c t a t a t t t t a a t a a g g a t t t g g a t c t c a c a a g a c a c c t t    2246
a g g c c t t a c a c a a g t t g t t c a g a t t c t t t g c c c c a g t t c t a a t c t a g t g a    2296
c a a a g g c a t a g a a t t c t c c t c c c a c a g g a a t g t a t t t c t a t t t t c a a g g t    2346
g t t a a t t a g t t c c a g t t t t t g g t t t t t g t c g t t t t c c c c a t g t c c g a t g c t t    2396
a t a t t g g a t g a t t t c t g a t a a a a c c c t g a c t a t t c c a a t a a a c c c t a g g c a    2446
t t t t t g a a t t t                                                2457
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Appendix 5: SIRT3 and SIRT5 in Oocytes

SIRT3 and SIRT5 Oocyte Immunohistochemistry

Materials and Methods

SIRT3 and SIRT5 Oocyte Immunohistochemistry

Fixed human oocytes (immature and unfertilised) were examined for SIRT3 and SIRT5 protein localisation. After collection the oocytes were fixed in 4% paraformaldehyde and then incubated with 0.1M glycine *(Sigma-Aldrich)* in Dulbecco's PBS for 5 mins. Blocking was performed using 5% donkey serum (*Sigma-Aldrich)* for 2 hrs and then 5% goat serum *(Sigma-Aldrich)* for 2 hrs. Incubation of the primary antibody SIRT3 (*Abcam)* or SIRT5 *(Abcam)* (Table 1) was done for 2 hrs at 37°C in a 1:50 dilution. To determine if SIRT3 and SIRT5 localised to the mitochondria co-immunohistochemistry was performed with the mitochondrial protein Cytochrome C (Table 1, *[Abcam]*). The samples were incubated in the secondary antibodies Fluorescein (FITC)-conjugated AffiniPure Donkey Anti-Rabbit *(Jackson ImmunoResearch Laboratories)* and Rhodamine (TRITC)-conjugated AffiniPure Goat Anti-Mouse *(Jackson ImmunoResearch Laboratories)* 1:100 for 2hrs at 37°C (Table 1). Nuclear staining was performed using 3µM 4,6-Diamidino-2-phenyindole, dilactate (DAPI) *(Sigma-Aldrich)* and was applied for 1-2 mins. The oocytes were loaded onto a slide using confocal loading media and imaged using the Leica TCS SP5 Spectral Scanning Confocal Microscope *(Adelaide Microscopy, Adelaide University, Australia)*. A negative control using a SIRT3 and SIRT5 blocking peptide (Table 1, *[Abgent]*) and a positive control using mouse zygotes were also imaged.

Table 1: Antibodies used for immunohistochemistry

Primary Antibodies		
Sirtuin 3	Abcam (ab75434)	Rabbit polyclonal
Sirtuin 5	Abcam (ab13697)	Rabbit polyclonal
Cytochrome C	Abcam (ab13575)	Mouse monoclonal
Secondary Antibodies		
Goat Anti-Mouse IgG	Jackson ImmunoResearch (115-025-003)	TRITC
Donkey Anti-Rabbit IgG	Jackson ImmunoResearch (711-095-152)	FITC
Blocking Peptides		
Sirtuin 3	Abgent (BP6242a)	C-term Synthetic Peptide
Sirtuin 5	Abgent (BP6244a)	C-term Synthetic Peptide

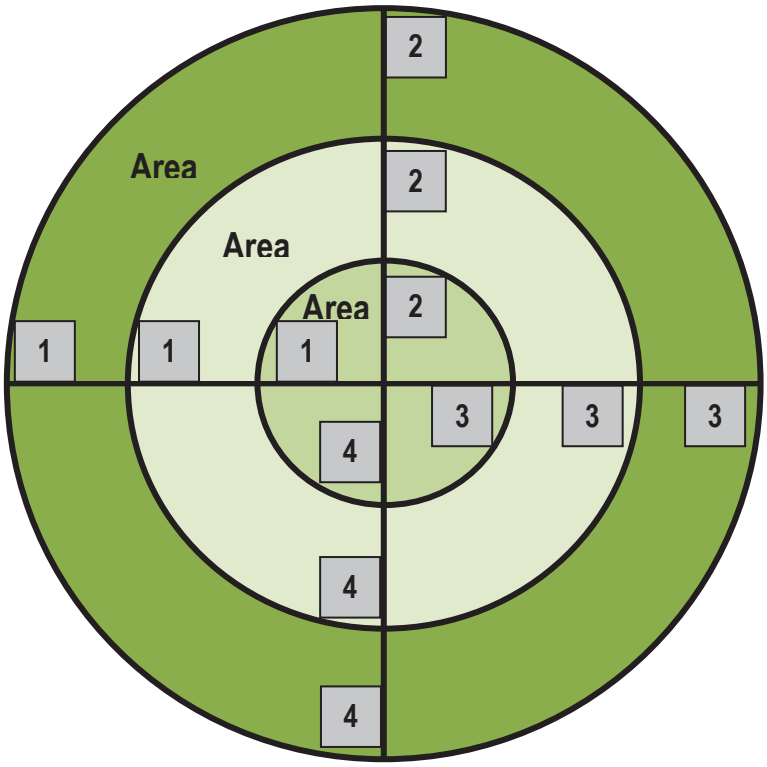
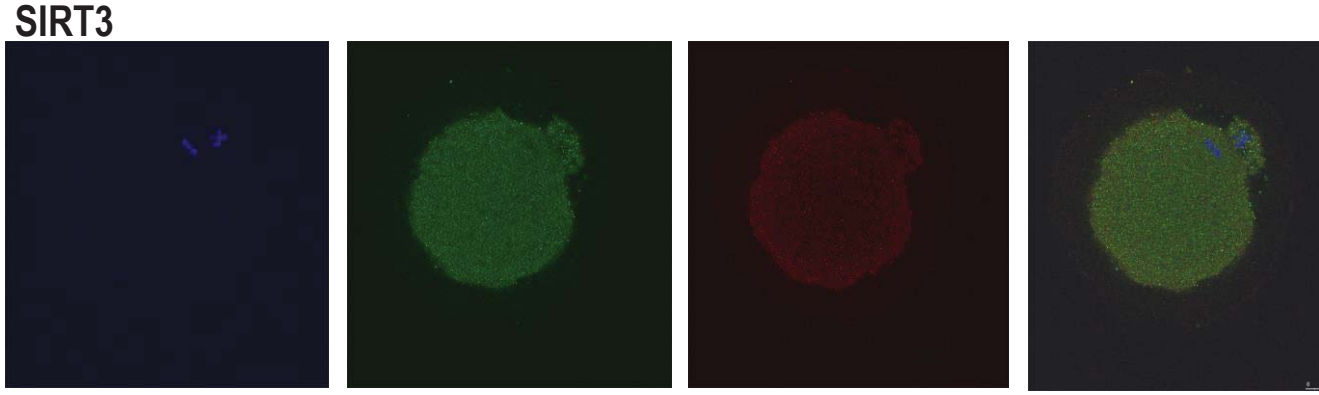


Figure 1: Template used for the analysis of SIRT3 and SIRT5 in oocytes, numbers 1-4 indicate where the fluorescent readings were determined (Mitchell et al., 2009).

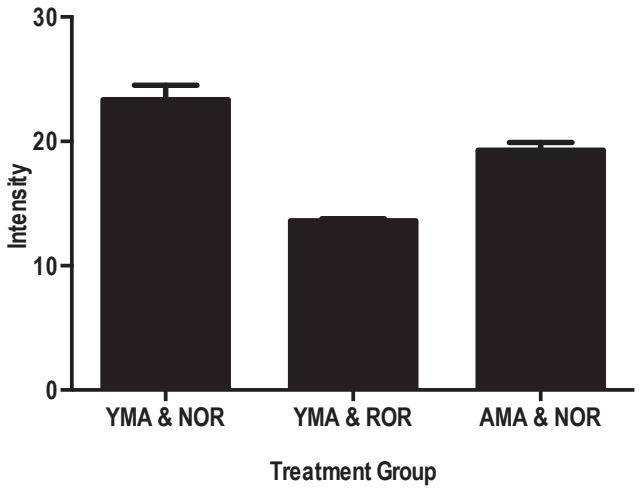
Assessment and Quantification of SIRT3 and SIRT5 in Oocytes

The images were assessed using a previously published method (*Mitchell et al., 2009*). All images were analysed using an image analysis program. Each oocyte was separated into quarters (numbered 1-4) and then separated into three different areas (Area 1-3) (Figure 1). SIRT3 and SIRT5 fluorescent stain intensity in each of the areas was determined and then averaged.

SIRT3 Oocyte Immunohistochemistry



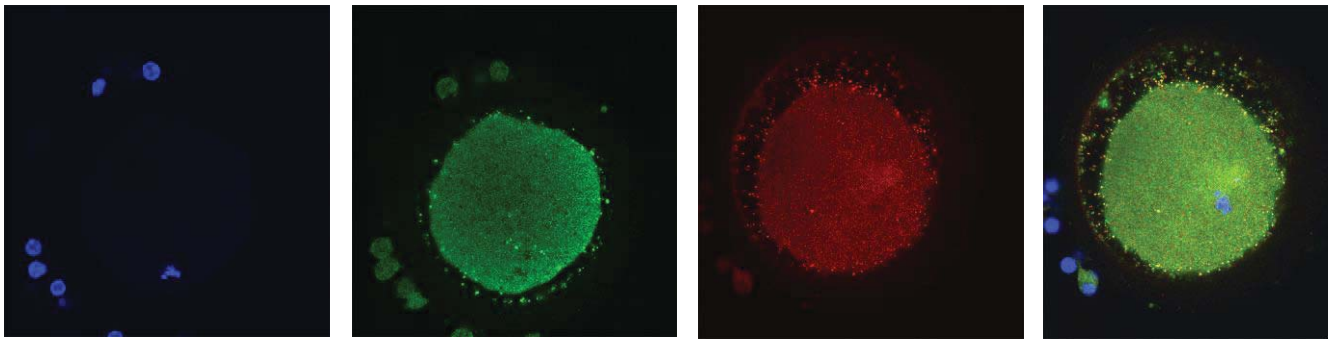
Co-localisation of SIRT3 protein to the mitochondria in the oocyte
n(YMA & NOR) = 4, n(ROR) = 2, n(AMA) = 3, a – blue indicates DAPI or nuclear staining, b – green indicates SIRT5 protein, c – red indicates Cytochrome C and was used as a mitochondrial marker and d – merged image. YMA – young maternal age, NOR – normal ovarian reserve, ROR – reduced ovarian reserve and AMA – advanced maternal age.



SIRT3 protein in oocytes
n(YMA & NOR) = 4, n(ROR) =2, n(AMA) = 3 YMA – young maternal age, NOR – normal ovarian reserve, ROR – reduced ovarian reserve and AMA – advanced maternal age. No significant differences detected, most likely due to small numbers.

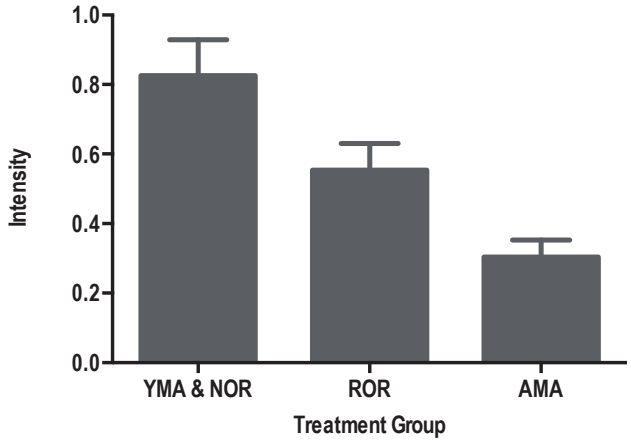
SIRT5 Oocyte Immunohistochemistry

SIRT5



A B C D

Co-localisation of SIRT3 protein to the mitochondria in the oocyte
n(YMA & NOR) = 4, n(ROR) = 2, n(AMA) = 3, a – blue indicates DAPI or nuclear staining, b – green indicates SIRT5 protein, c – red indicates Cytochrome C and was used as a mitochondrial marker and d – merged image. YMA – young maternal age, NOR – normal ovarian reserve, ROR – reduced ovarian reserve and AMA – advanced maternal age.



SIRT5 protein in the oocytes
n(YMA & NOR) = 4, n(ROR) =2, n(AMA) = 3 YMA – young maternal age, NOR – normal ovarian reserve, ROR – reduced ovarian reserve and AMA – advanced maternal age. No significant differences detected, most likely due to small numbers.

References

1) Mitchell, M., Schultz, S.L., Armstrong, D.T. and Lane, M. *Metabolic and Mitochondrial Dysfunction in Early Mouse Embryos Following Maternal Dietary Protein Intervention*. Biol Reprod, 2009, **80**(4): p.622-630

Published Version of Chapter Three

Women with reduced ovarian reserve or advanced maternal age have an altered follicular environment.

Leanne Pacella, Deirdre L. Zander-Fox, Ph.D., David T. Armstrong, Ph.D. and Michelle Lane, Ph.D.

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ORIGINAL ARTICLE: INFERTILITY

Women with reduced ovarian reserve or advanced maternal age have an altered follicular environment

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Objective: To determine whether altered follicular environment is associated with ovarian reserve or maternal age.
Design: Prospective study examining follicular fluid (FF) composition and follicular cell metabolism.
Setting: University research department and private IVF clinic.
Patient(s): Women (n = 54) undergoing routine IVF treatment were allocated to one of three groups based on ovarian reserve and maternal age.
Intervention(s): Surplus FF, granulosa cells (GC), and cumulus cells (CC) were collected.
Main Outcome Measure(s): Follicular fluid concentrations of carbohydrates, hormones, and selected ions. Metabolic analysis and gene expression of GCs and CCs.
Result(s): Compared to women <35 years with normal ovarian reserve, FF glucose levels were significantly decreased and lactate and progesterone (P4) concentrations significantly increased in women with reduced ovarian reserve or advanced maternal age, whereas GC and CC glucose uptake, lactate production, and phosphofructokinase platelet gene expression were significantly increased. Granulosa cell P4 production from women with reduced ovarian reserve or advanced maternal age was decreased; however, in CCs the reverse was observed with increased gene expression in P4 receptor, prostaglandin E receptor-2, cytosolic phospholipase A2, and tumor protein 53.
Conclusion(s): Women with either reduced ovarian reserve or advanced maternal age have altered follicular cell metabolism, FF metabolites, and P4 production. This perturbed environment may be responsible for impaired oocyte developmental competence and subsequent embryo development. (Fertil Steril® 2012;98:986–94. ©2012 by American Society for Reproductive Medicine.)
Key Words: Advanced maternal age, ovarian reserve, follicular cells, carbohydrates, progesterone

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In Western society the age of women trying to conceive is increasing, which correlates with decreased oocyte quality and pregnancy rates (PR) and increased pregnancy complications (1–4). This age-related decline in oocyte quality results from increased aneuploidy and reduced oocyte metabolic function (5, 6). Interestingly, reduced ovarian reserve (follicle number remaining in the ovarian pool), irrespective of age, is also associated with decreased PR and cumulative PRs (7, 8). Furthermore, women with reduced ovarian reserve have fewer oocytes collected, reduced embryo development, and increased miscarriage rates (7).

The ovarian follicle provides an important environment for the support and development of the oocyte and surrounding ovarian cells. Follicular fluid (FF) is comprised of factors that maintain the oocyte in meiotic arrest and aid in ovulation and fertilization (9). The oocyte and surrounding follicular cells play interdependent roles during follicular development, regulating growth, selection, and ovulation of a competent oocyte through signaling of paracrine factors and gonadotropins (10). These factors are synthesized by the follicular cells and oocyte and are secreted into the FF or

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originate from blood (9, 11). The FF microenvironment is integral to the growing oocyte and changes in follicular dynamics are reflected in FF composition.

Human FF contains carbohydrates, such as glucose, lactate, and pyruvate, with a recent study correlating concentration to IVF outcome and therefore, oocyte quality (12). Hormones are also important components of FF that act as key regulators of follicular cell function and development. Previous studies have demonstrated that high FF concentrations of FSH and LH are associated with increased fertilization, and embryos that result in successful IVF outcome are likely to have originated from follicles with high FF LH levels (11, 13). The concentration of steroid hormones is also predictive of implantation and pregnancy with increased estrogen (E2) associated with improved oocyte maturation and increased progesterone (P4) associated with increased abnormal oocyte fertilization rates (oocytes with 1 or >2 pronuclei [PN]), suggesting a link between hormonal regulation and oocyte competence (11).

In the antral follicle, differentiation of the somatic cells surrounding the oocyte into cumulus cells (CC) is essential for oocyte development and viability (14). Cumulus cells exhibit functional characteristics different to granulosa cells (GC) with higher rates of proliferation, low steroidogenic capabilities (especially P4), and low LH receptor expression (15, 16). In response to the LH surge, CCs undergo expansion and secrete macromolecules, whereas GCs undergo luteinization and increase P4 production (14). These changes are dependent on oocyte-secreted factors and, at present, few studies have addressed the effect of ovarian reserve or maternal age on cellular differentiation (17).

Thus far, little is understood as to how the follicular environment and follicular cell functions are altered as a result of reduced ovarian reserve or chronological age. This study aimed to determine the FF carbohydrate and hormone content, and the metabolic profile and steroidogenic activity of GCs and CCs in women with reduced ovarian reserve or advanced maternal age compared with young women with normal ovarian reserve.

MATERIALS AND METHODS

Patient Recruitment and Treatment Groups

Women (n = 54) undergoing IVF treatment at Repromed (Dulwich, South Australia, Australia) were recruited for participation in this study (exclusion criteria were donor/recipient cycles, preimplantation genetic screening, and patients with polycystic ovary syndrome [PCOS]). Each woman was only represented once within the study period. Samples were deidentified and randomly allocated to experimental protocols (Supplemental Fig. 1, available online), based on age (young maternal age: ≤35 years and advanced maternal age: ≥40 years) or ovarian reserve. Serum antimüllerian hormone (AMH) was used as a measure of ovarian reserve. The AMH range for young women (<35 years) with normal ovarian reserve was 1.4–4.2 μg/L and the reduced ovarian reserve group had serum AMH concentrations of <1.4 μg/L (these women were all <35 years). The AMH values for women of advanced maternal age (>40 years) were 0.4–1.4 μg/L and these values represent 25%–75% quartiles for this age group (18). Ethical approval was obtained from the Women's and Children's Hospital Research Ethics Committee (North Adelaide, South Australia, Australia) and written informed consent was obtained for use of surplus FF, GCs, and CCs.

Ovarian Simulation and FF Collection

All women underwent antagonist cycles using gonadotropin stimulation with recombinant FSH (Puregon [100–375 U]; Schering-Plough or Gonal F [150–350 U]; Merck Serono), administered from menstrual cycle day 2/3. On day 7 a GnRH antagonist (Orgalutran [250 μg]; Schering-Plough) was administered. Follicle growth was monitored by ultrasound and when ≥2 follicles ≥17mm were present, the hCG trigger (Ovidrel, 250 μg) was administered and oocytes collected 36 hours later. Follicular fluid was aspirated with a 17-gauge single-lumen needle, under sedation (no media flush), with the guidance of a transvaginal ultrasound from ovarian follicles. The surplus FF, containing no or minimal blood contamination, was centrifuged for 10 minutes at 300 × *g*, frozen and stored at −80°C. Each tube of FF contained one to two follicles (≥18 mm) and was processed separately. Consistent with previous findings by Robker et al. (19), there were no differences between FF concentrations of metabolites and hormones from follicles ≥18 mm (data not shown).

GC and CC Collection and Culture

Immediately after oocyte retrieval, GCs were removed from the surplus FF and washed into Dulbecco's phosphate-buffered saline (PBS) (Sigma-Aldrich). Cells were purified by density gradient centrifugation using 60%/40% silica solution (Spermgrad, Vitrolife) and spun for 30 minutes at 470 × *g*. Cumulus cells were collected by trimming cumulus–oocyte complexes of the outer layers of cells and washed through Dulbecco's PBS immediately after oocyte collection. Cell clumps were disassociated by pipetting, GC and CC counts were performed using a hamocytometer, and cell suspensions of 1×10^6 cells/mL were cultured in modified G-1 media (as described in Gardner et al. [20], minus lactate) at 37°C in 5% O_2/6% CO_2 for 4 hours. A modified G-1 media-only control was included.

Carbohydrate and Glycolytic Index Assessment

Follicular fluid glucose concentration was measured using the Optium Xceed diabetes monitoring system (Abbott Diabetes Care).

Follicular fluid pyruvate and lactate, and spent media glucose and lactate concentrations from cultured GCs and CCs were assessed using a microfluorometric assay. The concentration of each metabolite was determined using enzyme-linked reactions to pyridine nucleotides, as described previously (21). A standard curve ($r^2 > 0.98$) was run with each analysis and patient samples were assessed in triplicate and expressed as uptake or production per 1×10^6 cells/h.

Glycolysis was calculated on the basis of the breakdown of 1 molecule of glucose to 2 molecules of lactate expressed as a percentage.

Hormone Assessment

Estradiol, P4, LH, and FSH levels were analyzed by an ADVIAR Centaur XP (Siemens Healthcare Diagnostics), according to manufacturer's instructions by an externally accredited laboratory. The AMH concentration was determined using an Immunotech ELISA immunoassay (Beckman Coulter) according to manufacturer's instructions.

Ion, Lactate, Total Protein, Albumin, and Cholesterol Assessment

Follicular fluid was analyzed for ions (sodium, potassium, chloride, bicarbonate, and calcium), lactate, total protein, albumin, and cholesterol on a Siemens Advia Chemistry System (Siemens Healthcare Diagnostics) by an external accredited laboratory.

RNA Isolation and Quantitative Polymerase Chain Reaction in GCs and CCs

Granulosa cell and CC RNA was extracted as per manufacturer's instructions using an RNeasy Kit (Qiagen). RNA concentration was determined using the Nanodrop 1000 spectrophotometer (Thermo Scientific). Complementary DNA (cDNA) was synthesized using Superscript III Reverse Transcriptase (Invitrogen) as per manufacturer's instructions. Quantitative polymerase chain reaction (PCR) primers were designed to human sequences using NCBI Blast and synthesized by Geneworks (Hindmarsh) (Supplemental Table 1, available online). Samples were run in triplicate on the Corbett Rotor Gene 6000 (Corbett Life Sciences). For each reaction 2 μL of cDNA was added to a master mix containing 1 μL of each forward and reverse primer (diluted to 10 pmol/μL), 10 μL of SYBR Green (Applied Biosystems) and 6 μL of H_2O, giving a total volume of 20 μL. The PCR cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, 40 amplification cycles of 95°C for 15 seconds, and 60°C for 1 minute. Analysis was performed using delta, delta ct, normalized to 18S (22).

Statistical Analysis

Data are expressed as mean ± SEM. To determine significance between groups a Student's *t*-test was used for patient demographics and a one-way analysis of variance (ANOVA) with Tukey's post-hoc test was used for the remainder of analyses. A P value $<.05$ was considered statistically significant.

RESULTS

Patient Demographics

Mean maternal age, serum AMH levels, and etiology for young women with normal ovarian reserve, reduced ovarian reserve, and advanced maternal age are indicated in Table 1. No significant differences in body mass index (BMI) or fertilization rates were found between groups. Young women with normal ovarian reserve had significantly lower starting FSH doses, fewer previous cycles, and more oocytes collected compared with women with reduced ovarian reserve or advanced maternal age. Young women with normal ovarian reserve had increased number of embryos frozen and increased implantation rates compared with women with reduced ovarian reserve or advanced maternal age ($P<.05$; Table 1).

TABLE 1

Patient demographics and cycle outcomes.

	Young maternal age with normal ovarian reserve (n = 21)	Reduced ovarian reserve (n = 16)	Advanced maternal age (n = 17)
Age	30.9 ± 0.9[a]	32.5 ± 0.6[a]	41.5 ± 0.5[b]
Serum AMH (μg/L)	3.1 ± 0.3[a]	0.7 ± 0.1[b]	1.2 ± 0.1[c]
BMI	24.4 ± 1.2[a]	24.5 ± 1.6[a]	27.6 ± 0.5[a]
Starting FSH dose (IU)	190.5 ± 14.0[a]	303.5 ± 11.4[b]	307.2 ± 8.1[b]
No. of previous fresh cycles (IVF/ICSI)	0.8 ± 0.2[a]	2.6 ± 0.7[b]	3.2 ± 0.7[b]
No. of oocytes collected	11.4 ± 0.9[a]	8.0 ± 1.3[b]	7.8 ± 1.3[b]
Fertilization rate (%)	63.5 ± 6.3[a]	63.4 ± 6.5[a]	50.0 ± 6.6[a]
Mean no. of embryos transferred	1.2 ± 0.1[a]	1.3 ± 0.2[a,b]	1.6 ± 0.1[b]
Mean no. of embryos frozen	3.4 ± 0.7[a]	1.2 ± 0.4[b]	0.6 ± 0.3[b]
Pregnancy rate (%)	57.9[a]	50.0[a]	13.3[b]
Implantation rate (%)	45.5[a]	36.3[a]	8.3[b]
Utilization of oocytes (%)[d]	45.0[a]	27.2[b]	28.0[a]
Infertility diagnosis (%)[e]			
Ovulatory	4.8[a]	9.1[a]	0.0[a]
Tubal	9.5[a]	9.1[a]	5.9[a]
Endometriosis	19.1[a]	18.2[a]	0.0[a]
Unexplained	9.5[a]	9.1[a]	0.0[a]
Male factor	71.4[a]	81.8[a]	70.6[a]

Note: Data expressed as mean ± SEM. AMH = antimüllerian hormone; BMI = body mass index; ICSI = intracytoplasmic sperm injection.
Exclusion criteria: donor/recipient cycles, preimplantation genetic screening, and patients with polycystic ovary syndrome.
[a–c] Significant differences between treatment groups, $P<.05$.
[d] Utilization of oocytes was calculated as follows: (frozen embryos + embryos transferred)/oocytes.
[e] Infertility diagnosis: multiple diagnoses for some patients.

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Carbohydrate Concentrations in FF

Follicular fluid glucose concentration was decreased and lactate and pyruvate concentrations increased in young women with reduced ovarian reserve and women with advanced maternal age compared with young women with normal ovarian reserve ($P<.05$; Table 2). Furthermore, FF glucose concentration was decreased and lactate concentration increased in women with reduced ovarian reserve compared with women of advanced maternal age ($P<.05$; Table 2).

Hormone Concentrations in FF

Follicular fluid E_2 concentration was higher in women with reduced ovarian reserve and lower in women with advanced maternal age, compared with young women with normal ovarian reserve ($P<.05$; Table 2). Compared with young women with normal ovarian reserve, FF P4 concentration was increased and AMH concentration was decreased in the reduced ovarian reserve and advanced maternal age groups ($P<.05$; Table 2). No significant differences were found in FF concentrations of LH or FSH between groups.

Ion, Total Protein, Albumin, and Cholesterol Concentrations in FF

Follicular fluid concentrations of sodium, potassium, chloride, bicarbonate, calcium, protein, albumin, and cholesterol were not different between groups (Table 2).

Glucose Uptake and Lactate Production by Cultured GCs and CCs

To assess the follicular cell contribution to the differences in FF glucose and lactate concentration, the carbohydrate metabolism of GCs and CCs was assessed. In both cell types the rate of glucose uptake and lactate production in women with reduced ovarian reserve or advanced maternal age was increased compared with young women with normal ovarian reserve. Granulosa cell and CC glucose uptake and lactate production was increased in the reduced ovarian reserve compared with the advanced maternal age groups ($P<.05$; Fig. 1A and B).

The Glycolytic Index of the GCs and CCs

To further assess the differences in glucose uptake and lactate production between GCs and CCs, the glycolytic index was determined. Compared to GCs an increased glycolytic index (increase in lactate per glucose consumed) was exhibited in CCs, irrespective of treatment group ($P<.05$; Fig. 1C). No significant differences existed in the glycolytic index of GCs in the reduced ovarian reserve or advanced maternal age groups compared with the young with normal ovarian reserve group. However, an increased glycolytic index was observed in the GCs from women with reduced ovarian reserve compared with the advanced maternal age group ($P<.05$; Fig. 1C). In CCs young women with normal ovarian reserve had an elevated glycolytic index compared with the reduced ovarian reserve and the advanced maternal age groups ($P<.05$; Fig. 1C).

Glycolytic Gene Expression in the GCs and CCs

To further assess the differences in glycolysis, gene expression of the glycolytic enzymes, phosphofructokinase platelet and lactate dehydrogenase A were assessed. Levels of phosphofructokinase platelet gene expression were similar between CCs and GCs within treatments. Phosphofructokinase platelet levels were increased in GCs and CCs in the

TABLE 2

Follicular fluid concentrations of carbohydrates, hormones, and ions in young women with either normal ovarian reserve or reduced ovarian reserve and in women of advanced maternal age.

		Young maternal age with normal ovarian reserve (n = 10)	Reduced ovarian reserve (n = 8)	Advanced maternal age (n = 12)
Carbohydrates	Glucose (mmol/L)	3.9 ± 0.2^{a}	2.3 ± 0.2^{b}	2.9 ± 0.2^{c}
	Lactate (mmol/L)	2.6 ± 0.1^{a}	6.0 ± 0.6^{b}	3.9 ± 0.5^{c}
	Pyruvate (mmol/L)	0.6 ± 0.1^{a}	0.9 ± 0.1^{b}	0.7 ± 0.1^{b}
Hormones	E_2 (pmol/L)	$1{,}634.4 \pm 149.6^{a}$	$3{,}971.9 \pm 106.0^{b}$	770.3 ± 117.1^{c}
	P4 (nmol/L)	$2{,}917.9 \pm 229.1^{a}$	$3{,}765.1 \pm 200.2^{b}$	$4{,}701.0 \pm 547.6^{b}$
	AMH (μg/L)	5.8 ± 0.4^{a}	1.4 ± 0.1^{b}	1.1 ± 0.1^{b}
	LH (IU/L)	1.2 ± 0.2^{a}	1.0 ± 0^{a}	1.4 ± 0.2^{a}
	FSH (IU/L)	6.2 ± 0.7^{a}	5.3 ± 0.7^{a}	6.1 ± 0.6^{a}
Ions	Sodium (mmol/L)	129.6 ± 2.7^{a}	124.0 ± 3.6^{a}	124.6 ± 5.2^{a}
	Potassium (mmol/L)	4.1 ± 0.1^{a}	4.1 ± 0.2^{a}	4.0 ± 0.2^{a}
	Chloride (mmol/L)	103.2 ± 1.7^{a}	99.4 ± 2.4^{a}	100.0 ± 2.9^{a}
	Bicarb (mmol/L)	19.0 ± 0.6^{a}	17.6 ± 0.8^{a}	17.4 ± 1.7^{a}
	Calcium (mmol/L)	1.76 ± 0.1^{a}	1.61 ± 0.1^{a}	1.68 ± 0.1^{a}
	Protein (g/L)	45.8 ± 2.8^{a}	36.2 ± 7.5^{a}	38.4 ± 6.9^{a}
	Albumin (g/L)	30.6 ± 1.9^{a}	24.2 ± 4.9^{a}	26.2 ± 4.8^{a}
	Cholesterol (mmol/L)	0.66 ± 0.1^{a}	0.5 ± 0^{a}	0.67 ± 0^{a}

Note: Data expressed as mean ± SEM. AMH = antimüllerian hormone.
[a–c] Significant difference between treatment groups ($P<.05$).

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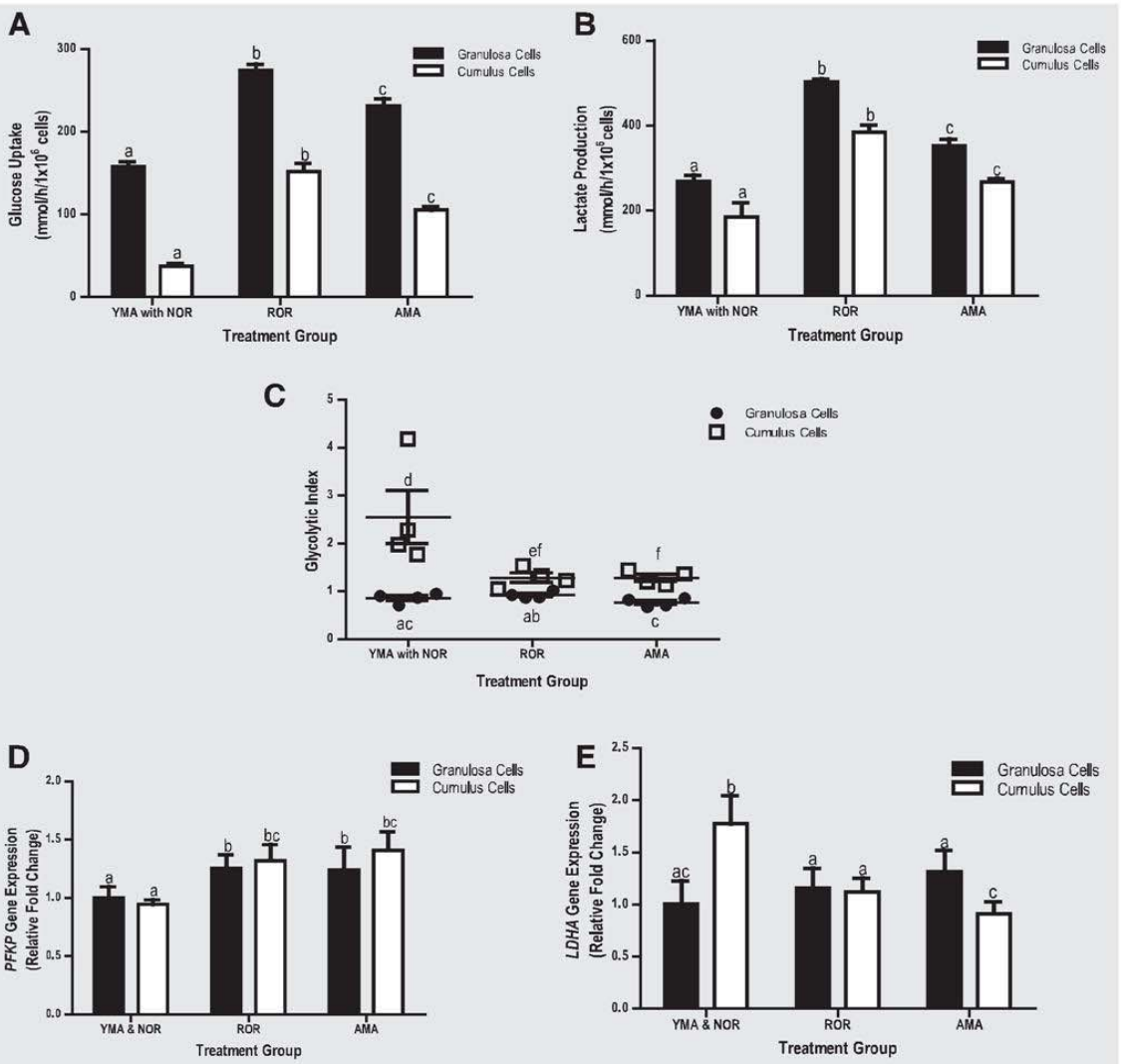
FIGURE 1



Comparison of granulosa cell (GC) and cumulus cell (CC) glucose uptake (A), lactate production (B), glycolytic index of GCs and CCs (C) and gene expression of phosphofructokinase platelet (PFKP) (D) and lactate dehydrogenase A (LDHA) (E). (A–C) n = 4 patients per treatment group; (D, E) n = 5 patients per treatment group. (A, B) a–c: significant differences between treatment groups for cell type ($P<.05$). (C) a–f: significant differences between treatment groups. (D, E) a–c: significant differences between treatment groups. AMA = advanced maternal age; NOR = normal ovarian reserve; ROR = reduced ovarian reserve; YMA = young maternal age.

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reduced ovarian reserve and advanced maternal age groups compared with young women with normal ovarian reserve concomitant with the observed increase in glucose uptake ($P<.05$; Fig. 1D). In contrast, lactate dehydrogenase A gene expression was decreased in the CCs from women with reduced ovarian reserve with a further decrease observed with advanced maternal age groups ($P<.05$; Fig. 1E), such that the normal increase in gene expression with differentiation of the CCs was lost in the reduced ovarian reserve and advanced maternal age groups.

Progesterone Production by the GCs and CCs

As there were differences in FF P4 concentration between groups, P4 secretion by the follicular cells was determined. Spent media concentrations of P4 produced by the GCs was

significantly lower in the reduced ovarian reserve and advanced maternal age groups compared with young women with normal ovarian reserve ($P<.05$; Fig. 2A). Conversely, CC P4 production was increased in the reduced ovarian reserve and advanced maternal age groups compared with young women with normal ovarian reserve. Cumulus cell P4 production in the reduced ovarian reserve group was elevated compared with the advanced maternal age group ($P<.05$; Fig. 2B).

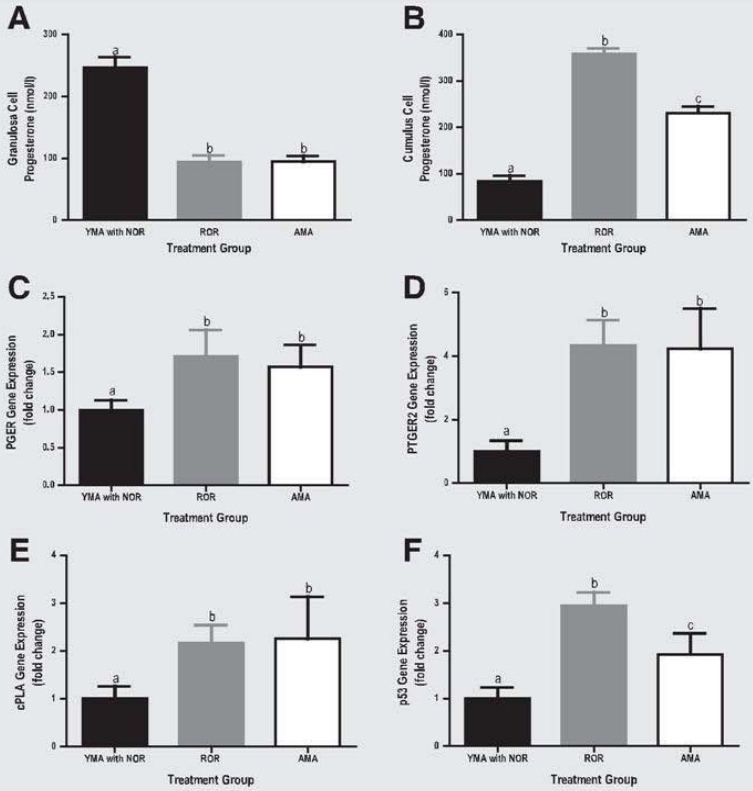
Progesterone Receptor, Prostaglandin and Phospholipase Activity, and Apoptotic Marker Gene Expression in CCs

To assess the molecular differences in P4 production and apoptosis, gene expression was performed on CCs for genes involved in the regulation of P4, prostaglandins and apoptosis. Gene expression levels for P4 receptor, prostaglandin E receptor-2, cytosolic phospholipase A2, tumor protein 53 were higher in CCs of women with reduced ovarian reserve or of advanced maternal age compared with young women with normal ovarian reserve ($P<.05$; Fig. 2C–2F). However, no differences were found in gene expression of prostaglandin endoperoxide synthase 2, secretory phospholipase A2, or retinoblastoma 1 (data not shown).

DISCUSSION

The results from this study have established that carbohydrate (glucose, lactate, and pyruvate) and hormone (P4, E2, and AMH) levels are altered in the FF of women with reduced ovarian reserve or advanced maternal age. Cumulus cells from these women did not display the changes in both metabolic and steroidogenic activity that were observed in the young women with normal ovarian reserve, reflecting a phenotype more commonly associated with GCs, suggesting altered or lack of differentiation in response to the LH surge. These data would suggest that there are metabolic and molecular changes to the follicular cells resulting in changes to the FF content that may be responsible for the reduced oocyte competence that were observed in the present study. There

FIGURE 2



Progesterone production by cultured granulosa cells (GCs) (A) and cumulus cells (CCs) (B). Gene expression of the P4 receptor (PGER) (C), prostaglandin E receptor-2 (PTGER2) (D), cytosolic phospholipase A2 (cPLA2) (E), and tumor protein 53 (p53) (F) in CCs. (A, B) n = 6 patients per treatment group; (C–F) n = 3 patients per treatment group. (A, B) a–c: significant differences between treatment groups ($P<.05$). (C–F) a–c: significant differences between treatment groups ($P<.05$). AMA = advanced maternal age; NOR = normal ovarian reserve; ROR = reduced ovarian reserve; YMA = young maternal age.

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has been some contradictory evidence in the literature regarding the ability of AMH to predict pregnancy outcomes, with some studies demonstrating a relationship with PRs (7, 8) and similar to our findings other studies demonstrating little effect (18). However, in the present study, implantation rates were reduced in the advanced maternal age group, and the mean number of embryos that were able to be frozen or used was significantly less in both the reduced ovarian reserve and advanced maternal age groups compared with the young women with normal ovarian reserve group. These findings indicate that there was a decrease in the percentage of viable oocytes in both the reduced ovarian reserve and advanced maternal age groups, which is similar to the reduction in cumulative PR previously reported (7).

Follicular fluid is not only a serum transudate but is comprised of hormones and carbohydrates that are synthesized by follicular cells (23). Ions, in particular, are components that are able to be filtered from blood serum. In the present study the levels of ions, as well as albumin, were similar to that previously reported for serum and reflects the levels reported previously for FF, which were not altered by reduced ovarian reserve or advanced maternal age (24).

However, in direct contrast to the ionic composition, FF carbohydrate composition was altered in women with reduced ovarian reserve or advanced maternal age. The decrease in glucose and concomitant increase in FF lactate concentrations reflects the altered metabolic profile of GCs and CCs in these women. These cells take up more glucose and produce higher lactate quantities, thus increasing FF lactate levels. Follicular fluid pyruvate concentrations were also elevated in women with reduced ovarian reserve and advanced maternal age, possibly reflective of increased metabolic rate. Previous studies in GCs of patients with PCOS report a down-regulation in insulin-dependent glucose uptake and lactate production with no change in LH-mediated glucose metabolism (25). In contrast, we report an increase in glucose metabolism, possibly LH mediated, resulting in increased lactate production in the reduced ovarian reserve and advanced maternal age groups. Similar to women with reduced ovarian reserve, obese women are also known to have elevated FF glucose and lactate concentrations (19, 26). The consequences of this up-regulation in glycolysis are currently unknown; however, a consequence of increased follicular lactate production may be a decrease in FF pH. Follicular fluid is considered to be alkaline and decreased pH is related to reduced oocyte fertilization (7, 24, 27).

In young women with normal ovarian reserve CC metabolism underwent an up-regulation in glycolytic index; however, no such change occurred in the women with reduced ovarian reserve or advanced maternal age. This increase in glucose consumption increased the levels of the allosteric glycolytic regulator phosphofructokinase platelet. Furthermore, lactate dehydrogenase A, which converts pyruvate to lactate in the glycolysis pathway, similarly reflected the changes in glycolysis and lactate production with increases in CCs in young women with normal ovarian reserve. Similarly, CC lactate dehydrogenase A was reduced in women with reduced ovarian reserve and advanced maternal age where glycolysis, specifically lactate production, was also reduced. Changes in glycolytic enzyme levels between GCs and CCs are at least in part mediated by the oocyte suggesting that oocyte signaling is impaired, possibly affecting the transition of GC to CC phenotype. This transition within the follicle has been shown to require endocrine signals from the follicle, but is also reliant on paracrine signaling from the oocyte (28). This bidirectional communication between CCs and the oocyte is essential for maintaining oocyte competence (29). It is therefore plausible that oocyte signaling from women with reduced ovarian reserve or advanced maternal age may be reduced, affecting the capacity of the cells surrounding the oocyte to differentiate into the CC phenotype. However, it is plausible that impairment of the follicular cells may be affecting their ability to differentiate. In animal models, reduced levels of oocyte-secreted factors, such as growth differentiation factor-9 and bone morphogenetic protein-15, result in decreased oocyte competence and changes in CC function, such as increased apoptotic markers (30). It has previously been demonstrated that GC viability is reduced with declining ovarian reserve and in our study CCs have increased gene expression of the apoptotic marker tumor protein 53 (31). Therefore, the levels of oocyte-secreted factors in women with reduced ovarian reserve or advanced maternal age warrant further investigation.

Studies have demonstrated that increased FF P4 or decreased E2 concentrations are associated with reduced oocyte competence (11, 13). Not surprisingly in older women, whose oocyte competence is reduced, there were elevated levels of P4 and lower levels of E2 in FF. However, in women with reduced ovarian reserve, although there was an increase in P4 levels, E2 was also increased. Because E2 is secreted by the theca cells and GCs this would indicate functional differences in steroidogenic capacity in these cells (32). Furthermore, CCs from women with reduced ovarian reserve and women of advanced maternal age had different steroidogenic profiles, particularly the expression of P4 receptor, prostaglandin E receptor-2, and cytosolic phospholipase A2, which regulate P4 and prostaglandin actions and production, were up-regulated compared with the young women with normal ovarian reserve. Similar to these findings a recent study by Skiadas et al. (33), also reported altered gene expression in the GCs of young women with reduced ovarian reserve compared with women with a higher ovarian reserve, particularly in genes that are involved with P4 production/response. Therefore, similar to the metabolic changes, the hormonal profile of CCs is altered in women with reduced ovarian reserve and advanced maternal age reflecting a more granulosa-like physiology, further suggesting that these cells may have failed to differentiate completely into a CC phenotype.

The increase in FF concentration of P4 and CC P4 production in the reduced ovarian reserve and advanced maternal age groups may be attributed to the increased gene expression of both P4 receptor and prostaglandin E receptor-2. In addition, the increase in gene expression of cytosolic phospholipase A2 in CCs in women with reduced ovarian reserve or advanced maternal age may result in greater phospholipolytic activity, presumably yielding increased levels of arachidonic acid, thereby augmenting the availability of substrate for prostaglandin E_2 (PGE_2) synthesis. The resulting increase in PGE_2, acting through the

elevated prostaglandin E receptor-2, would increase cyclic adenosine 3′:5′ monophosphate (cAMP) concentration, which, in turn, could augment P4 synthesis (34). Linoleic acid, another product of the phospholipid lipolysis, may contribute directly to the reduced oocyte quality observed in women with reduced ovarian reserve or advanced maternal age. This fatty acid has recently been reported to inhibit cumulus expansion and meiotic maturation in isolated bovine cumulus–oocyte complexes, resulting in impaired embryo development (35).

Interestingly, FF from women with reduced ovarian reserve and advanced maternal age contained lower AMH levels compared with the young women with normal ovarian reserve. The AMH is secreted by early GCs in the primary and small antral follicles (36). However, AMH expression still exists in CCs of the preovulatory follicle with lower expression in GCs, suggesting that these cells still have the capacity to secrete AMH into the FF at these later stages of folliculogenesis (37). Previous studies have demonstrated a positive correlation between FF AMH concentration and successful oocyte fertilization, suggesting that AMH production and activity is reduced in GCs and CCs from these patients (8, 37, 38). Andersen et al. (39) reported that FF FSH can act as a negative regulator of AMH expression; however, in the present study FF FSH and LH concentrations were not different between the groups. However, AMH null mice have increased oocyte degeneration, defined as the presence of oocyte remnants within follicles, suggesting that oocytes in the follicles from women with reduced ovarian reserve may be degrading and serves as an explanation for the decline in oocyte number seen in these women (7, 40).

These results demonstrate an association between perturbed ovarian follicular cell metabolism in women with reduced ovarian reserve and advanced maternal age. However, the mechanisms appear to differ between these two groups of women with differences observed in metabolic and steroid measures as well as in their cycle outcomes. However, although it is possible that the elevated FSH dose received by women with reduced ovarian reserve or advanced maternal age may be contributing to this altered follicular metabolism, the dose was similar in these two groups, implying that other factors are also involved. Interestingly, in the present study FF FSH concentration was not different between the three treatment groups, thus suggesting that an FSH dose effect on the immediate environment of the follicular cells may not be occurring. In this study, the altered follicular environment was characterized by what resembled a lack of differentiation of GCs into CCs, which has been implicated to result from reduced oocyte paracrine factors. This bidirectional communication between the oocyte and surrounding cells is important for oocyte viability. Therefore it is possible that the decrease in overall oocyte quality, as demonstrated in the reduction of oocyte utilization, seen in women with reduced ovarian reserve or advanced maternal age may be due to the perturbed follicular cell metabolism/environment or the reverse may be true with the altered follicular cell metabolism/environment potentially causing the decrease in overall oocyte quality. Therefore the exact causes resulting in these perturbations still remain to be elucidated. Knowledge of these perturbations could lead to changes in timing of oocyte retrieval or improving media requirements for these poorer prognosis women undergoing IVF treatment.

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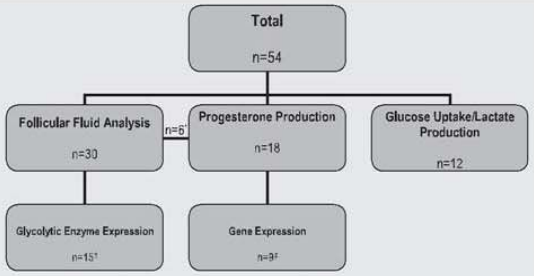
SUPPLEMENTAL FIGURE 1



Random allocation of patients to each experimental protocol. *Six patients were randomly allocated to both the follicular fluid (FF) analysis and P4 production experimental protocols; ‡ 9 patients were randomly allocated to both the P4 production and gene expression experimental protocols; † 15 patients were randomly allocated to both the FF and glycolytic enzyme expression experimental protocols.

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SUPPLEMENTAL TABLE 1

Primer designs for quantitative polymerase chain reaction.

Gene	Accession No.	Sequence	Amplicon size
Progesterone receptor (PGER)	NM_001202474	F: CCAGGGACAGGACCCCTCCG R: GCTGCCTCCAGCACCCCTTG	106
Prostaglandin E receptor-2 (PTGER2)	NM_000956	F: TCCTGGCTATCATGACCATCAC R: AGCTTGGAGGTCCCATTTTTC	107
Cytosolic phospholipase A2 (cPLA)	NM_001004426	F: GCAATGCTCGGTGCAACAT R: GGCTGCTGTCCATGCTGAT	108
Tumor protein 53 (p53)	NM_000546	F: CCCCTCCTCAGCATCTTATCC R: ACCTCAGGCGGCTCATAGG	109
Prostaglandin endoperoxide synthase 2 (PTGS2 or COX2)	NM_000963	F: ATCATTCACCAGGCAAATTGC R: TGCCTGCTCTGGTCAATGG	99
Secretory phospholipase A2 (sPLA2)	NM_000300	F: AGGAGAAGGGCTGCAACATTC R: CACAGAGGTTCACATGGCAGAA	102
Retinoblastoma 1 (RB1)	NM_000321	F: CAAGCAACCTCAGCCTTCCA R: GACAGAAGGCGTTCACAAAGTG	122
Phosphofruktokinase, platelet (PFKP)	NM_002627	F: AAGTACTTGGAAGAGATCGCCACACAGA R: CTGATCATCGGTGGATTCGAGGC	77
Lactate dehydrogenase (LDHA)	NM_005566	F: AACACCAAAGATTGTCTCTGGCAAAGAC R: TATCACGGCTGGGGCACGT	80
18S	AF176811	F: AGAAACGGCTACCACATCCAA R: CCTGTATTGTTATTTTTCGTCACTACCT	92

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Published Version of Chapter Four

Mitochondrial SIRT5 is present in follicular cells and is altered by reduced ovarian reserve and advanced maternal age.

Leanne Pacella-Ince, Deirdre L. Zander-Fox, Ph.D. and Michelle Lane, Ph.D.

Reproduction Fertility and Development. (Published online 27 August 2013)

Pacella-Ince, L., Zander-Fox, D.L. and Lane, M. (2013). Mitochondrial SIRT5 is present in follicular cells and is altered by reduced ovarian reserve and advanced maternal age. *Reproduction, Fertility and Development* 26(8) 1072-1083.

Published Version of Chapter Five

Mitochondrial SIRT3 and its target glutamate dehydrogenase are altered in follicular cells of women with reduced ovarian reserve or advanced maternal age.

Leanne Pacella-Ince, Deirdre L. Zander-Fox, Ph.D. and Michelle Lane, Ph.D.

Human Reproduction (in press)

Pacella-Ince, L., Zander-Fox, D.L. and Lane, M. (2014). Mitochondrial SIRT3 and its target glutamate dehydrogenase are altered in follicular cells of women with reduced ovarian reserve or advanced maternal age. *Human Reproduction* 29(7) 1490-1499.
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