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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STUDY QUESTION: Is the activity of sirtuin 3 (SIRT3) altered in granulosa and cumulus cells from young women with reduced ovarian reserve or women of advanced maternal age?

SUMMARY ANSWER: SIRT3 mRNA and active protein in granulosa and cumulus cells were decreased in women with reduced ovarian reserve and advanced maternal age.

WHAT IS KNOWN ALREADY: Young women with reduced ovarian reserve or women of advanced maternal age have reduced oocyte viability, possibly due to altered granulosa and cumulus cell metabolism. The mitochondrial SIRT3 protein may be implicated in these processes as it is able to sense the metabolic state of the cell and alter mitochondrial protein function post-translationally.

STUDY DESIGN, SIZE, DURATION: This is a prospective cohort study, in which women (n = 72) undergoing routine IVF/ICSI were recruited and allocated to one of three cohorts based on age and ovarian reserve (as assessed by serum anti-Mullerian hormone level). Women were classified as young (≤35 years) or of advanced maternal age (≥40 years).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Granulosa and cumulus cells were collected. SIRT3 mRNA and protein levels and protein activity was analysed in granulosa and cumulus cells via quantitative PCR, immunohistochemistry and western blotting, and deacetylation activity, respectively. Activity of the glutamate dehydrogenase (GDH) enzyme, a known target of SIRT3, was assessed, and acetylated proteins in mitochondria isolated from granulosa and cumulus cells were separated by immunoprecipitation and acetylation of GDH assessed by western blotting. Data for women with good prognosis (young women with normal ovarian reserve) were compared with those from young women with reduced ovarian reserve and those of advanced maternal age.

MAIN RESULTS AND THE ROLE OF CHANCE: SIRT3 mRNA and active protein were present in granulosa and cumulus cells and co-localized to the mitochondria. SIRT3 mRNA in granulosa cells was decreased in young women with reduced ovarian reserve and advanced maternal age versus young women with normal ovarian reserve (P < 0.05). SIRT3 mRNA in cumulus cells was decreased in women of advanced maternal age versus young women with normal ovarian reserve only (P < 0.05). Granulosa cell GDH activity was decreased in young women with reduced ovarian reserve and in women of advanced maternal age (P < 0.05), whereas cumulus cell GDH activity was reduced in the advanced maternal age group only (P < 0.05). The acetylation profile of GDH in mitochondria revealed increased acetylation of GDH in granulosa and cumulus cells from women of advanced maternal age (P < 0.05) while young women with reduced ovarian reserve had increased GDH acetylation in granulosa cells only (P < 0.05).

LIMITATIONS, REASONS FOR CAUTION: Although patients were allocated to groups based on maternal age and ovarian reserve and matched for BMI, other maternal factors may also alter the ‘molecular health’ of ovarian cells.

WIDER IMPLICATIONS OF THE FINDINGS: Our data suggest that SIRT3 post-translational modification of mitochondrial enzymes in human granulosa and cumulus cells may regulate GDH activity, thus altering the metabolic milieu surrounding the developing oocyte. Owing to the
association between the decline in oocyte quality and pregnancy rates in women of advanced maternal age and the possible association with reduced ovarian reserve, knowledge of perturbed SIRT3 function in granulosa and cumulus cells may lead to novel therapies to improve mitochondrial metabolism in the oocyte and follicular cells in women undergoing IVF treatment.

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**Key words:** sirtuin 3 / reduced ovarian reserve / advanced maternal age / glutamate dehydrogenase / ovarian follicular cells

### Introduction

It is well understood that oocyte quality and quantity decline with increasing maternal age, as indicated by increased oocyte aneuploidy and a reduction in metabolic output, for example glucose or pyruvate metabolism (Pellestor et al., 2003; Eichenaub-Ritter et al., 2004; Eichenaub-Ritter, 2012; Christopikou et al., 2013), which translates to decreased pregnancy rates (Dew et al., 1998; Baird et al., 2005; Alviggi et al., 2009; Yan et al., 2012). However, a decline in fertility and pregnancy is also observed with a decline in ovarian reserve independent of maternal age. Oocytes from women with reduced ovarian reserve display decreased fertilization rates and increased blastocyst aneuploidy rates (including a significantly increased risk of trisomy) and the women have increased rates of miscarriage (Levi et al., 2001; Lekamge et al., 2007; Haadmsa et al., 2010; Katz-Jaffe et al., 2013). However, the reason for this decline in oocyte quality in women with reduced ovarian reserve is unclear.

Oocyte developmental competence is highly dependent on the bidirectional dialogue between the granulosa/cumulus cells and the oocyte through the presence of transzonal projections, gap junctions and the secretion of paracrine factors (Gilchrist et al., 2008). Interruption of the transfer of nutrients (glucose, amino acids and nucleotides), proteins and ions as well as second messenger molecules between these follicular support cells, and the oocyte results in a loss in oocyte viability (Buccione et al., 1990; Sugita et al., 2005; Gershon et al., 2008). 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 that are differentially expressed compared with younger women (Grondahl et al., 2010; McReynolds et al., 2012). Further, granulosa cells from women with reduced ovarian reserve have altered mRNA levels for genes associated with metabolic pathways (Greensied et al., 2009; May-Panloup et al., 2012; Skiadas 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 observed decline in oocyte quality.

A family of metabolic sensor proteins, called the sirtuins, which are mammalian homologues 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 ageing and mitochondrial function as well as playing a role in epigenetic gene silencing, DNA repair and recombination and microtubule organization (North et al., 2003; Mao et al., 2011; Peng et al., 2011). These proteins are nutrient-sensing nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylases or desuccinylases. They are characteristically upregulated 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 Sirtuin 3 (SIRT3) is reported to be another mitochondrial protein which is able to sense metabolic state and post-translationally alter mitochondrial function. The exact location of SIRT3 within mitochondria differs between tissue types, with one study demonstrating exclusive localization to the mitochondria and another demonstrating localization to both the nucleus and mitochondria (Lombard et al., 2007; Scher et al., 2007). Its expression is particularly high in mitochondria rich tissues such as the brain, heart, liver and brown adipose tissue (Shi et al., 2005; Lombard et al., 2007; Ahn et al., 2008). Surprisingly, Sirt3 null mice appear phenotypically normal despite tissues (liver, brown adipose tissue, brain and heart) showing hyperacetylation (Lombard et al., 2007; Ahn et al., 2008). SIRT3 has been shown to target mitochondrial metabolic enzymes, such as glutamate dehydrogenase (GDH), and its deacetylase activity is stimulated upon caloric restriction (Lombard et al., 2007; Schlicker 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 et al., 2008; Schlicker et al., 2008; Bao et al., 2010; Cimen et al., 2010; Finley et al., 2011; Kendrick et al., 2011). 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 SIRT3 in follicular cells was investigated in women with good prognosis attending for IVF and compared with that in young women with reduced ovarian reserve and advanced maternal age, as cells from these women display altered metabolic profiles.

### Materials and Methods

**Patient recruitment and treatment groups**

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: $\leq 35$ years or advanced maternal age: $\geq 40$ years) and ovarian reserve [as measured by serum anti-Mullerian hormone (AMH) levels] and randomly allocated to an experimental protocol (Supplementary data, Fig. S1). Previous studies have established that serum AMH correlates well with antral follicle count and thus can be utilized as a...
marker of ovarian reserve (Tremellen and Kolo, 2010; Hansen et al., 2011; Rosen et al., 2012; Wiweko et al., 2013). The serum AMH age ranges for young women with normal ovarian reserve and young women with reduced ovarian reserve were 1.8–5.6 and 0.4–1.5 μg/l, respectively. Women of advanced maternal age had serum AMH concentrations ranging between 0.6 and 1.5 μg/l, and these concentrations were representative of the 25–75th percentage quartiles for this age group (Tremellen and Kolo, 2010). Exclusion criteria were donor/recipient cycles, preimplantation genetic screening/PGD cycles and patients diagnosed with polycystic ovary syndrome. Samples were de-identified, and each woman was represented once within the study period. Ethical approval 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 (Pasella et al., 2012). HCG trigger (Ovidrel (250 mcg) Merck Serono, Frenchs Forest, NSW, Australia) was administered when at least two follicles reached ≥ 17 mm diameter, and cumulus-enclosed oocytes were collected 36 h 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, Gothenburg, Sweden) followed by 30 min centrifugation at 470 g at room temperature. Using an 29 gauge insulin needle, following oocyte retrieval cumulus oocyte complexes (COCs) were manually trimmed of excess cumulus and granulosa cells, and blood clots attached to the COCs were also avoided. Cumulus cell masses were then washed through Dulbecco’s PBS and were dispersed by gentle pipetting. Granulosa and cumulus cells were diluted to a concentration of 1 × 10⁶/ml, and cell counts were performed using a haemocytometer.

RNA isolation and quantitative PCR 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, MA, USA), and RNA was considered to be of acceptable quality if absorbance for A260/A280 was 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 5 ng/μl and subsequently stored at −20°C until required. Human SIRT3 quantitative PCR (qPCR) primers (S’ to 3’) were designed using the NCBI Blast Program (accession number: NM_012239; Amplicon size: 104; F: TGCCCCAGAGGTTCTTGCT: R: CTCGGTCACAGCTGGCAAAAG) and were synthesized by Geneworks (Hindmarsh, South Australia, Australia). Primers were validated by gel electrophoresis and sequenced (results not shown). Each 20 μl qPCR reaction contained 2 μl cDNA and a master mix containing 10 μl SYBR Green (Applied Biosystems, Mulgrave, Victoria, Australia), 1 μl each of the SIRT3 forward and reverse primers at a concentration of 10 pmol/μl and 6 μl H₂O. O. Samples were run in triplicate, and the PCR cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 amplification cycles of 95°C for 15 s and 60°C for 1 min 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: AGAAGGCTCACCACATCCAA, R: CCTGATTTGTTATTTTCTGT CACTACCT, Qiagen; Livak and Schmittgen 2001).

Immunohistochemistry for SIRT3 in 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 0.1 M glycine (Sigma-Aldrich) in Dulbecco’s PBS for 5 min and blocked using 5% donkey serum (Sigma-Aldrich) followed by 5% goat serum (Sigma-Aldrich) each for 2 h. The localization 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, IL, USA) set at 37°C for 2 h was utilized for incubation of the SIRT3 primary antibody (Abcam, Saphire Bioscience, Waterlo, New South Wales, Australia) and the cytochrome C (Abcam) primary antibody in a 1:50 dilution. Incubation of the secondary antibodies, fluorescein isothiocyanate-conjugated AffiniPure Donkey Anti-Rabbit (Jackson Immunoresearch Laboratories, Abacus ALS, Brisbane Queensland, Australia) and Rhodamine (TRITC)-conjugated AffiniPure Goat Anti-Mouse (Jackson ImmuunoResearch Laboratories) diluted 1:100 using the ThermoBrite for 2 h at 37°C. Nuclei were stained for 1–2 min using 3 μM 4′,6-diamidino-2-phenyindole, dilactate (DAPI; Sigma-Aldrich) and imaged using a Leica TCS SPS 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 100 × stock solution, Sigma-Aldrich), radio-immunoprecipitation assay buffer and a loading buffer were added to each sample of granulosa or cumulus cells, diluted to 1 × 10⁶/ml (a Bradford assay was performed to ensure equal amounts of protein, results not shown) and were subsequently heated for 10 min at 70°C. A 20 μl aliquot of the sample was added to the pre-cast polyacrylamide gel wells (4–15% Mini-Protein TGX Pre-cast Gel, Bio-Rad, Gladville, New South Wales, Australia) and run at a constant 150 V for 60 min. Transfer onto a 100% pure nitrocellulose membrane was performed by running the gel and membrane at a constant 100 V for 60 min 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, Victoria, Australia)) overnight at 4°C followed by incubation with the SIRT3 antibody (as used for immunohistochemistry) diluted to 1:1000 for 3 h 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 h. This was followed by application of the ECL detection reagents for 5 min. The membrane was exposed to Hyperfilm ECL in a darkroom. A protein standard (Precision Plus Protein Dual Colour Standards, Bio-Rad), alpha tubulin loading control, positive control [recombinant SIRT3; (Abnova, Saphire Bioscience)] and negative control (no primary antibody) were also prepared. Quantification of the bands was performed by image analysis using densitometry normalized to the loading control. The 69 kDa recombinant SIRT3 protein corresponds to all amino acids (1–400) and 44 kDa 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 (r² > 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 of Sirt3 protein activity we used nicotinamide [0, 5, 10, 25 and 50 mM (Sigma-Aldrich)] and Suramin [0, 5, 10, 20 and 40 mM (supplied with activity kit)]. The stimulators of SIRT3 used were resveratrol [0, 5, 10 50 and 100 mM (Sigma-Aldrich)] and SIRT1720 [0, 75, 150, 300 and 600 μM (Gelleckhem, 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 (1 x 10⁶/ml), were collected and purified as outlined above and cultured in G-1 media (Vitrolife) with either 10 mM nicotinamide or 10 mM resveratrol at 37°C in 5%O₂/6%CO₂ for 24 h. A G-1 media-only control was included.

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 50 mM imidazole (Sigma-Aldrich), 25 mM ammonium acetate (Sigma-Aldrich), 100 μM ADP (Roche Diagnostic Australia, Dee Why, New South Wales, Australia), 100 μM NADPH (Roche Diagnostic Australia) and 0.05% bovine serum albumin (Sigma-Aldrich) with 0.2 μl of sample (cells diluted to 1 x 10⁶/ml) added to each reaction. Reactions were initiated by addition of 2 mM α-ketoglutaric acid. Fluorescence was assessed after 10 min at 37°C and each sample was run in triplicate. A standard curve (r² > 0.98) was obtained for each analysis. A positive control of GDH (Roche Diagnostics, Mannheim, Germany), and the negative control lactate dehydrogenase (Roche Diagnostics) and no sample were also included.

Immunoprecipitation of acetylated proteins in mitochondria extracted from 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 (data not shown). Immunoprecipitation of mitochondrial isolated from granulosa and cumulus cells was performed using the Dynabead Protein G Kit (Invitrogen, Life Technologies, Mulgrave, Victoria, Australia) according to manufacturers’ instructions. The anti-acylated antibody (Abcam) was first bound to the Dynabeads by incubation of the beads and antibody (1:100) for 10 min. The mitochondrial isolated sample was added to the beads and incubated for a further 10 min. The Dynabeads bound to the acetylated proteins were resuspended in 50 μl of washing buffer, and 10 μl of elution buffer was added. The Dynabeads (bound to the antibody and sample) were then stored at ~80°C until western blot analysis, when the Dynabeads were thawed, 10 μl of loading buffer with protein inhibitor cocktail was added and then denatured by heating the sample for 10 min at 70°C. The supernatant (containing the acetylated proteins) was removed and loaded into the prepared polyacrylamide gel (4–15%). 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 ± 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 analysis of variance with Tukey’s post hoc test was used. A P-value < 0.05 was considered statistically significant.

Results

Patient demographics

Patient demographics as well as IVF cycle details and outcomes are outlined in Table I. Serum AMH correlated with antral follicle count (Supplementary data, Fig. S2). Young women with normal ovarian reserve had lower starting FSH doses compared with young women with reduced ovarian reserve and women of advanced maternal age (all P < 0.05, Table I). Compared with both the young women with 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 I). The young women with normal ovarian reserve also had an increase in pregnancy rate and implantation rate compared with the young women with reduced ovarian reserve and advanced maternal age groups (P < 0.05, Table I). The young women with reduced ovarian reserve had an increase in fertilization rate and implantation rate compared with the advanced maternal age women (P < 0.05, Table I). No significant differences were found for either BMI or infertility diagnosis.

Granulosa and cumulus cell SIRT3 mRNA level

SIRT3 mRNA levels were significantly elevated in granulosa cells compared with cumulus cells in young women with normal ovarian reserve (P < 0.05, Fig. 1). 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 (Fig. 1). Granulosa cell SIRT3 gene expression was significantly decreased in young women with reduced ovarian reserve and was further decreased in women of advanced maternal age compared with young women with normal ovarian reserve (P < 0.05, Fig. 1). Similarly, cumulus cell SIRT3 mRNA levels in women with advanced maternal age were reduced compared with young women with normal ovarian reserve (P < 0.05, Fig. 1).

SIRT3 protein localization and levels in granulosa and cumulus cells

SIRT3 was found to co-localize with the mitochondria in both granulosa and the cumulus cells (Supplementary data, Fig. S3). Similar to the mRNA data, granulosa cell SIRT3 protein levels detected by western blot analysis were elevated compared with the cumulus cells in young women with normal ovarian reserve (Fig. 2A) with this difference again not evident in young women with reduced ovarian reserve or the advanced maternal age women. Compared with 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, Fig. 2B). Granulosa cell
SIRT3 protein levels were further decreased in the advanced maternal age cohort compared with young women with reduced ovarian reserve.

Cumulus cell SIRT3 protein levels were significantly decreased in women of advanced maternal age compared with young women with normal ovarian reserve (P < 0.05, Fig 2B). In contrast to the gene expression data, women of advanced maternal age also had decreased SIRT3 protein levels compared with young women with reduced ovarian reserve (P < 0.05, Fig 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 (Fig 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 V_{max} conditions and found to be present in both granulosa and cumulus cells from all women. SIRT3 protein deacetylation activity was highest in granulosa cells in young women with normal ovarian reserve (P < 0.05, Fig. 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 with young women with normal ovarian reserve (P < 0.05, Fig. 3). Women of advanced maternal age had decreased cumulus cell SIRT3 protein deacetylation activity compared with young women with normal ovarian reserve or young women with reduced ovarian reserve (P < 0.05, Fig. 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 (Fig. 3).

**Acetylation of GDH in mitochondria from granulosa and cumulus cells**

SIRT3 is able to deacetylate GDH therefore acetylation of GDH in granulosa and cumulus cells was determined by western blot analysis of the
mitochondrial acetylated protein fraction. Compared with 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 \((P < 0.05, \text{Fig. 4})\). Young women with normal ovarian reserve and young women with reduced ovarian reserve had similar GDH acetylation levels in cumulus cell (Fig.4). GDH acetylation in cumulus cells was highest in the advanced maternal age women and significantly higher than that in young women with normal ovarian reserve and young women with reduced ovarian reserve \((P < 0.05, \text{Fig. 4})\).

**Activity of the SIRT3 target glutamate dehydrogenase in granulosa and cumulus cells**

To assess the impact of increased GDH protein acetylation on activity, GDH \(V_{\text{max}}\) activity which catalyses the reaction between glutamate and \(\alpha\)-ketoglutarate, was determined. In agreement with SIRT3 deacetylation activity, granulosa cell GDH activity was decreased in young women reduced ovarian reserve and in women of advanced maternal age compared with young women with normal ovarian reserve \((P < 0.05, \text{Fig. 5A})\). Granulosa cell GDH activity in women of advanced maternal age was decreased compared with young women with reduced ovarian reserve, reflective of what was observed in granulosa cell SIRT3 deacetylation activity \((P < 0.05, \text{Fig. 5A})\). Similar to cumulus cell SIRT3
deacetylation activity, cumulus cell GDH activity was decreased in women of advanced maternal age compared with young women with normal ovarian reserve and young women with reduced ovarian reserve ($P < 0.05$, Fig. 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 (Fig. 5A).

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, respectively compared with the other inhibitors AGK7, suramin and sirtinol and the other activator SRT170 (Supplementary data, Fig. S4). The dose–response established that 10 mM nicotinamide and 10 mM resveratrol were the optimal concentrations in altering SIRT3 protein deacetylation activity. GDH activity in granulosa cells was increased versus control in the presence of resveratrol ($P < 0.05$, Fig. 5B). In contrast, nicotinamide caused a decrease in GDH activity versus control in granulosa cells ($P < 0.05$, Fig. 5B). Together, these data confirm 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 specialized with pyruvate being the main energy substrate, unlike somatic cells where energy is produced via aerobic respiration of glucose (Downs and Utecht, 1999; Wilding et al., 2001). Thus, cumulus cells have an essential role as they metabolize glucose into pyruvate which is transported into the oocyte, where it can be utilized. Thus, communication between the oocyte and its surrounding cells is essential for the development of a viable oocyte (Goud et al., 1998; Canipari, 2000; Sugiuara et al., 2005), and 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 with young women with normal ovarian reserve (Greenseid et al., 2009; May-Panloup et al., 2012; Skiadas et al., 2012). Similarly, proteomic analysis of cumulus cells from women of advanced maternal age has differentially expressed metabolic proteins compared with cumulus cells from younger women (McReynolds et al., 2012), and oocytes from women of advanced maternal age have altered expression of genes involved in the electron transport chain (Grondahl 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 mRNA and active protein are present in granulosa and cumulus cells and co-localize to the mitochondria, consistent with what has been reported in other tissues (Schwer et al., 2002; Michan and Sinclair, 2007; Hallovs et al., 2008). Furthermore, SIRT3 mRNA 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 recognized as a fundamental mechanism for regulating the activity of mitochondrial proteins and overall mitochondrial function (Hirschey 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 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 tricarboxylic acid (TCA) cycle may be targets of deacetylation (Hirschey 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 being unique to fed mice and 24% unique to fasted mice (Lombard et al., 2007). Interestingly, the acetylation of mitochondrial proteins also increased in mice on a long-term, high-fat diet (Hirschey 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 of advanced maternal age where it has been demonstrated that mitochondrial function is impaired (Van Blerkom et al., 1995; Wilding 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 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 with the cumulus cells, suggesting that the transition from granulosa to 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 were 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 extent of metabolic or steroidogenic changes associated with differentiation and instead demonstrated traits associated with granulosa cell function (Pacella et al., 2012). This altered cumulus cell function may have implications for COC metabolism and pyruvate supply to the oocyte thereby altering oocyte metabolism and affecting its developmental potential.

One of the most well-characterized confirmed targets of SIRT3 is GDH, a mitochondrial enzyme involved in both TCA activity and maintenance of the oxidative state.Activation of GDH is thought to promote the synthesis of ATP by allowing amino acids to be utilized as fuel for the TCA cycle (Frigerio et al., 2008). GDH has been demonstrated to co-localizes with SIRT3 in the mitochondrial matrix and in SIRT3 knockout mice GDH is hyperacetylated compared with wild-type (Lombard et al., 2007). SIRT3 can directly deacetylate GDH thus controlling mitochondrial metabolism, with incubation of SIRT3 with NAD+ increasing deacetylation of GDH therefore increasing its activity, and GDH activity is positively correlated with SIRT3 levels (Schlicker 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 SIRT 3 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, we observed increased acetylation of GDH in mitochondria from both the granulosa and cumulus cells of women of advanced maternal age compared with young women with normal ovarian reserve, whereas young women with reduced ovarian reserve had increased granulosa cell GDH activity in the mitochondria compared with 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 heat shock protein 70, which is involved 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 the regulation of manganese superoxide dismutase, which has been implicated in ageing and involved in the regulation of reactive oxygen species (Schlicker et al., 2008; Law et al., 2009; Mao and St Clair, 2009; Chen et al., 2011). Owing to the importance of these cellular processes in oocyte and embryo development as well as their link to ageing, 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 with 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 with 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 (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 caused by SIRT3 via post-translational protein modification may be a causative factor in the decline of 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 of women undergoing IVF treatment.

**Supplementary data**

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors’ roles
L.P.-I. was involved in study design, study execution, analysis and manuscript drafting and critical discussion, D.Z.-F. was involved in study design, manuscript drafting and critical discussion and M.L. was involved in study design, analysis, manuscript drafting and critical discussion.

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Conflict of interest
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